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Titel der Masterarbeit / Title of the Master's Thesis

„Glyphosate causes DNA damage in multiple organs of mice (liver, testes, kidneys and colon) but not in bone marrow“

verfasst von / submitted by

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Table of Contents

1. INTRODUCTION

Primary aim of the present thesis was to find out if glyphosate, the most widely used herbicide worldwide, has genotoxic properties. The clarification of this question is of importance in regard to ongoing discussions concerning the classification of the compound as a carcinogen.

It is known that damage of the genetic material is a hallmark of human cancer [1]. Several test procedures have been developed for the detection of genotoxic carcinogens since B. Ames postulated the use of mutagenicity assays for the identification of chemicals which cause malignant transformation in 1971 [2].

The following chapters give an overview concerning on the history, production and use of the compound (chapter 1.1) and on previous and ongoing expert discussions concerning its carcinogenic and mutagenic properties (chapter 1.2). Subsequent paragraphs specify in more detail the aim of the present study and the experimental systems which were used to investigate the DNA-damaging activities of the compound. The following parts of the thesis describe the materials and methods (chapter 2) and the results (chapter 3). The last section discusses the relevance of the current findings. Raw data of the individual experiments can be found in the appendix.

1.1 History and mode of action of Glyphosate

Glyphosate was synthesized by the Swiss chemist Henri Martin in 1950. Subsequently, Monsanto (St. Louis, Missouri, USA) tried to use it as potential water softening agent [3]. The herbicidal properties of the compound were discovered by J. E. Franz [4]; the most commonly used commercial formulation “Roundup” is on the market since 1974 [5]. The compound consists of an amine in the middle of the molecule and carboxylic and phosphonic acidic sites at both ends (Figure 1).

The inhibition of plant growth is due to the fact that glyphosate inhibits the biosynthesis of aromatic amino acid [6]. The herbicide binds and block the enzyme 5-enolpyruvyl-shikimate-3-phosphate-synthase (EPSPS), which catalyzes phosphoenol pyruvate to shikimate-3-phosphate [7].

Since pure glyphosate is only poorly soluble in water, different chemicals are added to increase its efficiency. The formulation “Roundup” is at present widely used and contains, apart from glyphosate, an additional surfactant (ethoxylated tallowamine) and isopropylamine.

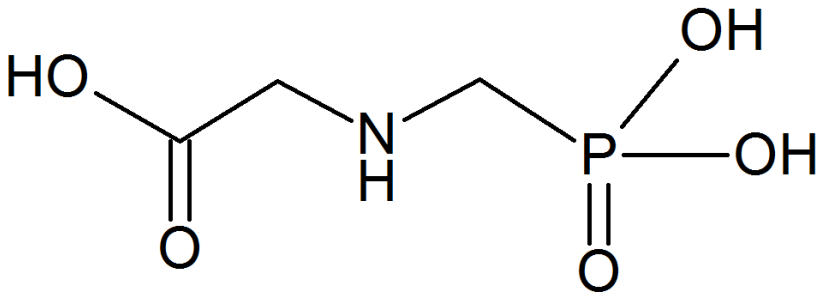


Figure 1: Chemical structure of glyphosate (N-(phosphonomethyl)glycine)

1.2 Discussions regarding the carcinogenic/mutagenic properties of glyphosate

The carcinogenic and mutagenic properties of glyphosate were evaluated by the German health ageny "Bundesinstitut für Risikobewertung" (BfR). In 2013 the first (interims) report was published; the final report appeared on March 2015 [8], shortly after the evaluation of the herbicide by the International Agency for Research and Cancer (IARC), World Health Organization [9]. The evaluation of the health risks of the BfR and the IARC differs strongly in regard to the classification of the mutagenic and carcinogenic properties of the compound. In August 2015, an addendum was published by the BfR which addressed the conflicting conclusions by the two agencies [10]. In November 2015, the European Food Safety Authority (EFSA) published a document which reflects the opinion of the BfR [11]. This document is an important milestone for the authorization of the drug in Europe.

1.2.1 Evaluation of the carcinogenic properties of glyphosate by the different agencies

Glyphosate was tested in regard to its carcinogenic properties in two studies in mice and rats already in 1970. It was criticized that the US-toxicologist William Dykstra, who worked for the United States Environmental Protection Agency (U.S. EPA), ignored in his expertise positive findings in rats [12]. In 1984, a mouse study was realized in which kidney tumors were detected [13]. The results were followed by discussions concerning the statistical significance of the findings.

The U.S. EPA stated in a comprehensive document that glyphosate is "non-carcinogenic" and categorized it as "class E"; also, the BfR came to the conclusion that it is devoid of carcinogenic activities, while the IARC experts placed it in "class 2A" (possibly carcinogenic to humans). The BfR argued repeatedly that its decision is not only based on results that were published in peer reviewed journals but included also industrial reports (which are not available for the public). In 2016, Christopher Portier and 94 other cancer experts published critical statement

concerning the tumorgenic properties of glyphosate [14] in which they stress that tests with human studies were inadequately interpreted by EFSA and that the positive findings in studies with mice concerning tumors, hemangiosarcomas and malignant lymphomas, were ignored.

A detailed description of the available information concerning the carcinogenic effects of glyphosate is beyond the scope of this thesis [15, 16]. In conclusion, it can be stated that the discussion is still in progress and that the assumption that the compound is safe, was criticized intensely by a large number of experienced scientists.

1.2.2 Mutagenic properties of glyphosate

As mentioned above, damage of the genetic material is one of the key mechanisms which leads to cancer. The "multi-step" hypothesis of cancer development is based on the assumption that mutations of somatic cells lead to initiation, when these cells divide during the "promotion-phase" (which can be also stimulated by non-mutagenic compounds), primary tumors are formed which metastasize in the subsequent progression phase [17].

In order to investigate the genotoxic properties of chemicals, a panel of test systems was developed, comprising gen-mutation assays with bacterial indicators (Salmonella/microsome assays) and mammalian cells (thymidine kinase (TK^r) and hypoxanthine phosphorybosyl transferase (HPRT) test) as well as experiments, which concern induction of chromosomal aberrations (including micronucleus experiments). DNA damage can be also detected by indirect approaches, reflecting induction of DNA repair processes and fragmentation as a consequence of formation of single- and double strand beaks. For routine testing of new chemicals, standardized tests are used which are included in internationally excepted guidelines (e.g. OECD guidelines). If compounds cause positive results *in vitro*, they are further studied in genotoxicity experiments with rodents, provided that these findings are positive, long term carcinogenicity trials with mice and rats are conducted which are time and cost effective. Table 1 describes a number of test-models which are commonly used for routine screening.

Table 1: Overview on mutagenicity test procedures which are used for routine testing of chemicals (OECD guidelines)

Test system	**Test principle**	**Remarks**	**Ref.**
In vitro studies			
Salmonella/microsome assay (OECD guideline #471 [18])	Induction of his+ revertants in a panel of tester strains with different specificity	Extremely large database available (more than 10.000 compounds tested); a high number of false positives is obtained with this test-system; Specificity: 74% [19]; Sensitivity: 70% [20], performed with and without metabolic activation (addition of liver S9)	Ames et al. [21]
Gene mutation assays with mammalian cell lines (OECD guideline #476 [22])	Based on the detection of resistance towards anti-metabolites due to gen-mutations (TG^r-thioguanine resistance, HPRT-assay)	Only 3000-4000 chemicals at present rarely used but only few tests were conducted with glyphosate	Aaron et al. [23]
Detection of chromosomal aberrations (OECD guideline #473 [24]) and micronuclei (OECD guideline #487[25])	performed with a number of different cell lines; most of them are metabolical not competent and lack phase 1 and phase 2 enzymes	Metaphase analyses of chromosomes are time consuming and costly and were largely replaced by micronucleus experiments	Ishidate et al. [26] Nesslany et al. [27]
In vivo studies			
Micronucleus test with bone marrow cells (OECD guideline #474 [28])	Based on the detection of MN which reflects structural/chromosomal aberrations in bone marrow cells	Most frequently used *in vivo* test for routine screening of chemicals, may false positives are obtained with this method; in some cases, it is not clear if the active compound reaches the target tissue, therefore the ratio PCE to NCE are determined	Hayashi [29]
Single Cell Gel Electrophoresis assays (OECD guideline #489 [30])	Based on the detection of DNA migration in an electric field, detection of single- and double strand breaks and apurinic sites	Higher sensitivity as MN experiments; false positive results may be obtained as a consequence of acute toxicity	Singh et al. [31]

1.2.3 Evaluation of glyphosate by EFSA/BfR and IARC

A detailed description of the database which was used by EFSA for the evaluation of the genotoxic properties of glyphosate can be found in the final addendum (published 2015) [10]. It describes results of bacterial tests, a few (in total 9) tests with mammalian cells (but no results of *in vitro* experiments) as well as several negative chromosomal aberration (CA) tests. *In vivo* studies include predominantly MN assays (in total 4) and CA trials (in total 5). Findings of other experiments (e.g. unscheduled DNA synthesis (UDS) and Single Cell Gel Electrophoresis (SCGE) assays) were not used in the classification; furthermore, tests (≥ 40) with non-mammalian organisms (fish, reptiles, amphibians) were also ignored.

It is mentioned in a separate table of the EFSA and also in the renewal assessment of the BfR that Bolognesi et al. [32] published a study which indicated that DNA adducts were induced by glyphosate in mice. Furthermore, a SCGE study with BALB/C mice of Manas et al. [33] is described which found evidence for a positive effect with an oral dose of 400 mg/kg bw. per day. It is stated that the induction of DNA strand breaks was observed at a dose which is close or in excess of the *i.p.* LD_{50} of glyphosate in mic, therefore the positive results may be due to secondary cytotoxicity effects [34]. A similar statement was made by Williams et al. [35], in regard to studies obtained by Bolognesi et al. [32] and Peluso et al. [36] who found induction for single strand breaks (alkaline elution method). The effects could be "indicative of events of cytotoxicity that reduces or retards rates of DNA replication, giving the appearance of breakage events. The fact that these events were transitory, being no longer evident 24h after exposure also suggests an indirect effect of exposure." [35].

The evaluation of the BfR/EFSA is partly based on expert reviews that were written by scientists who worked for Monsanto. Also, in these review papers, results with non-mammals are completely ignored as the authors explain that the overall weight of these studies is low [35, 37].

Two comments describe the reason for the different opinion of BfR and IARC; the first document [14] represents the position of the later agency and states that evidence from human *in vitro* studies in regard to induction of cancer was ignored and that the results of long term cancer studies with animals were misinterpreted. In a later paper, Portier et al. [38] describes the results of an evaluation of regulatory documents (in total 22 cancer studies with animals) and comes to the conclusion that also this investigation found evidence for carcinogenic properties. Furthermore, it is also stated in his first critical article that genotoxicity studies with human and human cells were ignored by BfR/EFSA and that results of genotoxicity trials, which were not publicly available, were used by these agencies to decide that the compound is not genotoxic [11]. Therefore, it is not possible to decide if this conclusion is justified.

1.3 Aim of the present study

1.3.1 General background

As mentioned in the last paragraph, the classification of glyphosate by EFSA/BfR is mainly based on negative results of *in vivo* MN assays which were obtained in bone marrow cells with rodents and also on negative findings from bacterial tests [10]. *In vitro* tests with mammalian cells (SCGE and chromosomal aberration assays) (in particular with liver derived cell lines) which yielded partly positive effects, were not taken into consideration, possibly due to criticism which can be found in the reviews of the Monsanto hired experts [37, 39, 40]. Also positive findings from SCGE experiments with mice and UDS studies were ignored.

The comet study of Manas et al. (2013) [33] is indeed not conclusive as it was not performed according to the current OECD guideline (#489) [30]; i.e. no positive controls were included and only two doses were tested. A further study which was published recently by Milic et al. (2018) had a substantially better design and found evidence for DNA damage in the liver, while the effects in blood cells were inconclusive and dependend on the parameter which was used. Cytologic effects which may lead to false positive findings [41, 42] in SCGE experiments were not studied in other tests; this enhardens the interpretation of the results. According to OECD guideline (#489), it is mandatory to exclude that positive effects are caused by cytotoxicity, „to assess the biological relevance of a positive or equivocal result, information on cytotoxicity at the target tissue is required (see paragraphs 54-55). Where positive or equivocal findings are observed solely in the presence of clear evidence of cytotoxicity, the study would be concluded as equivocal for genotoxicity unless there is enough information that is supportive of a definitive conclusion“ [30].

The work which is described in the present thesis is the first comprehensive SCGE study, which was realized in full agreement with the current OECD guideline (#489) [30], i.e. multiple doses were tested in five animals per experimental group and positive as well as negative controls were included. Also, additional histopathological analyses were performed in organs of animals in which positive results were obtained. Furthermore, we studied additionally in the same experimental series also formation of micronuclei (MN) in polychromatic erythrocytes (PCE) in the bone marrow. Also, this experiment followed strictly the current OECD guideline (#474) [28]. The following chapters provide further background information concerning the history, use and methodological principles of these methods.

1.3.2 Single Cell Gel Electrophoresis experiments

The Single Cell Gel Electrophoresis (SCGE or comet) assay is based on the determination of DNA migration in an electric field. The first experiments were conducted under neutral conditions by Östling and Johansson in 1984. This older version detects only double strand breaks and is relatively insensitive. An improved protocol was developed by the US scientists R. Tice and N. Singh in the late 1980's [31]. They conducted the first experiments under alkaline conditions which detect single- as well as double strand breaks and alkali labile sites [43].

The migration of the genetic material to the anode leads to formation of so-called "comets" and their size an intensity is used as a measure of genotoxic damage. These parameters can be quantified by use of computer aided image analysis systems [44]. The recommended endpoint is the percentage DNA in tail [45, 46] which was determined in the present study. Figure 2 shows images comets, which are detected in SCGE experiments.

The SCGE technique is at present the most widely used method in genetic toxicology [47]. It is applied for *in vitro* experiments with primary and cultivated cells [48] but also for *in vivo* studies with rodents [49] and for human biomonitoring [50]. The main advantage is that no cultivation of the individual indicator cells is required in contrast to other methods and a variety of cells from different organs can be analyzed.

The current protocol for *in vivo* experiments is based on the work of Sasaki et al. [51] who developed procedures for the homogenization and subsequent isolation of nuclei for different organs. As described above an OECD-guideline (#489) was published for SCGE experiments with rodents. When positive results are obtained it is mandatory to conduct histopathological investigations to avoid false positive results due to cytotoxicity [52].

We included in the present study such experiments and monitored DNA damage in the liver, since several earlier investigations indicate that it is a target for DNA instability by glyphosate [32, 33, 42]. Positive results were also obtained with hepatic cells of non-mammalian species in a number of studies [53, 54] and in human derived liver cells *in vitro* (such as HepG2) [55]. The colon was included as the herbicide comes in direct contact with the gastrointestinal tract after consumption of contaminated foods; bone marrow cells were analyzed as they are the target tissue for MN experiments (see below); the kidneys were studied as higher concentrations of the parent compound and or its metabolites can be expected in this organ. Finally, testes were studied as positive results in this organ are indicative for potential damage of germ cells and brain tissue was monitored as DNA damage in this organ may by indicative for neurological dysfunctions [56].

It is notable that comets do not reflect persisting mutations. These structures disappear as a consequence of repair processes and the biological consequence of comet induction are not

fully understood at present. Recent analysis of human studies indicated that comet formation in peripheral lymphocytes is associated with increased mortality and induction of cancer (S. Bonassi - personal communication).

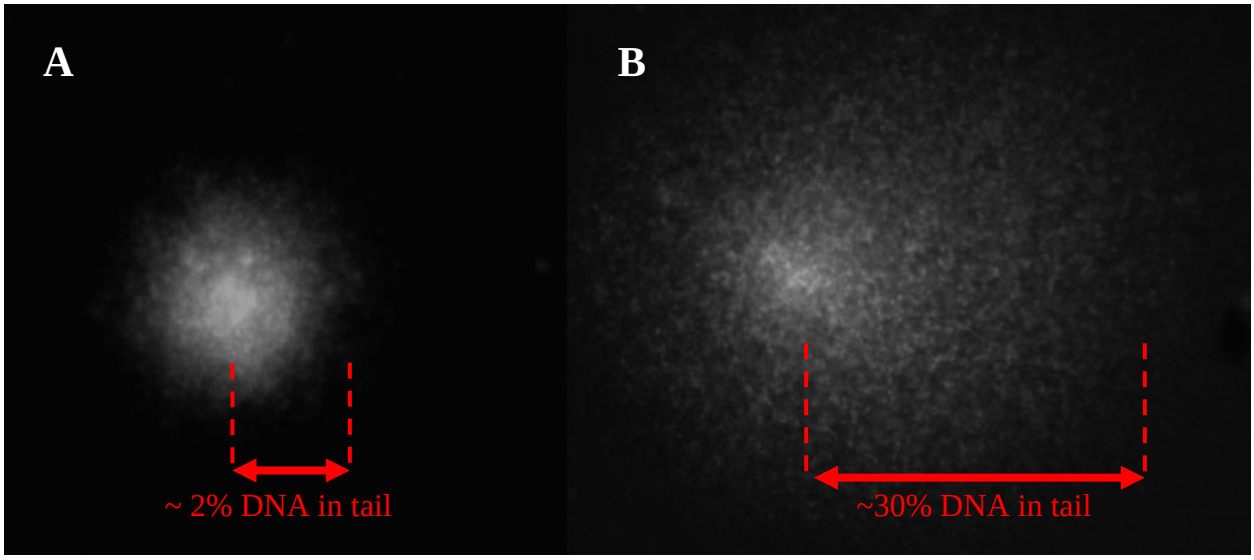


Figure 2: Photographic images of a comet. (A) shows a non-damaged/control cell with about 2% DNA in tail. (B) shows a damaged cell.

1.3.3 Bone marrow micronucleus assay

MN were initially termed "Howell-Jolly Bodies" according to the names of scientists who discovered them in blood cells of rats and cats. In 1959, H. Evans monitored the first MN in root tips of *Vicia Faba* after gamma-radiation [57] and hypothesized that they may represent chromosomal damage. Subsequently (in the 1970's) protocols were developed for MN experiments with blood and bone marrow cells by Boller and Schmid [58].

MN are formed as a consequence of structural and numerical chromosomal aberrations [59] and validated/standardized protocols [28] have been developed for routine screening of chemicals *in vitro* and for *in vivo* experiments with rodents. Figure 3 depicts the photographic images of polychromatic and normochromatic erythrocytes and of PCE with a MN.

The bone marrow MN assay is at present the most widely *in vitro* test for routine screening of chemicals; experiments with lymphocytes of humans are frequently used for occupational monitoring of workers [60] and lifestyle exposures [61]. Furthermore, protocols have been developed for the evaluation/determination of MN in exfoliated cells from buccal [62] and nasal mucosa and also for cervical and urothelial cells [63]. These experiments can be used for the detection of DNA damage by chemicals but also for the prediction/diagnosis of specific forms of

cancer and other diseases [64]. An analysis of available data from human studies with lymphocytes by Bonassi et al. [65] showed that MN in peripheral blood cells (lymphocytes) is a reliable biomarker for the prediction of increased cancer risks in humans.

As mentioned above, the classification of glyphosate by EFSA and BfR is predominantly based on the results of MN experiments with bone marrow cells of mice and rats. In total, 9 industrial studies were regarded as valid. Only two of them reported a positive result in mice. Notably, a study of Bolognesi et al. [32], which was published in the scientific literature, yielded a positive result but was not included due to unspecified methodological deficiencies.

MN formation was included in the present study in order to compare the results which were obtained with the herbicide in the same tissue in SCGE experiments with MN induction under identical experimental conditions.

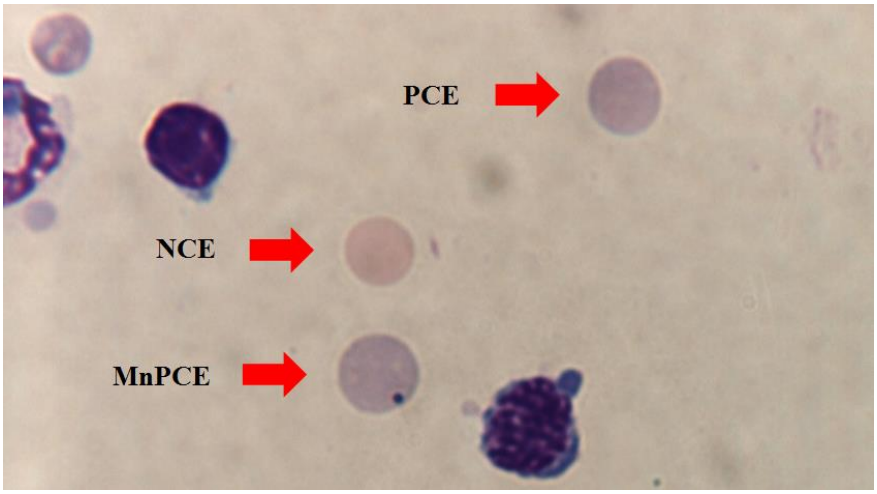


Figure 3: Morphological characteristics of a micronucleus in polychromatic erythrocytes (magnification 1000 x, 10% Giemsa stain). The figure shows MN, which is a DNA-containing extracellular body in the cytoplasm, in PCE and normochromatic erythrocytes.

1.3.4 Design of the study

As mentioned above, the study design followed the current OECD guidelines for SCGE #489 [30] and MN experiments #474 [28].

In the main experiment, five animals were used per group and three doses of glyphosate were tested, furthermore positive and negative controls were included. The treatment period was in the present study 14 days and the maximal dose in the main experiment was 1000 mg/kg bw. per day as suggested by the OECD. We calculated the daily dose on the basis of the water intake [66] which was measured every three days. The body weights were determined before, during and at the end of the supplementation period. Liver weights were determined in all groups at the end of the study. Ethyl methanesulfonate (EMS) was used as a positive control in SCGE experi-

ments and was administered *i.p.* (single dose, 300 mg/kg bw.) 3 h before they were sacrificed. Slides were evaluated by use of a computer aided image analyses system, the percentage of DNA was monitored as suggested by several authors [44, 46, 67].

MN were scored in the main experiment in PCE of the different treatment groups. In addition, also the frequencies of normochromatic erythrocytes (NCE) were determined and the rates PCE/NCE were calculated. Cyclophosphamide (CP) was used as a positive control; the animals received a single *i.p.* dose (50 mg/kg bw.) 24 h before they were sacrificed.

To verify the positive results in liver and kidneys, further SCGE experiments were conducted; the animals were treated ether with 400 or with 1000 mg/kg bw. per day for an identical period as in the main study. Again EMS (300 mg/kg bw) was used as a positive control for the SCGE experiment. We analyzed also the MN ratio in this experiment.

All data were analyzed with established statistical methods which were suggested for MN and SCGE studies [45, 68-72].

2. MATERIALS AND METHODS

2.1 Test compound

2.1.1 Characteristics of the test compound

Table 2 summarizes the characteristics of the test compound

Table 2. Chemical properties of glyphosate (N-(Phosphonomethyl)glycin)

Name	Glyphosate
IUPAC name	N-(Phosphonomethyl)glycin
Molecular formula	$C_3H_8NO_5P$
CAS number	1071-83-6
EC number	213-997-4
Purity	96% (Sigma-Aldrich, Schnelldorf Germany)
Molecular weight	169.073 g/mol
Aggregate state	Solid
Color	white crystalline powder
Solubility	Soluble in H_2O (1.01 g/100 mL (20 °C))
Stability	Stable under storage conditions

N-(Phosphonomethyl)glycin (purity 96%) was purchased from Sigma-Aldrich (Schnelldorf, Germany), stored at room temperature and dissolved in drinking water immediately before treatment of the animals. Different concentrations were used: 150, 400 or 1000 mg/kg bw. per day. Glyphosate is stable in water; normal light does not have any photodegrading effects on the substance [73].

2.1.2 Positive controls

Table 3 lists both substances which were used as positive controls

Table 3. Chemicals used for positive controls

Compound	**Abbreviation/ Formula**	**Company**	**Product number**	**CAS-No.**
Ethyl methanesulfonate	EMS	Sigma-Aldrich	M0880	62-50-0
Cyclophosphamide monohydrate	CP	Sigma-Aldrich	C0768	6055-19-2

Ethyl methanesulfonate (EMS, purity 100%) was purchased from Sigma-Aldrich (Schnelldorf, Germany), stored at -20°C and dissolved in PBS before treatment of the animals. A concentration of 300 mg/kg bw. was used as a positive control in the SCGE experiment (one *i.p*. treatment).

Cyclophosphamide monohydrate (CP, purity 100%) was purchased from Sigma-Aldrich (Schnelldorf, Germany), stored at -20°C shortly before use and dissolved. A concentration of 50 mg/kg bw. was used as a positive control for the MN experiments experiment (one *i.p*. treatment).

2.2 Chemicals used in Single Cell Gel Electrophoresis experiments

Table 4 lists all chemicals which were used for the SCGE experiment.

Table 4. Chemicals used in SCGE experiments

Compound	Abbreviation/ Formula	Company	Product number	CAS-No.
2-Amino-2-(hydroxymethyl)-1,3-propanediol	Trizma® base	Sigma-Aldrich	T6066	77-86-1
4-(1,1,3,3-Tetramethylbutyl)-phenylpolyethylene glycol	Triton™ X-100	Sigma-Aldrich	T9284	9002-93-1
Dimethylsulfoxid	DMSO	Carl Roth	4720.3	67-68-5
Dulbecco's Phosphate Buffered Saline (10x) without Ca & Mg	PBS	PAA	H15-011	
Ethylenediaminetetra-acetic acid disodium salt dihydrate	Na_2EDTA	Sigma-Aldrich	E5134	6381-92-6
Low Melting Point Agarose	LMPA	Thermo Fisher Scientific	15517-014	16520-100
Normal Melting Point Agarose	NMPA	Serva	11404.04	9012-36-6
Propidium iodide solution (1 mg/mL)	PI	Sigma-Aldrich	P4846	25535-16-4

2.2.1 Preparations of solutions for Single Cell Gel Electrophoresis experiments

Normal melting point agarose (NMPA)

1.5 g normal melting point agarose was diluted in 100.0 mL ddH_2O. Clean microscopic slides were coated with the prepared NMPA, dried overnight and stored at room temperature in a dark place until the experiment was performed.

Low melting point agarose (LMPA)

125.0 mg of low melting point agarose was diluted in 25.0 mL PBS and was used to embed cells on the coated slides. Solution was then stored at room temperature.

Homogenizing buffer (HB)

HB was prepared according to Sasaki et al. [74], i.e. 4.383g/L NaCl and 8.93g/L Na_2EDTA were dissolved in 1L ddH_2O and adjusted to pH 7.5 with 10M NaOH, HB was stored at 4°C and 2-4 mL was used to homogenize organs to obtain cells..

Phosphate Buffered Saline (PBS)

PBS (10x) stock solution was diluted 10-fold with ddH_2O and stored at 4°C.

Lysis solution

NaCl (146.1 g), Na_2EDTA (37.2 g), Trizma® base (1.2 g) and NaOH (7.0 g) were dissolved in 1.0 L ddH_2O and stirred until the solution became clear. Subsequently, the pH was adjusted to 10.0 with NaOH (10.0 M). The solution was stored at 4 °C. Before use, the required amount of lysis solution was calculated and 1.0 % of Triton™ X-100 and 10.0 % of dimethyl sulfoxide (DMSO) were added. The lysis solution was filled into Coplin jars and stored in the dark at 4 °C.

Alkaline electrophoresis buffer

84.0 mL of NaOH (10.0 M) and 14.0 mL of Na_2EDTA (0.2 M) were added to 2702.0 mL cold ddH_2O and mixed with a magnetic stirrer. The pH of the solution $\geq$ 13.0 was checked before use.

Preparation of slides

The slides were dripped into 1.0 % NMA which was dissolved in PBS. The slides were dried overnight and stored at room temperature until further use.

2.3 Chemicals used in micronucleus experiments

Table 5 lists all chemicals which were used for the MN experiment.

Table 5. Chemicals used in MN experiments

Compound	Abbreviation/ Formula	Company	Product number	CAS-No.
Fetal bovine serum	FBS	Sigma-Aldrich	F7524	
Methanol 80%	CH_3OH	Sigma-Aldrich	322415	67-56-1
Giemsa Stain, Modified Solution	Giemsa	Sigma-Aldrich	48900	51811-82-6
Entellan		Merck	107961	

2.3.1 Preparations of solutions for micronucleus experiments

Fetal bovine serum (FBS)

FBS was freshly prepared and stored at 4°C on the same day and immediately used.

Methanol (MetOH)

80% MetOH was prepared and stored at -20°C for fixation on the next day.

Staining

Giemsa solution was prepared 1:10 with ddH_20.

2.4 Chemicals used for histopathology

Table 6 lists chemicals which were used for histopathology.

Table 6. Chemicals used for histopathological analyses

Compound	Abbreviation/ Formula	Company	Product number	CAS-No.
Formaldehyde solution 4%, neutral buffered		SAV LP GmbH	FN-1000-4-1	50-00-0
Ethanol 70%	EtOH	Sigma-Aldrich		64-17-5
Paraffin				

2.5 Animals

The mice were kept in Makrolon-III cages under standard conditions (room temperature 22 ± 2°C; humidity 45 +/- 5%; 12 hours light/dark cycle) and were fed with standard chaw and received water *ad libitum*. The animals were acclimatized for 14 days before the start of the experiment.

2.6 Treatment of the animals

Two experiments were conducted. In the main experiments, five animals were tested per group and received glyphosate supplemented drinking water *ad libitum* for a period of 14 days. The mice received 150, 400 or 1000 mg/kg bw. per day of the herbicide *p.o.* via drinking water. In addition, negative and positive control groups were included in the main and in the confirmation study. For SCGE experiments EMS (dose: 300 mg/kg bw.) was injected *i.p.*; CP (dose: 50 mg/kg bw.) was given *i.p.* and was used as a positive control for the main MN study. Both compounds were administered only once. Glyphosate solutions were freshly prepared every three days and the pH of the drinking water was adjusted to 7.2 by addition of 10M NaOH. The control groups received only the pH adjusted drinking water. Body weights as well as water consumption were monitored in intervals of 3 days.

2.7 Tissue collection

After a period of 14 days, the animals were sacrificed by cervical dislocation. Half of the liver, brain, colon, one testis and one kidney were transferred to 4% formaldehyde and stored at 2°C for additional histopathological examinations.

2.8 Single Cell Gel Electrophoresis experiments

All solutions were prepared according to the protocol of Tice et al. [51] The experiments were conducted under alkaline conditions (pH ≥ 13) and followed the current OECD Guideline. The processing of the organs were made according to the method developed by Sasaki et al. [75].

For experiments with colon cells, they were directly scratched from the epithelial layer and resuspended in 500 µL chilled HB. Bone marrow cells were obtained by flushing the femur with 500µL PBS immediately after removal [74]. Half of the brain, 1g of the liver, one testis and one kidney were minced in 4.0 mL chilled homogenization buffer (pH 7.5), homogenized at 550 rpm on ice by use of a Potter Elvehjem-type homogenizer (B. Braun, Melsungen, Germany) and filtered with a 40 µm strainer (EASYstrainer from Greiner). Subsequently, the homogenates were centrifuged (700 rpm, 10 min, 4 °C) and the pellets resuspended with 1.0 mL chilled HB. The cell suspensions were then mixed with 0.5% LMPA and spread on pre-coated agarose slides (1.5% NMPA). Lysis was performed in the dark at 4°C for at least 60 minutes. After unwinding under alkaline conditions (pH ≥ 13) for 30 minutes, electrophoresis was carried out for 30 min (1.0 V/cm, at 4° C). Subsequently, slides were washed twice with ddH_2O for 8 minutes. From each organ, 3 slides were prepared per animal. Overall 15 Slides per dose and organ were evaluated.

Air-dried slides were stained with propidium iodide (10 µg/mL) and coded. The percentage DNA in the tail was measured with a computer aided image analysis system (Comet IV, Perceptive Instruments Ltd., Burry St. Edmunds' UK). 50 nuclei were evaluated randomly from each slide (150 nuclei per organ/animal). A schematic overview on the steps of the SCGE experiment can be seen in Fig. 4.

2.9 Histopathological analyses of organs using HE-staining

Organs which yielded significant results in SCGE experiments (liver, kidneys, colon and testes) as well as the negative controls, were evaluated for histopathological examinations. After dehydration, tissues were embedded in paraffin wax and 2 µm thick sections were cut and stained with hematoxylin and eosin (HE). The histopathological evaluation was done under blinded conditions.

2.10 Micronucleus experiments

In vivo MN assays were conducted according to OECD guideline #474. A schematic overview on the different steps of the bone marrow MN experiment can be seen in Fig. 5.

After sacrificing the animals, bone marrow was extracted from both femurs; bone marrow cells were collected by flushing each femur with 0.3 mL FBS. Subsequently, the cell suspensions were centrifuged (400 *g*, 10 minutes, 37°C). Then the supernatants were removed and the pellets were resuspended in 1.5 µL FBS. Subsequently, a cytospin channel centrifugation was performed (600 *g*, 100 µL per spot, 5 minutes). After drying of the slides, fixation was conducted at 4°C with 80% cold methanol for 1 h. The slides were then stained with 10% Giemsa (Fluka Chemie GmbH, Buchs, Switzerland) for 20 min, air-dried and coded [76].

The MN rates were determined in 2000 PCE per animal, using a brightfield optical microscope (magnification of 1000 x, oil immersion, Nikon Optiphot-2, Tokyo, Japan). The ratio of PCE to total erythrocytes was evaluated in 500 cells per animal.

Treatment of the animals: 14 days (150, 400, 1000 mg/kg bw. day), neg. control, pos. control (EMS 300 mg/kg)

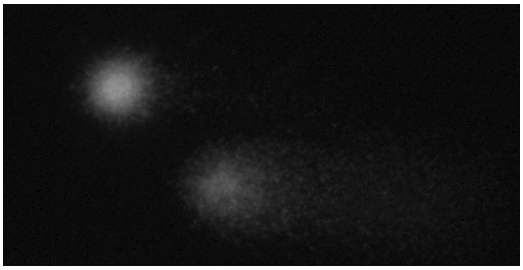
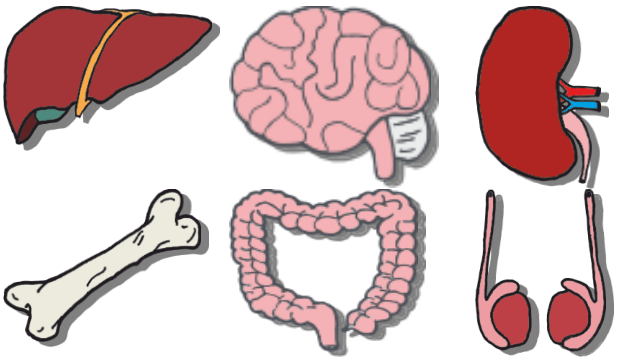
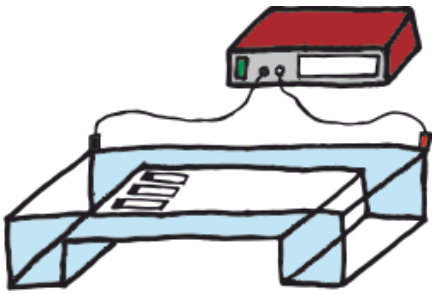


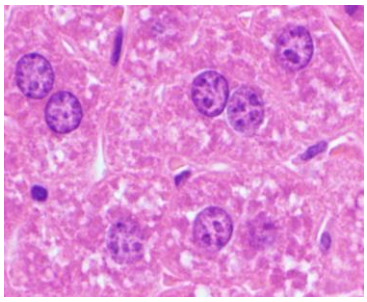
Image analysis with a computer aided image analysis system and statistical evaluation



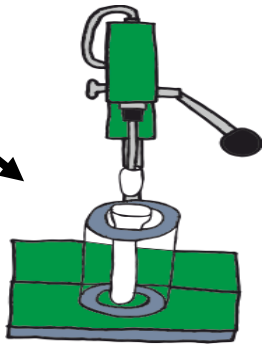
Removal of inner organs



Unwinding and electrophoresis at pH ≥ 13, 1.0 V/cm, 30 min



Histopathological examination



Homogenization of tissues (550 rpm, 5 sec.) with a Potter Elvehjem-type homogenizer

Figure 4: Schematic overview on different steps of SCGE assays. 1. Mice were treated with glyphosate in drinking water (treatment time = 14 days, 150, 400 and 1000 mg/kg bw. per day). 2. Organs (liver, brain, kidney, bone marrow, colon, testes) were removed. 3. Liver, kidney, brain and testes were homogenized; colon cells were directly scratched from the mucosa; bone marrow cells were obtained by flushing the femurs. 4. Parts of the organs were collected for histopathological examinations. 5. Electrophoresis was carried out for 30 min (1.0 V/cm). 6. Slides were evaluated with an automated image analysis system.

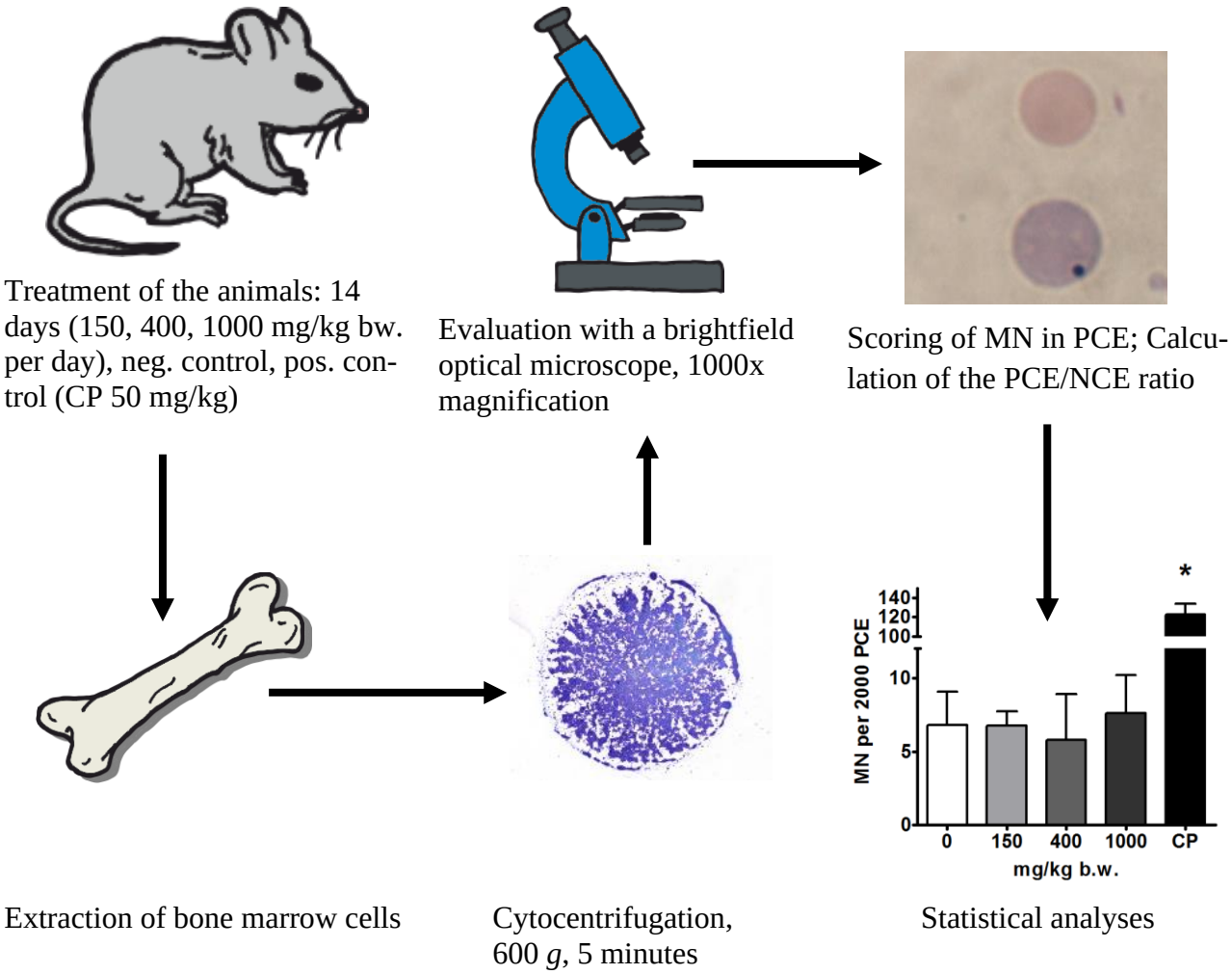


Figure 5: Schematic overview on the steps of bone marrow MN experiments. 1. Mice were treated with glyphosate in drinking water (treatment time = 14 days, 150, 400 and 1000 mg/kg bw. per day). 2. bone marrow cells were collected by flushing the femurs with PBS. 3. Cytocentrifugation was performed at 600 *g*, with 100 µL per spot for 5 minutes. 4. MN rates as well as ratios of PCE/NCE were determined after evaluation of the cells with a brightfield optical microscope (1000x magnification, oil immersion).

2.11 Statistical analyses

Due to the nature of data obtained in SCGE experiments, the median percentage of DNA in tails for each slide were determined and the means of the median values were calculated for each animal according to OECD Guideline #489 [30]. Statistical analyses of SCGE experiments were conducted using one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. For MN experiments, the Mann-Whitney-U Test was used. For all comparisons, P-values ≤ 0.05 were considered significant. Statistical analyses were performed using Graphpad Prism (PRISM 5, Version 5.02, 2008, San Diego, CA, USA).

3. RESULTS

The following chapters describe the weight gain and daily water intake of the animals in both studies (chapter 3.1), the findings of the main study (chapter 3.2) and of the confirmation studies (chapter 3.3). The last paragraph contains information about the results of the histopathological analyses.

3.1 Weight gain and water consumption

Tables 7 and 8 summarize information concerning alterations of the body weights of the animals after supplementation of the drinking water with glyphosate and also alterations of water intake and liver weights. It is evident that the daily water intake was not altered after supplementation with different amounts of the herbicide; also the body weights were not affected. Liver weights were slightly increased (by 7.5%) in the group which consumed the highest dose of the herbicide (1000 mg/kg bw. per day) after 14 days of the supplementation phase; however, this effect was statistically not significant.

Table 7: Water consumption and alterations of body and liver weights in the main study[1)]

Treatment (n = 5)	Daily water intake (mL)	Body weights at the start of the study (g)	Body weights at the end of the study (g)	Liver weights at the end of the study (g)
Controls	5.5 ± 0.6	28.2 ± 1.8	29.0 ± 1.4	1.7 ± 0.1
Group 1 (150 mg/kg bw. per day)	5.5 ± 0.4	28.0 ± 1.6	29.6 ± 2.3	1.8 ± 0.3
Group 2 (400 mg/kg bw. per day)	5.7 ± 0.5	28.2 ± 1.9	27.8 ± 2.3	1.6 ± 0.2
Group 3 (1000 mg/kg bw. per day)	6.0 ± 0.5	28.2 ± 1.8	28.8 ± 1.6	1.9 ± 0.1

[1)] Asterisks indicate statistical significance ($p \leq 0.05$, ANOVA followed by Dunnett's multiple comparison test) between comparative groups.

Table 8: Water consumption and alterations of body and liver weights in the follow up study[1)]

Treatment (n = 3)	Daily water intake (mL)	Body weights at the start of the study (g)	Body weights at the end of the study (g)	Liver weights at the end of the study (g)
Controls	5.6 ± 0.3	27.0 ± 1.0	27.3 ± 0.6	1.8 ± 0.2
Group 1 (400 mg/kg bw. per day)	5.6 ± 0.5	27.0 ± 1.0	28.0 ± 1.0	2.0 ± 0.3
Group 2 (1000 mg/kg bw. per day)	5.9 ± 0.5	26.7 ± 0.6	28. 7 ± 0.6	1.8 ± 0.1

[1)] Asterisks indicate statistical significance ($p \leq 0.05$, ANOVA followed by Dunnett's multiple comparison test) between comparative groups.

3.2 Results of the main study

3.2.1 Results of the Single Cell Gel Electrophoresis Assays

As described in Section 28, SCGE tests reflect formation of single and double-strand breaks and apurinic [31]. The results of the main experiment with mice, which consumed glyphosate supplemented drinking water for a period of 14 days are summarized in Figure 6 (A-F). EMS (dose: 300 mg/kg bw.) was used as a positive control. The animals were treated once with the alkylating agent *i.p.* and were sacrificed 3 hours after the treatment.

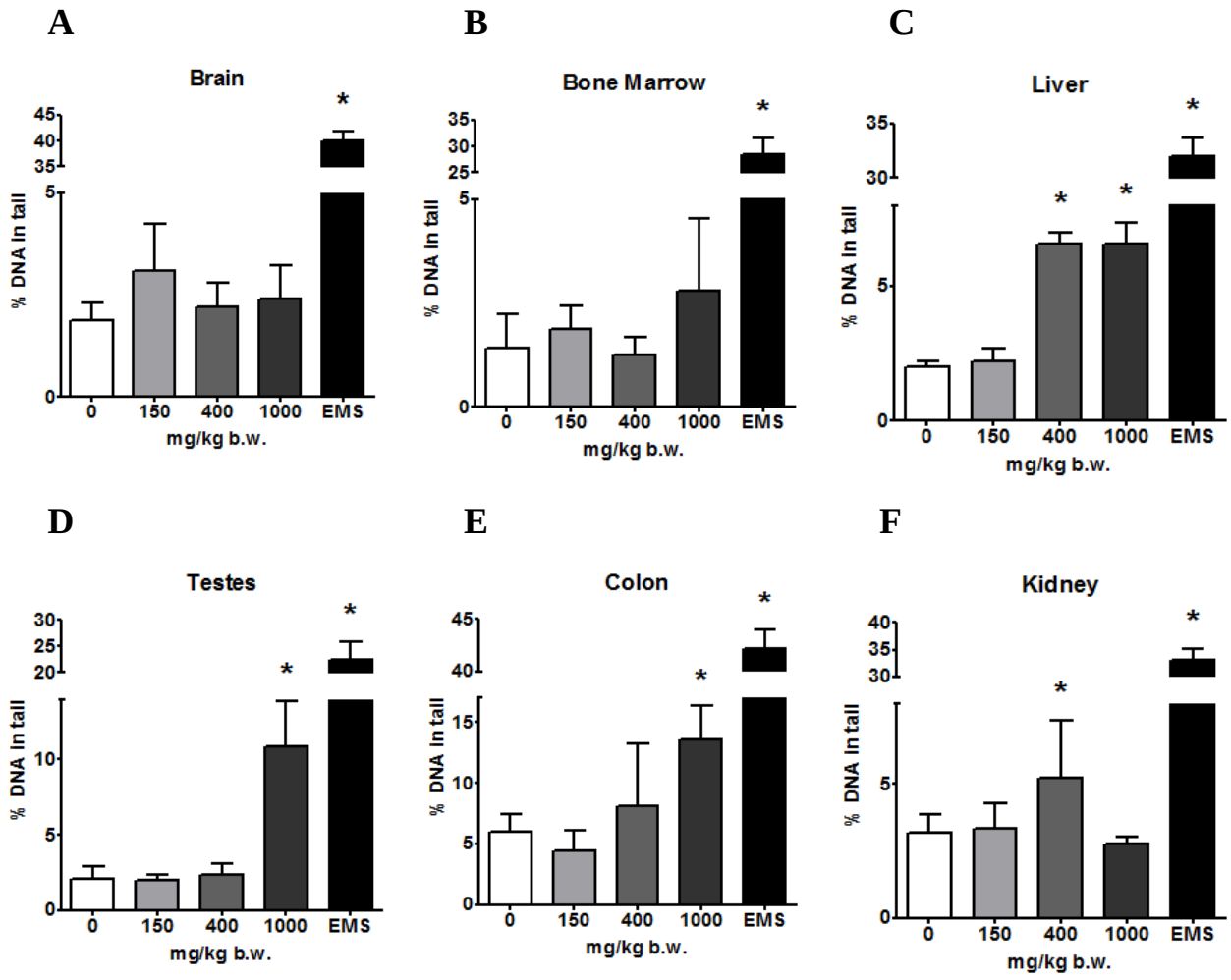


Figure 6: Induction of DNA migration in male BALB/c mice after consumption of glyphosate in drinking water (14 days, 150, 400 and 1000 mg/kg bw. per day). EMS (dose 300 mg/kg bw., *i.p.* administration) was used as positive control. After the animals were sacrificed, the organs were removed and DNA migration was monitored as described in Materials and Methods. From each organ, 3 slides were made and 50 cells were evaluated per slide; bars indicate means ± SD of results obtained with 5 animals per experimental group; asterisks indicate statistical significance ($p \leq 0.05$, ANOVA followed by Dunnett's multiple comparison test).

It can be seen that the herbicide caused no significant effects in brain and bone marrow cells while clear formation of comets was observed in liver, testes, colon and kidneys. Only in the colon a linear dose response was observed.

The strongest effect with the highest dose (1000 mg/kg bw.) was observed in the testes (428.6%) followed by the liver (226.9%) and colon (125.5%) (numbers in parenthesis indicate the increase of DNA migration in % compared to the control group). Only in two organs (kidneys and liver) clear induction of comet formation was found with a lower dose (400 mg/kg bw.), DNA damage was increased in these organs by 66.8 and 226.9% compared to the control group.

3.2.2 Histopathological findings

Figure 7 depicts results of histopathological examinations in organs of animals in which positive results were obtained in SCGE experiments. In the liver of all animals (A-B), glycogen was present in hepatocytes in a mild to moderate extent regardless of the treatment. No lesions indicative of acute toxicity (necrosis, apoptosis) were detectable in this organ. The kidneys of all animals were normal (C-D), necrosis and apoptosis were not seen. In the testes of two animals (1000 mg/kg bw. and negative control) slightly reduced spermatogenesis was observed in single seminiferous tubules (E-F). Testes of all other animals showed no lesions and no lesions indicative for acute toxicity (necrosis, apoptosis) were detectable. The colon of all animals (G-H) showed scattered lymphocytes in the lamina propria, the epithelia of all animals were normal; necrosis or apoptosis were not observed.

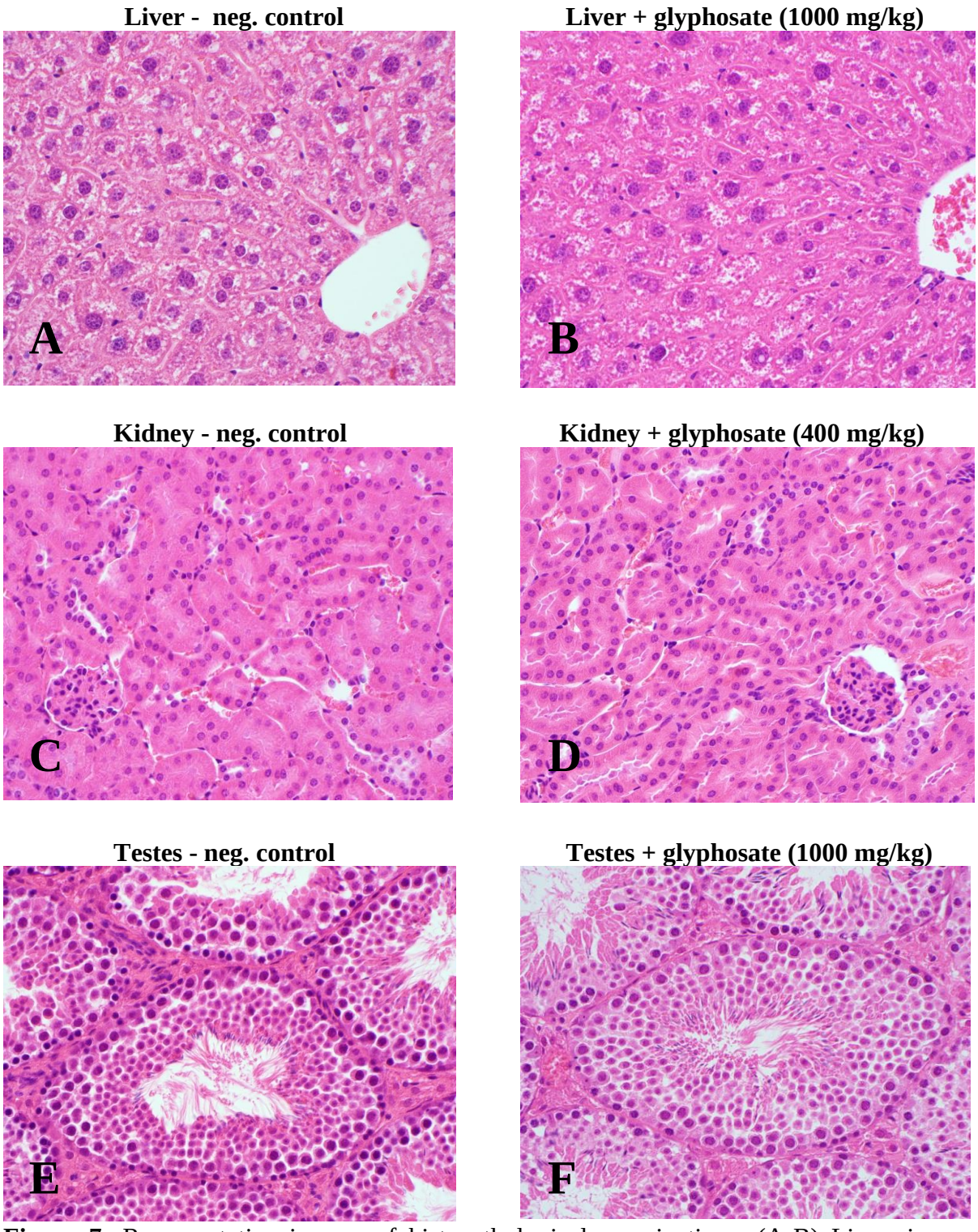


Figure 7: Representative images of histopathological examinations. (A-B) Liver; images of physiologic glycogen storage in hepatocytes of control and 1000 mg/kg bw. animals. HE-staining, magnification 400x. No lesions indicative of acute toxicosis (necrosis, apoptosis) were detectable in any liver. (C-D) Kidneys; representative images of normal tubules and a glomerulum in control in animals which were treated with 400 mg/kg bw. per day. HE-staining, magnification 400x. Necrosis or apoptosis were not present in any kidney. (E-F) Testes; representative images of normal seminiferous ducts in control and in animals which 1000 mg/kg bw. animals. HE-staining, magnification 400x. No lesions indicative of acute toxicosis (necrosis, apoptosis) were detectable. (G-H) Colon; representative images of normal epithelium of control and 1000 mg/kg bw. animals. HE-staining, magnification 400x. Necrosis or apoptosis were not detectable.

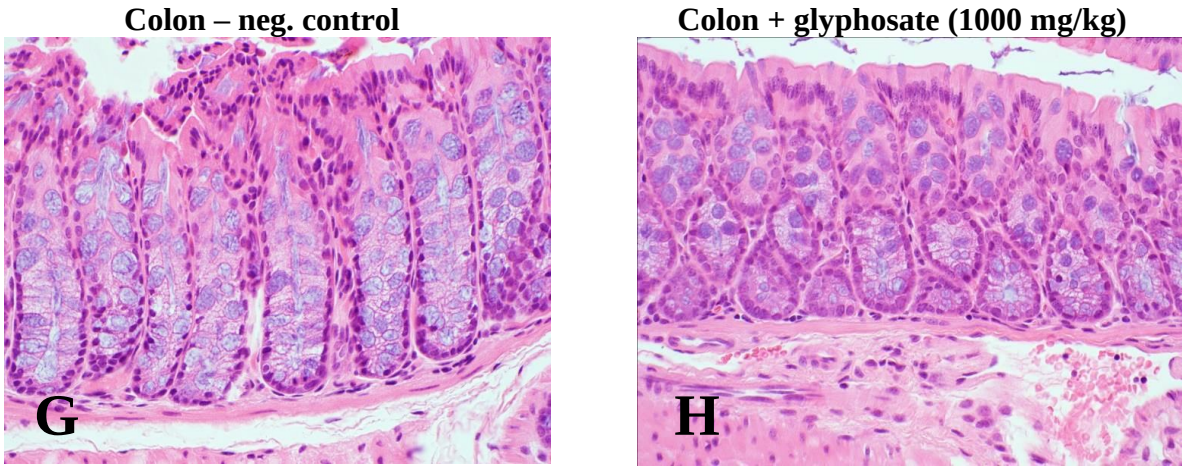


Figure 7: continued

3.2.3 Results of micronucleus experiments with bone marrow cells

The findings of MN experiments are summarized in Figure 8 A-B. It can be seen that the rates of MN in PCE (B) were in both experimental groups similar. Also, the ratio of PCE/total erythrocytes (A) were more or less identical.

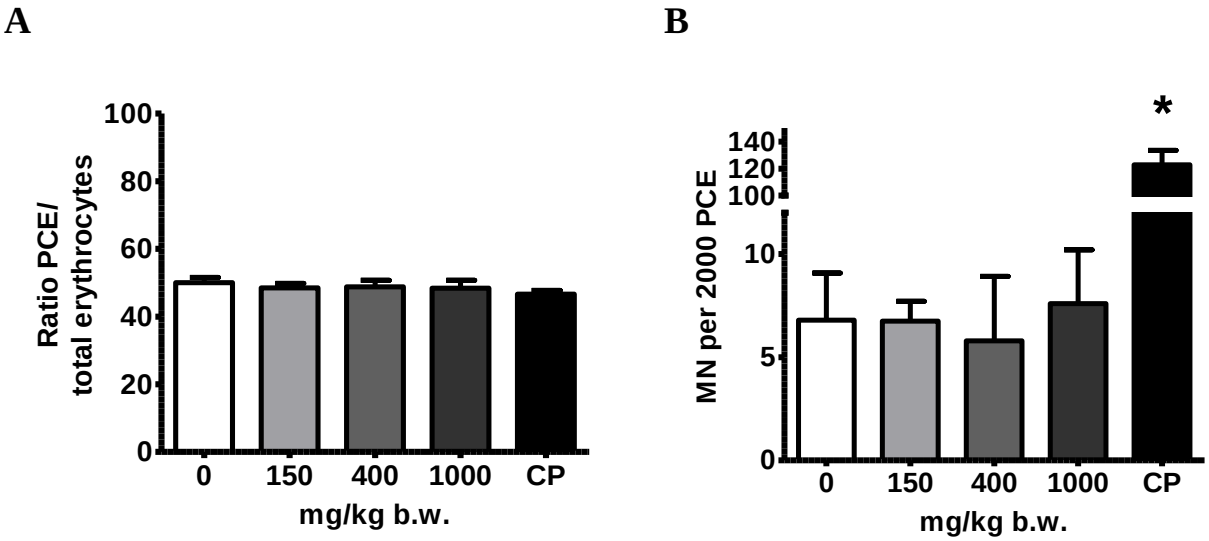


Figure 8: Impact of oral treatment with glyphosate of the ratio of PCE/total erythrocytes ratios (A) and on MN rates in PCE (B). The animals received the herbicide in drinking water (14 days, 150, 400 and 1000 mg/kg bw. per day). Bone marrow cells were isolated and processed as described in Materials and Methods (stain: 10% Giemsa). From each animal at least 2000 cells were evaluated. Bars indicate means ± SD of results obtained with 5 animals per experimental group; asterisks indicate statistical significance ($p \leq 0.05$, Mann-Whitney-U Test).

3.3 Results of the repeat experiment

According to the current OECD guideline [30], a clear positive result is obtained when at least one the tested doses exhibits a statistic significant increase and when the increase is dose related. The later criterium is not fulfilled in experiments with liver and kidneys, therefore we conducted a repeat experiment to confirm the initial findings. The results are summarized in Figure 9. In experiments with kidneys and liver, two groups of mice were treated with 400 and 1000 mg/kg bw. per day, in addition a negative and positive control was included. As in the main study, the animals were treated for a time period of 14 days and positive and negative controls were included. It can be seen that clear positive findings were obtained in both organs.

3.3.1 Results of the Single Cell Gel Electrophoresis assays

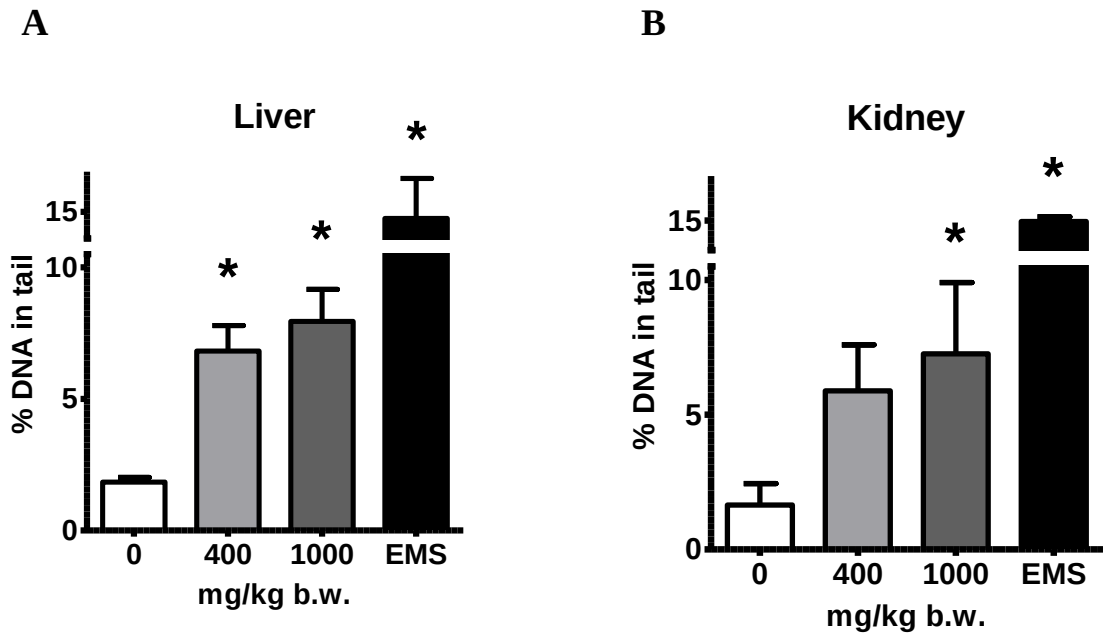


Figure 9: Induction of DNA migration in male BALB/c mice after consumption of glyphosate in drinking water (14 days, 400 and 1000 mg/kg bw. per day). EMS (dose 300 mg/kg bw., *i.p.* administration) was used as positive control. After the animals were sacrificed, the organs were removed and DNA migration was monitored as described in Materials and Methods. From each organ 3 slides were made and 50 cells were evaluated per slide; bars indicate means ± SD of results obtained with 3 animals per experimental group; asterisks indicate statistical significance ($p \leq 0.05$, ANOVA followed by Dunnett's multiple comparison test).

3.3.2 Results of micronucleus experiments with bone marrow cells

We evaluated also MN formation in the groups which had been tested with 400 mg/kg bw. per day and in the control group in the SCGE conformation study (see chapter 3.3.1). The results are summarized in Figure 10 A-B. It can be seen that the rates of MN in PCE (B) were in both experimental groups similar. Also, the ratio of PCE/total erythrocytes (A) were identical.

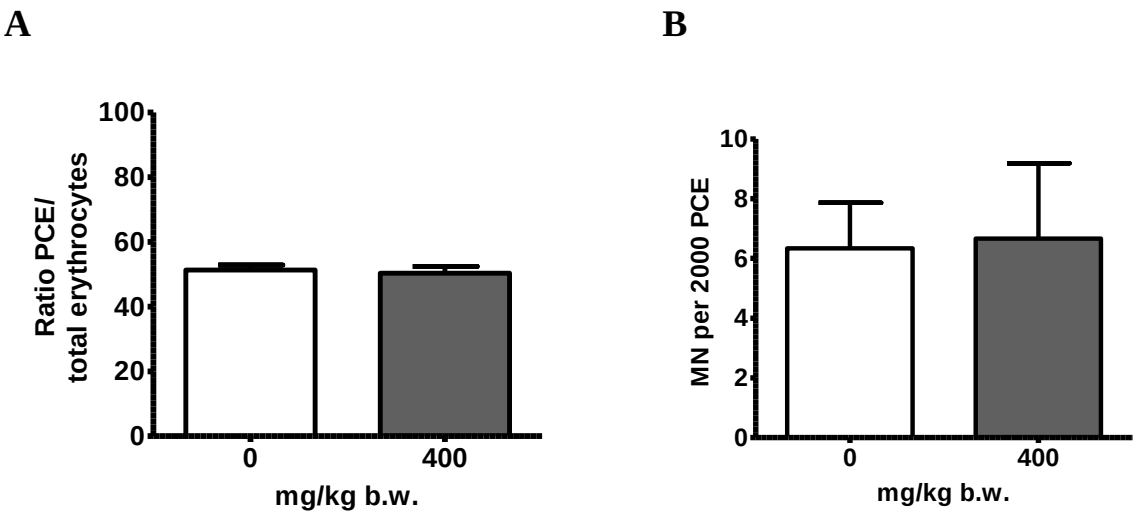


Figure 10: Impact of oral treatment with glyphosate of the ratio of PCE/total erythrocytes ratios (A) and on MN rates in PCE (B). The animals received the herbicide in drinking water (14 days, 400 mg/kg bw. per day). Bone marrow cells were isolated and processed as described in Materials and Methods (stain: 10% Giemsa). From each animal at least 2000 cells were evaluated. Bars indicate means ± SD of results obtained with 3 animals per experimental group; asterisks indicate statistical significance ($p \leq 0.05$, Mann-Whitney-U Test).

4. DISCUSSION

As mentioned above, this is the first SCGE study with glyphosate, which was realized in agreement with the current OECD guideline (#489). It is stated in this document that in addition to the liver, additionally organs of the gastro-intestinal tract should be included, as they come in direct contact with the test compound. This suggestion is also in agreement with a statement of the European Chemicals Agency (ECHA, 2015), which mentions that duodenum/jejunum should be evaluated [77]. We focused in the present study on colon cells, as we conducted several earlier investigations with this organ [78-80]. The following chapters describe (i) the impact of the supplementation of the drinking water with glyphosate on water consumption and body weights of the animals; (ii) histopathological findings in inner organs; (iii) results of SCGE experiments in inner organs of mice after treatment with different doses of the herbicide and (iv) results of MN experiments with bone marrow cells which were realized under identical conditions as the SCGE assays. (v) The final chapter summarizes the main findings and discusses the consequences of the results in regard to the classification of the compound as a mutagen and carcinogen.

4.1 Impact of supplementation of the drinking water and growth of the animals

We found no evidence for an effect, on water consumption (page 19, Table 7 and 8) in groups which were treated in the range between 150 and 1000 mg/kg bw. over a period of 14 days. This observation is in agreement with earlier observations; for example, Manas et al. [33] reported no difference between the consumption levels of groups which received only water and those which consumed glyphosate supplemented drinking water (40 & 400 mg/kg bw.) for a period of 14 days.

Also, the weights of the animals were not significantly affected by oral intake of glyphosate (page 19, Table 7 and 8). In this context, it is notable that Greim et al. [81] mention an industrial feeding study with Swiss Albino mice which received 1454 mg/kg bw. per day; no alterations of the body weight gains compared to the control group were observed after the feeding period (18 months).

The liver weights increased in the present investigation in all groups in a similar manner; and no differences were observed (page 19, Table 7 and 8). Also in a long-term carcinogenicity trial (NTP, 1992) with the same mouse strain which was tested in the present study no differences of the body weights were found after oral consumption in chow (highest dose 1500 mg/kg bw. per day, feeding time 13 weeks).

4.2 Results of histopathological analyses

In order to exclude that acute cytotoxic effects lead to false positive findings in SCGE experiments, it is mandatory to evaluate histopathological changes [46, 82]. The results of the evaluation of the histological slides after HE-staining are described in chapter 3.2.2. No evidence for cytotoxic effects was found in different organs in which glyphosate induced clear DNA damage.

Pathological alterations were also monitored in an older NTP-study with male and female B6C3F$_1$ mice (NTP, 1992) [83]. The animals received a maximal dose of 9710 mg/kg bw. per day glyphosate in the chow and no cytotoxic effects were seen in bone marrow, colon, liver, kidney, testes and brain and several other organs (which were not evaluated in the present study). Likewise, no effects were detected in a 18-month feeding study with CD-1 mice, which is described in a review of Greim et al. [81].

4.3 Results of SCGE experiments with different organs

It is notable that DNA migration which was observed in different organs of mice in the present study is in agreement with results of earlier SCGE studies. For example, similar results were reported in liver and kidneys of CD-1 mice by Prokopiev et al. [84]. Also, in C57BL6/J animals a similar extent of DNA migration as in the present study was reported by Setayesh et al. [78] in colon, liver and brain of untreated mice.

EMS was used in the study as a positive control. This agent is a cytostatic drug and causes ethylation of different DNA bases [85] and it is frequently used as a positive control in *in vitro* and *in vivo* experiments. The effects which we found in different organs are in agreement with earlier findings from rodent experiments in which similar exposure doses were used [86, 87].

We detected in the present main study significant induction of DNA damage in three out of six organs (liver, testes and colon) at the highest concentration (1000 mg/kg bw.) and in two out of six organs (liver and kidneys) with a lower dose (400 mg/kg bw.). In bone marrow and also in the brain negative results were obtained under all experimental conditions.

According to the current OECD guideline [30], a clear positive result is obtained when (i) at least one does is significantly higher as the negative control, (ii) the effect is dose-related (and shows a linear trend) and (iii) "any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations".

In order to confirm the findings in liver and kidneys a repeat experiment was conducted (as suggested by the OECD) in which the animals were treated with the higher doses (400 and

1000 mg/kg bw. per day) for the same time period as in the main experiment (14 days). The results confirmed the observations of the first experiment (see Fig. 9).

The most pronounced effect was observed with the highest dose in the testicular tissue (5.3-fold increase over the background) followed by the liver (3.3-fold) and colon (2.4-fold). In the kidneys only a moderate effect was seen which reached significance at the intermediate dose (400 mg/kg).

According to our knowledge, only liver and lymphocytes were studied in earlier SCGE experiments with rodents. Manas et al. [33] reported a clear effect in hepatic tissue in mice after treatment with 400 mg/kg while no effect was seen with a lower dose (40 mg/kg). The effect which was detected was less pronounced as that found in the present study. Also in a rat study clear formation of comets was observed in hepatic tissue (dose 10 mg/kg bw. per day) after oral administration; while the effects in leucocytes depended on the parameter (tail length vs. tail intensity) [42].

Several other findings indicate that the liver and kidneys are target organs for the induction of DNA damage by glyphosate. For example, Bolognesi et. al. [32] found DNA damage in alkaline elution experiments after *i.p*. administration (300 mg/kg) in the liver of mice and Peluso et al. [36] detected increased levels of DNA adducts in hepatic tissue of mice after *i.p.* injection of 600 mg/kg Roundup. Furthermore, it is notable that a study with liver-derived human cells (HepG2) detected comet formation by the pure herbicide [88]. Also, with a formulation (Roundup), positive findings were obtained with these cells [89]. Only one study was published which concerned DNA damage in kidneys by glyphosate and positive findings were seen mice after *i.p*. administration of 300 mg/kg in the alkaline elution method [32]; also the levels of 8-OHdG were clearly increased after the treatment. Furthermore, evidence for formation of ^{32}P-adducts was detected in the same organ when the animals were exposed *i.p*. to Roundup [36].

According to our knowledge no further results from genotoxicity experiments with cells from other organs are currently available. The positive *in vivo* findings which we obtained in testes indicate that also sperm cells may be affected. In this context, it is notable that a few studies have been published, which concerned the impact of glyphosate on sperm. Anifandis et al. [90] published an investigation in which they treated human sperm cells *in vitro* with high toxic doses; no sperm motility and no impact on DNA integrity (fragmentation) was observed; on the other hand, a clear positive result was obtained in an *in vivo* feeding study with rats in which oral exposure to the herbicide caused significant DNA migration in SCGE assays [41]; furthermore, it led to abnormal sperm morphology and increased the methodological MDA levels which are indicative for oxidative stress. Also in experiments with fruit flies, a clear positive effect was found in Sex-Linked Recessive Lethal assays with two formulations of glyphosate (Roundup and

Pondmaster) [91]. This test procedure detects lethal mutations in larval spermatocytes and spermatogonia.

SCGE assays were also conducted in a number of studies with aquatic organisms, reptiles, amphibians, birds and plants. The results of some investigations are described in Table 9. In most of them, positive results were obtained in a variety of different target cells.

Table 9: Results of selected SCGE experiments with non-mammalian indicator species.

Species	**Dose, time (h)**	**Target cells**	**Results[1)] LOEC**	**Remarks**	**Ref**
Aquatic species					
Bone fish (*Prochilodus lineatus*)	0.5 and 2.4 mg/L, negative control; 6, 24 and 96h	erythrocytes and gill cells	↑ gill cells with 0.5 and 2.4 mg/L only at 24h; ↑ LOEC erytrocytes with 0.5 and 2.4 mg/L at 6h and 24h	blood and gill cells responded different to DNA damage	Moreno et al. [92]
Zebrafish larvae (*Danio rerio*)	1.7, 5.0, 10.0, 23, 50 and 100 mg/L; 96h	single cells from zebrafish larvae (*in vitro*)	↑ 1.7 mg/L	no mortality was observed in zebrafish early-life stage with different concentrations (1.7 –100 mg/L)	Rodrigues et al. [93]
Tilapia (*Oreochromis niloticus*)	0.7, 7.0, 70 and 700 µM; 20h	erythrocytes	↑ genotoxicity at ≥ 7.0 µM	the increase in DNA migration was proportional to the glyphosate concentration; *in vivo*, the response was not concentration dependent	Alvarez-Moya et al. [94]
Oyster spermatozoa (*Ostrea edulis*)	0.5; 1.0; 1.5; 2.5; 5.0 µg/L 10.0 mL of sperm at 17 °C; 1h	spermatozoa	↔	out of three independent embryo-larval bioassays, only one assay revealed that glyphosate causes embryotoxic effect at a concentration of 2.5 µg/L	Akcha et al. [95]

Table 9: continued

Species	Dose, time (h)	Target cells	Results[1)] LOEC	Remarks	Ref
Reptiles					
Caiman eggs (*Caiman latirostris*)	Roundup: 50 to 1750 µg/egg; 70 days (*in ovo*)	erythro-cytes	↑ at ≥ 500 µg/egg	formulated product may not be safe, surfactants may considerably increase the toxicity	Poletta et al. [96]
Lizard eggs (*Salvator merianae*)	Roundup: 50 to 1600 µg/egg; 60 days (*in ovo*)	erythro-cytes	↑ at ≥ 200 µg/egg	no significant effects were detected in MN and nuclear abnormality assays	Schaumburg et al. [97]
Amphibians					
Bullfrog (*Rana catesbeiana*)	Roundup: 1,69, 6,75 and 27 mg/L; 24h	erythro-cytes	↑ at 6.75 and 27 mg/L	strong linear correlation between DNA damage and dose	Clements et al. [98]
frog (*Eleutherodactylus johnstonei*)	Roundup: 0.5, 0.9, 1.3 and 1.7 µg a.e./cm^2; 0.5, 1, 2, 4, 8, 16 and 24h	erythro-cytes	↑ ≥ 0.5 µg a.e./cm^2 at 1h and more	DNA damage did not increase with the duration of exposure – the highest percentage DNA in tail was seen after 8h	Meza-Joya et al. [99]
Birds					
Rooster (*Gallus gallus domesticus*)	50, 75, 100 and 125 mg/kg bw.; 45 days	blood lympho-cytes	↔	higher concentrations caused different clinical signs: ruffled feathers, tremors, anemic wattle and comb and reduced the frequency of crowing	Hussain [100]
Plants					
Tradescantia clone 4430 (*hybrid hirsutrifolia X acaulis*)	0.7, 7.0, 70 and 700 µM; 3h	stamen hair cells	↑ ≥ 0.7 µM	the genotoxic response was not proportional to the concentrations tested	Alvarez-Moya et al. [101]

[1)] ↑ significant increase; ↔ no changes; LOEC, lowest observed effective concentration

4.4 Results of micronucleus experiments

The findings of the present MN experiments show clearly that glyphosate does not cause a positive effect in PCE. The background levels which we found in untreated control animals are similar to those reported by other authors [102, 103].

CP was used in these experiments as a positive control as suggested in the OECD guideline #474 [28] and caused clear effects; i.e. the MN rates were 18-fold higher as in the controls. The compound was used in several earlier studies and similar results were reported [104, 105].

Our results are in agreement with industrial studies which were used by EFSA for the classification of glyphosate. The agency evaluated in total 9 rodent trials; only one reported a weak positive effect in females, another study found MN induction with 600 mg/kg bw. per day, but the MN rates were in the range of the historical controls. These studies are listed in Table 10 which describes also findings of published studies (included in the IARC monograph 112). It is notable that a Brazilian group published a meta-analysis of MN studies with a variety of different species [106]. The authors evaluated in total 81 studies and come to the conclusion that their results support the assumption that exposure to the herbicide and its formulations increases the MN frequencies. However, no such effects were detected when the compound was administered orally. A comprehensive list of bone marrow MN studies with rodents can be found in a review article of Kier and Kirkland [37]. The majority of investigations were conducted with mice (in total 11), only one used rats. With one exception, the results were clearly negative, only one study was positive, but the result was criticized, since MN rates were unusually high and historical control data were not presented.

Table 10: Results of selected MN experiments with glyphosate with bone marrow cells

Species	**Dosage**	**Results[1)] LOEC**	**Reference, study identification, owner**
Results of unpublished industrial studies (EFSA – final addendum, https://echa.europa.eu/documents/10162/13626/renewal_assessment_report_addenda_en.pdf)			
CD-1 Mice, 7 males	0, 150, 300, 600 mg/kg bw., single *i.p*. dose; sampling after 24 and 48h	↑ 600 mg/kg bw. (24h) within historical control	2006; ASB2012-11478; Nufarm
NMRI Mice, 5 males	0, 2000 mg/kg bw., single oral dose; sampling after 24 and 48h, 500 & 1000 mg/kg bw. sampling only after 24h	↔	2008; ASB2012-11483; Syngenta
CD-1 Mice, 5 males and 5 females	0, 5000 mg/kg bw., single oral dose; sampling after 24 and 48h	↔	1996; TOX2000-1996; Syngenta
Swiss Albino Mice, 6 males	0, 2000 mg/kg bw., two oral injections (24h interval); sampling after 24h	↔	2012; ASB2014-9277; Dow
Swiss Albino Mice, 5 males and 5 females	0, 50, 500, 5000 mg/kg bw./day, daily oral applications for 2 successive days; sampling 24h after second dose	males: ↔ females: weakly positive at highest dose	1993b; TOX9551100; ADAMA

Table 10: continued

Species	Dosage	Results[1)] LOEC	Reference, study identification, owner
Results of unpublished industrial studies (EFSA – final addendum, https://echa.europa.eu/documents/10162/13626/renewal_assessment_report_addenda_en.pdf)			
NMRI Mice, 5 males and 5 females	0 – 5000 mg/kg bw.; single *i.p.* dose; sampling after 24, 48, 72h	↔	1991c; TOX9552374; Cheminova
NMRI Mice, 7 males	0, 2000 mg/kg bw., single oral dose; sampling after 24 and 48h	↔	2012; ASB2014-9333; Syngenta
CD Rats, 5 males and 5 females	0, 500, 1000, 2000 mg/kg bw./day, single oral application; sampling after 24 and 48h	↔	2009b; ASB2012-11479; Helm
Results of selected published MN experiments with rodents			
4 Balb/C Mice	100, 200 and 400 mg/kg bw., two *i.p.* injections (24h interval)	↑ (with 400 mg/kg)	Manas et al. [88]
7-10 NMRI-Bom Mice	100, 150 and 200 mg/kg, single *i.p.* injection	↔	Rank et al. [107]
3 Swiss CD1 male Mice	300 mg/kg, two *i.p.* injections (24h interval)	↑	Bolognesi et al. [32]

[1)] ↑ significant increase; ↔ no changes; LOEC, lowest observed effective concentration

Notably, a number of further MN studies was realized with non-mammalian indicator organisms, for example with fishes [108, 109], reptiles [96, 110] and amphibians [111, 112]. In many of them, positive results were obtained, however, they were realized with formulations and not with pure glyphosate, therefore it is unclear if the results can be attributed to the herbicide.

The results of human comet- and MN biomonitoring studies were evaluated by Kier in 2015 [113]. The author discusses results of 19 genotoxicity studies with humans and all of them, except two reported positive results. Induction of MN was found in 4 trials in lymphocytes and in 3 trials in buccal cells; furthermore, DNA damage was detected in SCGE experiments with buccal cells in 3 investigations. Nevertheless, the authors state that the results "do not contradict conclusions from earlier experimental genotoxicity studies that typical glyphosate-based formulations (GBF) do not appear to present significant genotoxic risk…". The authors stress that most studies are not informative as the exposure levels to glyphosate-based formulations were either low, or as the participants were exposed additionally to other herbicides. Indeed, no study has been published so far, in which workers which are exposed solely to herbicide for example at production sites.

Recently, a study was published in Mexico [114] in which MN rates were evaluated in lymphocytes of glyphosate exposed agricultural workers. The authors found higher numbers of MN

as in the controls, but this effect did not reach significance. On the contrary, a clear increase of nuclear buds and nucleo-plasmatic bridges were detected. No firm conclusions can be drawn from these findings, as the corresponding rates of the nuclear anomalies, which were found in controls, were in all cases 0 per 1000 cells (which is in strong contrast with other findings; in most established investigations 3-12 MN were found per 1000 cells in non-exposed individuals [115]).

4.5 Relevance of the present findings and conclusions

The results of the present study contribute substantially to the current discussion concerning carcinogenic properties of glyphosate. As mentioned in the introduction, BfR and EFSA classified the herbicide as non-carcinogenic and non-mutagenic [11], while IARC placed it in category 2A ("Probably carcinogenic to humans") and came to the conclusion that it is mutagenic [116]. It is generally excepted that induction of DNA damage is a key mechanism of malignant transformation of cells [1] therefore, it is of high relevance, to clarify if a chemical is DNA-reactive or not. As mentioned in the introduction, the conclusion of the BfR/EFSA is predominantly based on negative results, which were obtained in bacterial gene mutation assays and in chromosomal aberration analyses and MN tests with bone marrow cells of rodents. Positive *in vivo* findings were not included in the evaluation due to methodological shortcomings; i.e. it was stated that it can be not excluded that they are due to acute cytotoxic effect [35].

The present experiments were conducted in agreement with the current OECD guidelines #489 and #474 for SCGE- and MN experiments [28, 30]. The results show that the compound causes significant DNA damage in four out of six inner organs. Positive findings were also obtained in an earlier SCGE study with mice in the liver [33], but no positive controls were included and only two doses were tested. In a newer investigation [42], mice were treated with four doses and with a positive control and clear effects were seen in hepatic tissue. However, none of these investigations studied cytotoxic effects which can be detected in histopathological analyses. Our findings show that the induction of DNA damage which was found in testes, liver, colon and kidneys, is not caused by cytotoxicity. Negative results were obtained in the present SCGE experiments only in brain and bone marrow. This observation is interesting as it indicates that PCE from bone marrow, which are used in MN experiments, are not a target for DNA damage of glyphosate. This assumption is also supported by the negative findings which were obtained in the present experiments in MN tests with bone marrow cells. These assays were conducted under identical experimental conditions, as the SCGE experiments.

Our findings indicate that the classification of glyphosate on the basis of the experimental evidence, which was used by BfR/EFSA, should be reconsidered. The question if glyphosate is a

DNA-reactive carcinogen is also relevant in regard to the definition of exposure limits, as it is generally assumed that genotoxins have no- or only very low thresholds [117]. Further investigations are needed to study its dose response characteristics and to find out, if the induction of DNA damage is caused by direct attack of the genetic materials as indicated by the observation of ^{32}P-adducts in hepatic tissue by Roundup [36], or via indirect modes of actions such as alterations of the redox status which was reported by Milic et al. [42].

It is mentioned in a critical review of an expert panel (several of them were sponsored by Monsanto) that MN tests with bone marrow cells are more reliable than SCGE experiments [39]. This assumption is possibly based on the fact that MN reflect structural and numerical chromosomal aberrations which are known to play a key role in carcinogenesis [65, 118, 119]. However, recent comparisons of the validity of MN tests and comet assays with rodents for the detection of genotoxic garcinogens showed that the sensitivity of the later procedure for the detection of carcinogens exceeds clearly that of the MN test with bone marrow erythrocytes, which is at present the most widely used method for routine screening of new chemicals [60]. The comet assay was initially recommended as a follow up of a negative or equivocal *in vivo* MN assay and as a mean to measure genotoxicity in target tissues other than the liver [120, 121]. Evaluation of the reliability of the *in vivo* MN assay in regard to the detection of rodent carcinogens indicated that the sensitivity and specificity are 40.0% and 60.5% respectively (data on 293 carcinogens and 86 non-carcinogens were included in this study) [122]. In an earlier comprehensive collaborative study, chemicals, which were classified by the IARC as group 1, 2A and 2B, were compaired in regard to their effects in MN experiments and positive findings were obtained with 68.6, 54.5 and respectively 45.6% [123]. More promising results were found in validation studies in which comet formation was used as an endpoint. A recent Japanese investigation with genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens and non-genotoxic non-carcinogens showed that with 82.5% of the tested chemicals (33 of 40) the expected results were obtained in SCGE experiments with cells from the liver; the corresponding value for the stomach was 82% [82]. The high sensitivity of *in vivo* SCGE experiments is also mentioned in a report of the 7th international Workshop on Genotoxicity Testing (IWGT). The authors evaluated results that were obtained with 78 carcinogens and 87% of them gave positive results in comet experiments with rodents [124]. These recent comparisons indicate that SCGE experiments *in vivo* (in the liver), are more reliable as MN experiments with bone marrow cells.

In the last years substantial evidence accumulated which indicates that glyphosate causes damage in the genetic material in the liver and also in other tissues but not in the bone marrow. This assumption is also confirmed by the results of the present study and the health agencies should therefore reconsider their decision in the light of the newer findings.

5. ABSTRACT

The genotoxic and carcinogenic properties of glyphosate, which is currently the most widely used herbicide worldwide, are controversially discussed. While the BfR and EFSA stated that the compound is not carcinogenic and not mutagenic, it was classified by IARC (WHO) as carcinogenic and DNA-reactive. The evaluation of the BfR/EFSA is based predominantly on negative results which were obtained in bacterial tests and micronucleus assays (MN) with bone marrow cells of mice which reflect chromosomal aberrations. Positive results of other *in vivo* experiments were not taken into consideration due to methodological shortcomings; i.e. it could be not excluded that these effects were caused by acute toxic properties.

Glyphosate was tested in the present study according to the current OCED guideline for induction of DNA damage in Single Cell Gel Electrophoresis assays (SCGE), which are based on the determination of DNA migration in an electric field in six inner organs of BALB/C mice after administration in drinking water for a period of 14 days. These assays detect single- and double strand breaks and apurinic sites. Additionally, MN tests were conducted under identical experimental conditions with bone marrow cells and histopathological examinations were conducted with different organs. The compound induced positive results in four organs (liver, testes, colon, kidneys); only in the colon a dose response was detected but positive findings were also obtained in a repeat experiment with kidneys and liver with the two higher doses. The evaluation of hematoxylin-eosin stained histological slides did not indicate cytotoxic damage. Therefore, it can be excluded that the results are due to cell damage. In MN experiments with bone marrow cells, no induction for chromosomal damage was observed in polychromatic erythrocytes and the ratio of poly- to normochromatic erythrocytes was not altered after administration of the compound. It is notable, that it was found also in earlier experiments that glyphosate causes DNA damage in liver and kidneys in SCGE experiments. However, these studies are not conclusive, since acute toxic effects were not studied.

In conclusion, the present experiments show, that glyphosate causes DNA damage in various inner organs, is not caused by acute toxicity, while no effects were found in bone marrow cells. The current experiments confirm the assumption of the IARC that the compound causes damage of the genetic material; since these effects lead to formation of malignant cells, they also support the assumption of carcinogenic properties of this chemical.

6. ZUSAMMENFASSUNG

Die kanzerogenen und gentoxischen Eigenschaften von Glyphosat, das derzeit am häufigsten verwendete Herbizid, werden kontroversiell diskutiert. Während das BfR und die EFSA die Substanz als nicht krebserregend und nicht mutagen einstuften, klassifizierte die IARC (WHO) das Herbizid als krebsauslösend und DNA schädigend. Die Bewertung der BfR/EFSA beruht vorwiegend auf Negativbefunden die in Bakterientests und in Kleinkerntests in Knochenmarkszellen von Mäusen erhalten wurden. Positive Ergebnisse anderer *in vivo* Studien wurden aufgrund methodischer Mängel als nicht aussagekräftig bewertet, da nicht auszuschließen ist, dass sie durch akut toxische Wirkungen verursacht wurden.

In der vorliegenden Studie wurde Glyphosat entsprechender geltender OECD Richtlinie auf Auslösung von DNA Schäden in sechs inneren Organen von Mäusen getestet. Zusätzlich wurden unter identen experimentellen Bedingungen Kleinkerntests mit Knochenmarkszellen durchgeführt. In den Experimenten wurden zusätzlich auch zytotoxische Effekte in histopathologischen Präparaten analysiert. Die Substanz induzierte positive Ergebnisse in vier Organen (Leber, Hoden, Dickdarm, Nieren); allerdings wurde im Dickdarm eine Dosiswirkungsbeziehung festgestellt. In einem Wiederholungsexperiment mit Nieren und Leber wurden mit den beiden höheren Dosen positive Resultate erhalten. Die Auswertung Hämatoxylin-Eosin gefärbter Gewebeschnitte ergab keine Hinweise auf Zelltoxizität. Im Kleinkerntest mit Knochenmarkszellen (polychromatische Erythrozyten) wurden keine Hinweise auf Auslösung von Chromosomenschäden gefunden. Das Verhältnis von polychromatischen Erythrozyten zu normochromatischen Erythrozyten wurde durch die Verabreichung der Substanz nicht beeinflusst. Auch in früheren SCGE Experimenten wurden Hinweise auf Auslösung von DNA Schäden in Leber und Niere detektiert. Diese Studien sind jedoch nicht aussagekräftig, da akut toxische Effekte nicht berücksichtigt wurden.

Zusammengefasst zeigen die Experimente, dass Glyphosat in diversen inneren Organen DNA Schäden verursacht, die nicht mit akut toxischen Wirkungen im Zusammenhang stehen. In Knochenmarkszellen löst die Substanz keine Schäden der Erbsubstanz aus. Die vorliegenden Ergebnisse unterstützen die Einstufung der IARC als erbsubstanzschädigend; da derartige Wirkungen zur Auslösung von Krebserkrankung führen, untermauern sie auch die Annahme krebserregende Eigenschaften dieser Verbindung.

7. LIST OF FIGURES

8. LIST OF TABLES

9. LIST OF ABBREVIATIONS

BfR	deutsches Bundesinstitut für Risikobewertung
CA	chromosomal aberration
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EMS	ethyl methanesulfonate
EPSP	5-enolpyruvyl-shikimate-3-phosphate-synthase
HB	homogenizing buffer
HPRT	hypoxanthine phosphoribosyl transferase
i.p.	intraperitoneal
IARC	International Agency for Research and Cancer
LMPA	low melting point agarose
LOEC	Lowest Observed Effect Concentration
MnPCE	micronucleus in polychromatic erythrocyte
NCE	normochromatic erythrocytes
NMPA	normal melting point agarose
OECD	Organisation for Economic Co-operation and Development
p.o.	peroral
PBS	phosphate buffered saline
PCE	polychromatic erythrocytes
SCGE	Single Cell Gel Electrophoresis
TK^r	thymidine kinase resistance
U.S. EPA	United States Environmental Protection Agency
UDS	Unscheduled DNA Synthesis
WHO	World Health Organization

10. REFERENCES

1. Hanahan, D. and R.A. Weinberg, *The Hallmarks of Cancer.* Cell, 2000. **100**(1): p. 57-70.
2. Ames, B.N., *The Detection of Chemical Mutagens with Enteric Bacteria*, in *Chemical Mutagens: Principles and Methods for Their Detection Volume 1*, A. Hollaender, Editor. 1971, Springer US: Boston, MA. p. 267-282.
3. Nandula, V.K., *Glyphosate resistance in crops and weeds: history, development, and management*. 2010: John Wiley & Sons.
4. Franz, J.E., M.K. Mao, and J.A. Sikorski, *Glyphosate: a unique global herbicide*. 1997: American Chemical Society.
5. Duke, S.O. and S.B. Powles, *Glyphosate: a once-in-a-century herbicide.* Pest Management Science: formerly Pesticide Science, 2008. **64**(4): p. 319-325.
6. Jaworski, E.G., *Mode of action of N-phosphonomethylglycine. Inhibition of aromatic amino acid biosynthsis.* Journal of Agricultural and Food Chemistry, 1972. **20**(6): p. 1195-1198.
7. Amrhein, N., et al., *The site of the inhibition of the shikimate pathway by glyphosate: II. Interference of glyphosate with chorismate formation in vivo and in vitro.* Plant physiology, 1980. **66**(5): p. 830-834.
8. BfR, *Bundesinstitut fuer Risikobewertung - Does Glyphosate cause cancer? https://www.bfr.bund.de/cm/349/does-glyphosate-cause-cancer.pdf*. 2015.
9. WHO, *IARC Monographs Volume 112: evaluation of five organophosphate insecticides and herbicides https://www.iarc.fr/wp-content/uploads/2018/07/MonographVolume112-1.pdf*. 2015.
10. RAR, *Final addendum to the renewal assessment report (RAR) on the active substance glyphosate prepared by the rapporteur Member State Germany in the framework of Commission Regulation (EU) No 1141/2010, compiled by EFSA in October 2015 https://gmwatch.org/files/Renewal_Assessment_Report_Glyphosate_Addendum1_RAR.pdf*. 2015.
11. EFSA, *Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2015.4302.* European Food Safety Authority Journal, 2015. **13**(11): p. 4302.
12. U.S.-EPA-Memorandum, *https://archive.epa.gov/pesticides/chemicalsearch/chemical/foia/web/pdf/103601/103601-125.pdf (Accessed on 04.03.2020)*. 18. February 1982.
13. U.S.-EPA-Memorandum, *https://archive.epa.gov/pesticides/chemicalsearch/chemical/foia/web/pdf/103601/103601-166.pdf (Accsessed on 04.03.2020)*. 10. February 1984.
14. Portier, C.J., et al., *Differences in the carcinogenic evaluation of glyphosate between the International Agency for Research on Cancer (IARC) and the European Food Safety Authority (EFSA).* J Epidemiol Community Health, 2016. **70**(8): p. 741-745.
15. Andreotti, G., et al., *Glyphosate Use and Cancer Incidence in the Agricultural Health Study*. JNCI: Journal of the National Cancer Institute, 2017. **110**(5): p. 509-516.
16. IARC, *International Agency for Research on Cancer Monographs Volume 112: evaluation of five organophosphate insecticides and herbicides.* World Health Organization, Lyon, 2015.
17. Duesberg, P. and R. Li, *Multistep Carcinogenesis: A Chain Reaction of Aneuploidizations.* Cell Cycle, 2003. **2**(3): p. 201-209.
18. OECD, T.N., *471: bacterial reverse mutation test.* OECD Guidelines for the Testing of Chemicals, Section, 1997. **4**.
19. Kirkland, D., et al., *Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: I. Sensitivity, specificity*

and relative predictivity. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2005. **584**(1): p. 1-256.
20. Ashby, J. and R.W. Tennant, *Chemical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP*. Mutation Research/Genetic Toxicology, 1988. **204**(1): p. 17-115.
21. Ames, B.N., J. McCann, and E. Yamasaki, *Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test.* Mutation Research/Environmental Mutagenesis and Related Subjects, 1975. **31**(6): p. 347-363.
22. OECD, T.N., *476: in vitro mammalian cell gene mutation test.* OECD Guidelines for the Testing of Chemicals, Section, 1997. **4**.
23. Aaron, C.S., et al., *Mammalian cell gene mutation assays working group report.* Mutation Research/Environmental Mutagenesis and Related Subjects, 1994. **312**(3): p. 235-239.
24. OECD, T.N., *473: In vitro mammalian chromosome aberration test.* OECD guidelines for the testing of Chemicals, section, 1997. **4**.
25. OECD, *Test No. 487: In vitro mammalian cell micronucleus test.* OECD Guidelines for Testing of Chemicals Section for Health Effects, 2014.
26. Ishidate, M., K.F. Miura, and T. Sofuni, *Chromosome aberration assays in genetic toxicology testing in vitro.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1998. **404**(1): p. 167-172.
27. Nesslany, F. and D. Marzin, *A micromethod for the in vitro micronucleus assay*. Mutagenesis, 1999. **14**(4): p. 403-410.
28. OECD, *Test No. 474: Mammalian Erythrocyte Micronucleus Test*. 2016.
29. Hayashi, M., *The micronucleus test—most widely used in vivo genotoxicity test—*. Genes and Environment, 2016. **38**(1): p. 18.
30. OECD, *Test No. 489: In Vivo Mammalian Alkaline Comet Assay*. 2016.
31. Singh, N.P., et al., *A simple technique for quantitation of low levels of DNA damage in individual cells.* Experimental Cell Research, 1988. **175**(1): p. 184-191.
32. Bolognesi, C., et al., *Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup.* Journal of Agricultural and Food Chemistry, 1997. **45**(5): p. 1957-1962.
33. Mañas, F., et al., *Oxidative stress and comet assay in tissues of mice administered glyphosate and ampa in drinking water for 14 days.* BAG. Journal of basic and applied genetics, 2013. **24**: p. 67-75.
34. RMS-Germany, *Glyphosate Addendum 1 to RAR - Assessment of IARC Monographs Volume 112 (2015): Glyphosate*. 2015.
35. Williams, G., R. Kroes, and I. Munro, *Safety Evaluation and Risk Assessment of the Herbicide Roundup and Its Active Ingredient, Glyphosate, for Humans.* Regulatory toxicology and pharmacology : RTP, 2000. **31**: p. 117-65.
36. Peluso, M., et al., *32P-postlabeling detection of DNA adducts in mice treated with the herbicide roundup.* Environmental and Molecular Mutagenesis, 1998. **31**(1): p. 55-59.
37. Kier, L.D. and D.J. Kirkland, *Review of genotoxicity studies of glyphosate and glyphosate-based formulations.* Critical Reviews in Toxicology, 2013. **43**(4): p. 283-315.
38. Portier, C.J., *A comprehensive analysis of the animal carcinogenicity data for glyphosate from chronic exposure rodent carcinogenicity studies.* Environmental Health, 2020. **19**(1): p. 18.
39. Brusick, D., et al., *Genotoxicity Expert Panel review: weight of evidence evaluation of the genotoxicity of glyphosate, glyphosate-based formulations, and aminomethylphosphonic acid.* Critical reviews in toxicology, 2016. **46**(sup1): p. 56-74.
40. Williams, G.M., et al., *A review of the carcinogenic potential of glyphosate by four independent expert panels and comparison to the IARC assessment.* Critical Reviews in Toxicology, 2016. **46**(sup1): p. 3-20.

41. Avdatek, F., et al., *Ameliorative effect of resveratrol on testicular oxidative stress, spermatological parameters and DNA damage in glyphosate-based herbicide-exposed rats.* Andrologia, 2018. **50**(7): p. e13036.
42. Mirta, M., et al., *Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate.* Archives of Industrial Hygiene and Toxicology, 2018. **69**(2): p. 154-168.
43. Collins, A.R., et al., *The comet assay: topical issues.* Mutagenesis, 2008. **23**(3): p. 143-151.
44. Kumaravel, T.S., et al., *Comet Assay measurements: a perspective.* Cell Biology and Toxicology, 2009. **25**(1): p. 53-64.
45. Lovell, D.P. and T. Omori, *Statistical issues in the use of the comet assay.* Mutagenesis, 2008. **23**(3): p. 171-182.
46. Burlinson, B., et al., *Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2007. **627**(1): p. 31-35.
47. Neri, M., et al., *Worldwide interest in the comet assay: a bibliometric study.* Mutagenesis, 2015. **30**(1): p. 155-163.
48. Burlinson, B., *The in vitro and in vivo comet assays*, in *Genetic Toxicology*. 2012, Springer. p. 143-163.
49. Sasaki, Y.F., et al., *The alkaline single cell gel electrophoresis assay with mouse multiple organs: results with 30 aromatic amines evaluated by the IARC and U.S. NTP1This paper is dedicated to the memory of the late Prof. Kiyosi Tutikawa, National Institute of Genetics, Mishima (Japan).1.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 1999. **440**(1): p. 1-18.
50. Collins, A., et al., *Comet assay in human biomonitoring studies: Reliability, validation, and applications.* Environmental and Molecular Mutagenesis, 1997. **30**(2): p. 139-146.
51. Tice, R.R., et al., *Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing.* Environmental and Molecular Mutagenesis, 2000. **35**(3): p. 206-221.
52. Maronpot, R.R., C.A. Hobbs, and S.-M. Hayashi, *Role of pathology peer review in interpretation of the comet assay.* Journal of toxicologic pathology, 2018. **31**(3): p. 155-161.
53. Guilherme, S., et al., *DNA damage in fish (Anguilla anguilla) exposed to a glyphosate-based herbicide – Elucidation of organ-specificity and the role of oxidative stress.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2012. **743**(1): p. 1-9.
54. Leveroni, F.A., J.D. Caffetti, and M.C. Pastori, *Genotoxic response of blood, gill and liver cells of Piaractus mesopotamicus after an acute exposure to a glyphosate-based herbicide.* Caryologia, 2017. **70**(1): p. 21-28.
55. Kašuba, V., et al., *Effects of low doses of glyphosate on DNA damage, cell proliferation and oxidative stress in the HepG2 cell line.* Environmental Science and Pollution Research, 2017. **24**(23): p. 19267-19281.
56. Setayesh, T., et al., *Impact of Weight Loss Strategies on Obesity-Induced DNA Damage.* Molecular Nutrition & Food Research, 2019. **63**(17): p. 1900045.
57. Evans, H.J., G.J. Neary, and F.S. Williamson, *The Relative Biological Efficiency of Single Doses of Fast Neutrons and Gamma-rays on Vicia Faba Roots and the Effect of Oxygen.* International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine, 1959. **1**(3): p. 216-229.
58. Boller, K. and W. Schmid, *Chemische Mutagenese beim Säuger. Das Knochenmark des Chinesischen Hamsters als in vivo-Testsystem. Hämatologische Befunde nach Behandlung mit Trenimon.* Humangenetik, 1970. **11**(1): p. 35-54.
59. Heddle, J.A. and M.F. Salamone, *Chromosomal Aberrations and Bone Marrow Toxicity.* Environmental Health Perspectives, 1981. **39**: p. 23-27.

60. Nersesyan, A., et al., *Use of the lymphocyte cytokinesis-block micronucleus assay in occupational biomonitoring of genome damage caused by in vivo exposure to chemical genotoxins: Past, present and future.* Mutation Research/Reviews in Mutation Research, 2016. **770**: p. 1-11.
61. Fenech, M. and S. Bonassi, *The effect of age, gender, diet and lifestyle on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes.* Mutagenesis, 2011. **26**(1): p. 43-49.
62. Thomas, P., et al., *Buccal micronucleus cytome assay.* Nature Protocols, 2009. **4**(6): p. 825-837.
63. Knasmueller, S., et al., *Use of nasal cells in micronucleus assays and other genotoxicity studies.* Mutagenesis, 2011. **26**(1): p. 231-238.
64. Bonassi, S. and M. Fenech, *CHAPTER 4. Micronuclei and Their Association with Infertility, Pregnancy Complications, Developmental Defects, Anaemias, Inflammation, Diabetes, Chronic Kidney Disease, Obesity, Cardiovascular Disease, Neurodegenerative Diseases and Cancer*. 2019. p. 38-78.
65. Bonassi, S., et al., *Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies.* Mutagenesis, 2011. **26**(1): p. 93-100.
66. Bachmanov, A.A., et al., *Food intake, water intake, and drinking spout side preference of 28 mouse strains.* Behavior genetics, 2002. **32**(6): p. 435-443.
67. Kumaravel, T. and A.N. Jha, *Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2006. **605**(1-2): p. 7-16.
68. Suzuki, T., et al., *Genotoxicity assessment of intravenously injected titanium dioxide nanoparticles in gpt delta transgenic mice.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2016. **802**: p. 30-37.
69. Diab, K.A., et al., *Genotoxicity of carbon tetrachloride and the protective role of essential oil of Salvia officinalis L. in mice using chromosomal aberration, micronuclei formation, and comet assay.* Environmental Science and Pollution Research, 2018. **25**(2): p. 1621-1636.
70. Jeon, H.L., et al., *Development of a test method for the evaluation of DNA damage in mouse spermatogonial stem cells.* Toxicological research, 2017. **33**(2): p. 107-118.
71. Chen, R., et al., *Assessment of Pig-a, Micronucleus, and Comet Assay Endpoints in Tg. RasH2 Mice Carcinogenicity Study of Aristolochic Acid I.* Environmental and molecular mutagenesis, 2019.
72. Bright, J., et al., *Recommendations on the statistical analysis of the Comet assay.* Pharmaceutical statistics, 2011. **10**(6): p. 485-493.
73. Lund-HØie, K. and H.O. Friestad, *Photodegradation of the herbicide glyphosate in water.* Bulletin of environmental contamination and toxicology, 1986. **36**(1): p. 723-729.
74. Sasaki, Y.F., et al., *Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (Comet) assay1This paper is dedicated to the memory of the late Prof. Kiyosi Tutikawa, National Institute of Genetics, Mishima (Japan).1.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 1997. **388**(1): p. 33-44.
75. Sasaki, Y.F., et al., *The comet assay with 8 mouse organs: results with 39 currently used food additives.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2002. **519**(1): p. 103-119.
76. Krishna, G., G. Urda, and J.C. Theiss, *Comparative mouse micronucleus evaluation in bone marrow and spleen using immunofluorescence and Wright's Giemsa.* Mutation Research Letters, 1994. **323**(1): p. 11-20.
77. ECHA, *Evaluation Under REACH: Progress Report 2015. Safer Chemicals – Focussing on What Matters Most. Reference: ECHA-15-R-20-EN.* European Chemicals Agency, Helsinki, Finland (2016), 2015.

78. Setayesh, T., et al., *Gallic acid, a common dietary phenolic protects against high fat diet induced DNA damage.* European Journal of Nutrition, 2019. **58**(6): p. 2315-2326.
79. Ferk, F., et al., *Xanthohumol, a prenylated flavonoid contained in beer, prevents the induction of preneoplastic lesions and DNA damage in liver and colon induced by the heterocyclic aromatic amine amino-3-methyl-imidazo [4, 5-f] quinoline (IQ).* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2010. **691**(1-2): p. 17-22.
80. Ferk, F., et al., *Potent protection of gallic acid against DNA oxidation: results of human and animal experiments.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2011. **715**(1-2): p. 61-71.
81. Greim, H., et al., *Evaluation of carcinogenic potential of the herbicide glyphosate, drawing on tumor incidence data from fourteen chronic/carcinogenicity rodent studies.* Critical Reviews in Toxicology, 2015. **45**(3): p. 185-208.
82. Uno, Y., et al., *JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2015. **786-788**: p. 45-76.
83. Chan, P. and J. Mahler, *NTP technical report on the toxicity studies of Glyphosate (CAS No. 1071-83-6) Administered In Dosed Feed To F344/N Rats And B6C3F1 Mice.* Toxicity report series, 1992. **16**: p. 1-D3.
84. Prokopiev, I., et al., *Genotoxicity of (+)- and (−)-usnic acid in mice.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2019. **839**: p. 36-39.
85. Sega, G.A., *A review of the genetic effects of ethyl methanesulfonate.* Mutation Research/Reviews in Genetic Toxicology, 1984. **134**(2): p. 113-142.
86. Patel, S., et al., *Cypermethrin-induced DNA damage in organs and tissues of the mouse: Evidence from the comet assay.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2006. **607**(2): p. 176-183.
87. Mathew, J. and J. Thoppil, *Genotoxicity of methyl parathion and antimutagenic activity of Salvia Officinalis L. (Sage) extracts in Swiss albino mice.* Asian Journal of Pharmaceutical and Clinical Research, 2012. **5**: p. 164-170.
88. Mañas, F., et al., *Genotoxicity of glyphosate assessed by the comet assay and cytogenetic tests.* Environmental Toxicology and Pharmacology, 2009. **28**(1): p. 37-41.
89. Gasnier, C., et al., *Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines.* Toxicology, 2009. **262**(3): p. 184-191.
90. Anifandis, G., et al., *The Effect of Glyphosate on Human Sperm Motility and Sperm DNA Fragmentation.* International journal of environmental research and public health, 2018. **15**(6): p. 1117.
91. Kale, P.G., et al., *Mutagenicity testing of nine herbicides and pesticides currently used in agriculture.* Environmental and Molecular Mutagenesis, 1995. **25**(2): p. 148-153.
92. Moreno, N.C., S.H. Sofia, and C.B.R. Martinez, *Genotoxic effects of the herbicide Roundup Transorb® and its active ingredient glyphosate on the fish Prochilodus lineatus.* Environmental Toxicology and Pharmacology, 2014. **37**(1): p. 448-454.
93. de Brito Rodrigues, L., et al., *Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2019. **842**: p. 94-101.
94. Alvarez-Moya, C., et al., *Comparison of the in vivo and in vitro genotoxicity of glyphosate isopropylamine salt in three different organisms.* Genetics and Molecular Biology, 2014. **37**: p. 105-110.
95. Akcha, F., C. Spagnol, and J. Rouxel, *Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos.* Aquatic Toxicology, 2012. **106-107**: p. 104-113.
96. Poletta, G.L., et al., *Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (Caiman latirostris) evidenced by the Comet assay and the*

Micronucleus test. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2009. **672**(2): p. 95-102.
97. Schaumburg, L.G., et al., *Genotoxicity induced by Roundup® (Glyphosate) in tegu lizard (Salvator merianae) embryos.* Pesticide Biochemistry and Physiology, 2016. **130**: p. 71-78.
98. Clements, C., S. Ralph, and M. Petras, *Genotoxicity of select herbicides in Rana catesbeiana tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay.* Environmental and Molecular Mutagenesis, 1997. **29**(3): p. 277-288.
99. Meza-Joya, F.L., M.P. Ramírez-Pinilla, and J.L. Fuentes-Lorenzo, *Toxic, cytotoxic, and genotoxic effects of a glyphosate formulation (Roundup®SL–Cosmoflux®411F) in the direct-developing frog Eleutherodactylus johnstonei.* Environmental and Molecular Mutagenesis, 2013. **54**(5): p. 362-373.
100. Hussain, R., *Exposure to Sub-Acute Concentrations of Glyphosate Induce Clinico-Hematological, Serum Biochemical and Genotoxic Damage in Adult Cockerels.* Pakistan Veterinary Journal, 2019. **39**: p. 181-186.
101. Alvarez-Moya, C., et al., *Evaluation of genetic damage induced by glyphosate isopropylamine salt using Tradescantia bioassays.* Genetics and Molecular Biology, 2011. **34**: p. 127-130.
102. Holden, H.E., J.B. Majeska, and D. Studwell, *A direct comparison of mouse and rat bone marrow and blood as target tissues in the micronucleus assay.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 1997. **391**(1): p. 87-89.
103. Witt, K.L., et al., *Phenolphthalein: induction of micronucleated erythrocytes in mice.* Mutation Research/Genetic Toxicology, 1995. **341**(3): p. 151-160.
104. Bokulić, A., et al., *The effect of apigenin on cyclophosphamide and doxorubicin genotoxicity in vitro and in vivo.* Journal of Environmental Science and Health, Part A, 2011. **46**(5): p. 526-533.
105. Agrawal, R.C. and S. Kumar, *Prevention of Cyclophosphamide-induced Micronucleus Formation in Mouse Bone Marrow by Indole-3-carbinol11ITRC communication no. 1967.* Food and Chemical Toxicology, 1998. **36**(11): p. 975-977.
106. Ghisi, N.d.C., E.C.d. Oliveira, and A.J. Prioli, *Does exposure to glyphosate lead to an increase in the micronuclei frequency? A systematic and meta-analytic review.* Chemosphere, 2016. **145**: p. 42-54.
107. Rank, J., et al., *Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test.* Mutation Research/Genetic Toxicology, 1993. **300**(1): p. 29-36.
108. Çavaş, T. and S. Könen, *Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (Carassius auratus) exposed to a glyphosate formulation using the micronucleus test and the comet assay.* Mutagenesis, 2007. **22**(4): p. 263-268.
109. Cavalcante, D.G.S.M., C.B.R. Martinez, and S.H. Sofia, *Genotoxic effects of Roundup® on the fish Prochilodus lineatus.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2008. **655**(1): p. 41-46.
110. Poletta, G.L., et al., *Genetic, enzymatic and developmental alterations observed in Caiman latirostris exposed in ovo to pesticide formulations and mixtures in an experiment simulating environmental exposure.* Ecotoxicology and Environmental Safety, 2011. **74**(4): p. 852-859.
111. Bosch, B., et al., *Micronucleus test in post metamorphic Odontophrynus cordobae and Rhinella arenarum (Amphibia: Anura) for environmental monitoring.* Journal of Toxicology and Environmental Health Sciences, 2011. **3**: p. 155-163.
112. Yadav, S.S., et al., *Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (Euflictis cyanophlyctis) in the presence and absence of predator stress.* Aquatic Toxicology, 2013. **132-133**: p. 1-8.

113. Kier, L.D., *Review of genotoxicity biomonitoring studies of glyphosate-based formulations.* Critical Reviews in Toxicology, 2015. **45**(3): p. 209-218.
114. Balderrama-Carmona, A.P., et al., *Herbicide biomonitoring in agricultural workers in Valle del Mayo, Sonora Mexico.* Environmental Science and Pollution Research, 2019.
115. Bonassi, S., et al., *HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei.* Environmental and Molecular Mutagenesis, 2001. **37**(1): p. 31-45.
116. Tarone, R.E., *On the International Agency for Research on Cancer classification of glyphosate as a probable human carcinogen.* European Journal of Cancer Prevention, 2018. **27**(1): p. 82-87.
117. Nohmi, T., *Thresholds of Genotoxic and Non-Genotoxic Carcinogens.* Toxicological research, 2018. **34**(4): p. 281-290.
118. Bonassi, S., et al., *An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans.* Carcinogenesis, 2007. **28**(3): p. 625-631.
119. Boffetta, P., et al., *Chromosomal Aberrations and Cancer Risk: Results of a Cohort Study from Central Europe.* American Journal of Epidemiology, 2006. **165**(1): p. 36-43.
120. Eastmond, D.A., et al., *Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme.* Mutagenesis, 2009. **24**(4): p. 341-349.
121. Guideline, I.H.T. *Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2 (R1)*. in *International conference on harmonization of technical requirements for registration of pharmaceuticals for human use, ICH Expert Working Group*. 2011.
122. Morita, T., et al., *Evaluation of the sensitivity and specificity of in vivo erythrocyte micronucleus and transgenic rodent gene mutation tests to detect rodent carcinogens.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2016. **802**: p. 1-29.
123. Morita, T., et al., *Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (Groups 1, 2A and 2B): The summary report of the 6th collaborative study by CSGMT/JEMS·MMS.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 1997. **389**(1): p. 3-122.
124. Kirkland, D., et al., *In vivo genotoxicity testing strategies: Report from the 7th International workshop on genotoxicity testing (IWGT).* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2019. **847**: p. 403035.
125. Remely, M., et al., *Vitamin E modifies high-fat diet-induced increase of DNA strand breaks, and changes in expression and DNA methylation of Dnmt1 and MLH1 in C57BL/6J male mice.* Nutrients, 2017. **9**(6): p. 607.
126. Remely, M., et al., *EGCG prevents high fat diet-induced changes in gut microbiota, decreases of DNA strand breaks, and changes in expression and DNA methylation of Dnmt1 and MLH1 in C57BL/6J male mice.* Oxidative medicine and cellular longevity, 2017. **2017**.
127. Hansen, M.K., et al., *In vivo Comet assay – statistical analysis and power calculations of mice testicular cells.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2014. **774**: p. 29-40.
128. Kumar, A., et al., *Sesamol ameliorates radiation induced DNA damage in hematopoietic system of whole body γ-irradiated mice.* Environmental and Molecular Mutagenesis, 2018. **59**(1): p. 79-90.

11. APPENDIX I

Table 11: SCGE data from the main experiment (three doses, positive and negative controls; 5 animals per group) with different organs.[1]

Dose (mg/kg bw.)	Animal ID.	Liver	Mean ± SD	Kidneys	Mean ± SD	Brain	Mean ± SD	Testes	Mean ± SD	Bone Marrow	Mean ± SD	Colon	Mean ± SD
neg. control	17	1.67	2.01 ± 0.19	5.11	3.16 ± 0.69	1.88	1.87 ± 0.43	2.94	2.06 ± 0.81	2.28	1.43 ± 0.81	3.01	6.0 ± 1.43
		2.64		3.90		1.22		1.70		0.66		3.95	
		2.39		2.93		0.60		1.54		0.85		11.48	
	18	2.20		3.49		1.50		2.27		2.75		3.89	
		1.66		2.85		4.33		3.71		1.37		6.91	
		2.08		2.79		1.28		4.21		1.99		5.97	
	21	2.28		4.25		1.84		1.83		0.29		2.34	
		2.80		2.58		2.45		1.26		0.28		7.33	
		1.39		0.98		0.83		2.71		0.42		8.22	
	26	2.54		3.06		3.26		1.04		3.25		2.50	
		1.30		3.30		1.92		1.21		3.23		3.98	
		1.74		0.91		0.81		2.66		0.70		5.96	
	31	2.13		1.83		3.41		1.34		1.28		3.66	
		1.10		7.03		0.89		1.95		1.39		10.82	
		2.27		2.45		1.81		0.55		0.66		9.93	

Table 11: continued

Dose (mg/kg bw.)	Animal ID.	Liver	Mean ± SD	Kidneys	Mean ± SD	Brain	Mean ± SD	Testes	Mean ± SD	Bone Marrow	Mean ± SD	Colon	Mean ± SD
150	15	3.06	2.23 ± 0.45	4.68	3.34 ± 0.95	6.56	3.08 ±1.12	1.20	1.94 ± 0.46	1.06	1.89 ± 0.55	7.61	4.40 ± 1.68
		2.66		1.50		1.63		1.46		1.66		6.03	
		1.55		2.22		6.41		2.56		1.94		4.07	
	19	3.05		3.14		2.85		1.27		1.68		8.40	
		2.41		1.99		4.07		2.08		2.81		4.09	
		2.63		3.31		1.66		2.71		0.49		7.02	
	22	2.76		3.56		0.67		0.98		2.50		4.47	
		2.57		1.77		1.76		2.23		3.42		1.21	
		2.22		4.60		2.95		3.57		1.86		4.99	
	23	2.26		2.55		2.13		0.75		0.30		4.67	
		1.62		3.31		2.66		1.56		3.28		3.55	
		1.86		2.43		5.64		1.44		0.30		1.51	
	28	1.38		5.08		1.76		2.16		2.54		2.99	
		1.67		3.93		2.64		2.74		1.63		2.77	
		1.82		5.93		2.78		2.31		2.82		2.66	

Table 11: continued

Dose (mg/kg bw.)	Animal ID.	Liver	Mean ± SD	Kidneys	Mean ± SD	Brain	Mean ± SD	Testes	Mean ± SD	Bone Marrow	Mean ± SD	Colon	Mean ± SD
400	10	6.58	6.57 ± 0.43	7.00	5.26 ± 2.10	1.45	2.21 ± 0.57	3.46	2.32 ± 0.73	1.24	1.27 ± 0.41	13.42	8.09 ± 5.19
		6.82		0.46		1.47		2.46		1.06		17.00	
		6.96		4.87		0.99		2.64		0.80		13.03	
	11	6.62		5.48		0.86		4.48		0.17		8.97	
		7.40		4.74		2.41		1.59		2.32		12.64	
		6.86		4.61		3.18		1.96		0.89		9.57	
	13	5.88		4.14		1.37		2.95		1.32		18.01	
		5.34		7.10		0.92		0.94		1.05		16.24	
		6.29		6.08		4.48		0.56		1.48		16.64	
	20	6.25		8.48		3.12		2.23		2.00		n. c.[2]	
		7.92		9.35		1.42		1.29		0.74		n. c.[2]	
		5.59		7.71		2.90		1.26		3.13		n. c.[2]	
	29	6.82		2.68		1.96		2.46		0.52		9.21	
		7.05		2.94		2.29		4.32		0.27		17.37	
		6.22		3.23		4.33		2.23		1.99		10.27	

Table 11: continued

Dose (mg/kg bw.)	Animal ID.	Liver	Mean ± SD	Kidneys	Mean ± SD	Brain	Mean ± SD	Testes	Mean ± SD	Bone Marrow	Mean ± SD	Colon	Mean ± SD
1000	6	6.77	6.57 ± 0.80	3.57	2.78 ± 0.25	6.05	2.40 ± 0.81	14.02	10.89 ± 3.0	2.09	2.79 ± 1.74	2.60	13.53 ± 2.83
		5.59		5.01		3.20		16.65		4.60		5.92	
		6.11		0.85		2.07		13.27		6.98		n. c.[2]	
	9	6.64		1.22		1.97		11.82		2.41		7.14	
		7.39		4.14		2.60		17.18		0.88		4.95	
		6.38		3.08		1.47		10.14		1.38		5.44	
	16	6.32		3.01		2.80		10.15		1.45		5.26	
		5.92		2.84		3.19		9.83		1.52		3.97	
		5.04		1.83		0.68		11.86		1.84		6.07	
	27	9.96		2.67		1.18		12.99		1.86		12.51	
		8.06		3.72		1.50		5.69		0.79		11.56	
		5.49		1.22		2.31		7.77		1.59		26.87	
	35	5.45		3.70		3.20		5.99		2.46		9.32	
		7.14		1.42		1.26		7.12		9.23		6.32	
		6.24		3.31		2.56		8.84		2.82		9.11	

Table 11: continued

Dose (mg/kg bw.)	Animal ID.	Liver	Mean ± SD	Kidneys	Mean ± SD	Brain	Mean ± SD	Testes	Mean ± SD	Bone Marrow	Mean ± SD	Colon	Mean ± SD
EMS (pos. control) 300	7	30.74	32.06 ± 1.55	36.69	33.09 ± 2.15	45.34	39.92 ± 1.98	19.86	22.49 ± 3.39	27.71	28.45 ± 3.09	n. c.[2]	42.20 ± 1.81
		31.56		29.69		35.07		19.49		28.95		n. c.[2]	
		27.71		35.87		40.94		13.59		28.80		n. c.[2]	
	12	30.64		28.81		42.34		22.61		25.33		40.55	
		30.49		28.07		37.87		25.13		n. c.[2]		46.25	
		36.99		33.49		38.02		16.93		32.22		47.42	
	24	30.05		34.60		38.53		23.47		38.20		50.26	
		28.16		36.54		43.60		28.03		24.50		32.50	
		34.69		36.51		38.25		25.99		33.29		39.02	
	30	33.80		34.47		42.85		23.77		30.06		42.86	
		34.40		34.96		44.10		28.47		33.64		47.04	
		29.94		30.13		40.69		24.72		24.79		36.51	
	32	32.02		30.39		37.51		21.74		16.20		45.49	
		35.21		30.34		36.17		23.64		22.97		44.29	
		34.46		35.79		37.48		19.91		31.38		34.20	

[1] Numbers indicate mean of median % DNA in tail as well as Mean ±SD per organ which were monitored with a computer aided image analysis, three slides were made and 50 cells were evaluated per slide; EMS, ethyl methanesulfonate

[2] n. c., not countable

Table 12: SCGE data from the confirmation experiment (one dose, positive and negative controls; 3 animals per group) with different organs.[1]

Dose (mg/kg bw.)	Animal ID.	Liver	Mean ± SD	Kidneys	Mean ± SD
0	3	1.68	1.83 ± 0.19	0.57	1.65 ± 0.79
		2.24		1.00	
		0.98		0.68	
	11	2.10		2.16	
		2.16		2.87	
		1.73		0.89	
	12	1.85		2.81	
		1.56		1.69	
		2.19		2.19	
400	1	5.96	6.82 ± 0.97	5.34	5.89 ± 1.71
		6.42		4.09	
		7.75		4.02	
	2	7.64		8.47	
		8.02		5.97	
		7.87		8.93	
	7	7.39		4.96	
		5.34		4.36	
		4.98		6.84	
1000	14	8.53	7.94 ± 1.22	6.15	7.26 ± 2.64
		10.79		6.76	
		8.42		6.64	
	15	7.50		6.09	
		7.27		5.25	
		8.51		3.84	
	16	7.56		9.10	
		5.43		10.97	
		7.47		10.50	
EMS (pos. control) 300	5	16.53	14.50 ± 2.30	15.95	14.97 ± 0.15
		18.23		15.02	
		16.38		13.46	
	6	16.68		12.15	
		15.92		14.81	
		13.21		18.41	
	8	8.69		13.69	
		11.46		14.67	
		13.45		16.56	

[1] Numbers indicate mean of median % DNA in tail as well as Mean ±SD per organ which were monitored with a computer aided image analysis; three slides were made and 50 cells were evaluated per slide; EMS, ethyl methanesulfonate

12. APPENDIX II

Table 13: MN results (main experiment; three doses, positive and negative controls; 5 animals per group) with bone marrow cells.[1]

		Ratio of PCE and NCE				Ratio of MN in PCE	
Dose (mg/kg bw.)	**Animal ID.**	**total counted erythrocytes**	**PCE**	**NCE**	**PCE to total erythrocytes in %**	**total counted PCE**	**MN**
0	17	500	240	260	48	2000	9
	18	500	262	238	52	2000	4
	21	500	256	244	51	2000	7
	26	500	246	254	49	2000	5
	31	500	252	248	50	2000	9
150	15	500	247	253	49	2000	8
	19	500	251	249	50	2000	6
	22	500	239	261	48	2000	7
	28	500	234	266	47	2000	6
	23	n. a.[2]	n. a.[2]	n. a.[2]	n. a.[2]	n. a.[2]	n. a.[2]
400	10	500	230	270	46	2000	4
	11	500	247	253	49	2000	3
	13	500	256	244	51	2000	5
	20	500	238	262	48	2000	6
	29	500	251	249	50	2000	11

Table 13: continued

		Ratio of PCE and NCE				Ratio of MN in PCE	
Dose (mg/kg bw.)	**Animal ID.**	**total count-ed erythro-cytes**	**PCE**	**NCE**	**PCE to total erythrocytes in %**	**total counted PCE**	**MN**
1000	6	500	240	260	48	2000	11
	9	500	244	256	49	2000	9
	16	500	228	272	46	2000	7
	27	500	260	240	52	2000	4
	35	500	234	266	47	2000	7
CP (pos. control) 50	8	500	230	270	46	2000	127
	14	500	233	267	47	2000	134
	25	500	241	259	48	2000	109
	33	500	237	263	47	2000	130
	34	500	225	275	45	2000	114

[1] Micronucleus (MN) rates were determined in 2.000 PCE per animal, using a brightfield optical microscope; the ratio of PCE to total erythrocytes were scored in 500 cells per animal; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes

[2] n. a., not available

Table 14: MN results (confirmation experiment; one dose and negative controls; 3 animals per group) with bone marrow cells.[1]

		Ratio of PCE and NCE				Ratio of MN in PCE	
Dose (mg/kg bw.)	**Animal ID.**	**total count-ed erythro-cytes**	**PCE**	**NCE**	**PCE to total erythrocytes in %**	**total counted PCE**	**MN**
0	3	500	248	252	50	2000	5
	11	500	263	237	53	2000	8
	12	500	255	245	51	2000	6
400	1	500	239	261	48	2000	7
	2	500	253	247	51	2000	4
	7	500	259	241	52	2000	9

[1] Micronucleus (MN) rates were determined in 2.000 PCE per animal, using a brightfield optical microscope; the ratio of PCE to total erythrocytes were scored in 500 cells per animal; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes

13. APPENDIX III

Table 15 summarizes the results of earlier SCGE studies which were obtained with different organs of untreated mice in our group [78, 125, 126] and by other authors [33, 84, 127]. It is evident, that the results which were obtained in liver, testes, colon and kidneys after glyphosate treatment are out of the range found in earlier trials in controls.

Table 15: DNA damage in different organs of untreated mice[1]

		Organ (% DNA in tail)					
Study (reference)	**Animals (n)**	**Brain**	**Bone Marrow**	**Liver**	**Testes**	**Colon**	**Kidney**
Setayesh et al., 2019 [78]	C57BL6/J mice (n = 5)	0.8 ± 0.2		4.6 ± 0.3		6.0 ± 1.0	
Remely et al., 2017 [126]	C57BL6/J mice (n = 15)			3.6 ± 1.4		4.5 ± 1.9	
Remely et al., 2017 [125]	C57BL6/J mice (n = 15)			3.7 ± 1.4		4.1 ± 1.7	
Manas et al., 2013 [33]	Balb/C mice (n = 6)			1.0 ± 0.8			
Kumar et al., 2017 [128]	C57BL6/J mice (n = 3)		2.5 ± 1.9				
Hansen et al., 2014 [127]	CD-1 mice (n = 5)				2.5 ± 0.6		
Prokopiev et al., 2019 [84]	CD-1 mice (n = 5)			2.2 ± 0.8			2.7 ± 0.4

[1]Values indicate % DNA in tail, mean ± SD were assessed on the basis of values indicated in bar diagrams in the respective articles.