**The Fertilized Avian Egg Fetal Liver Assays for Assessing DNA Damaging Potential of Chemicals: A Comparative Analysis with In Vitro and In Vivo Genotoxicity Assays and Rodent Carcinogenicity**

Running Title: **Detection of chemical DNA damage in avian fetal livers**

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# Abstract

The ability to produce direct DNA damage (genotoxicity) underlies the carcinogenic mode of action of various chemicals. As such, genotoxicity endpoints are typically evaluated in a regulatory-approved battery of in vitro tests with potential in vivo follow-up. Growing concern for animal welfare and implementation of new regulations which restrict the use of laboratory animals necessitated the introduction of New Approach Methodologies (NAMs). The avian egg-based (*in ovo*) models, the Chicken and related Turkey Egg Fetal Liver DNA Damage Assays, were developed as metabolically competent NAMs to potentially replace short-term in vivo genotoxicity assays for chemicals that are genotoxic in vitro. Both models utilize avian fetal livers for the evaluation of endpoints indicative of DNA damage produced by either direct or indirect mechanisms, specifically, the formation of nuclear DNA adducts and strand breaks. Moreover, avian embryos carry genetic and morphologic resemblance to mammals and can be used for an extensive evaluation of other endpoints including histopathology and tissue-specific genomic profiling. Avian fetal livers contain a full complement of metabolizing enzymes and are capable of bioactivation, detoxication, and elimination of xenobiotics. The comprehensive analysis of 87 and 59 chemicals assessed in the chicken and turkey models, respectively, revealed a stronger correlation with the results from in vivo assays demonstrating that *in ovo* models can detect the genotoxic potential of a broader range of compounds compared to in vitro assays with S9 supplementation. In conclusion, fertilized avian egg fetal liver assays offer a promising alternative to traditional in vivo genotoxicity assays.

# 1. Introduction

## Genotoxicity Assessment

A wide variety of chemicals can produce DNA damage, also referred to as genotoxicity, which is considered to be an initiating event in the process of carcinogenesis leading to mutations, chromosomal abnormalities and genomic instability (Kobets et al. 2018c; Kobets and Williams 2018; Preston and Williams 2005). Thus, evaluation of genotoxicity in the short term in vitro and in vivo assays is required for the safety assessment of most consumer products (Corvi and Madia 2017; ICH 2012; Recio and Kobets 2023; Williams 1989). The genotoxicity endpoints in these assays assess potential of a chemical to produce mutations (mutagenicity) and/or chromosome aberrations (clastogenicity/aneugenicity). Since these endpoints can be elicited by DNA damage (Kobets et al. 2018c; Kobets and Williams 2018; Preston and Williams 2005), assessment of DNA damage itself provides a mechanistic endpoint for genotoxicity (Williams 1984).

An example of an in vitro assay for DNA damage is the hepatocyte DNA repair assay (HPC/DRA) (Butterworth et al. 1987; Williams et al. 1982; Williams et al. 1989). This assay utilizes a cell type with the broadest representations of in vivo biotransformation processes and monitors a specific response to DNA damage. The liver is the primary metabolic organ, which participates in biotransformation of the majority of xenobiotics, natural and synthetic (Moscovitz and Aleksunes 2013). It is exposed to the largest amount of chemicals absorbed from the gastrointestinal tract via the portal circulation (first pass) and has the greatest biotransformation capacity (Larrey and Branch 1983). The HPC/DRA can be conducted with HPCs derived from any species (Maslansky and Williams 1895; McQueen and Williams 1983; McQueen and Williams 1987), including humans (McQueen et al. 1988). Usually, mouse and rat HPCs are used, although to study specific biotransformation activities special genetic breed such as the New Zealand white rabbit which has a genetic polymorphism in acetylation can be utilized (McQueen et al. 1982; McQueen et al. 1983). Testing in the HPC/DRA has revealed responses to a wide structural variety of genotoxin (McQueen et al. 1988; McQueen and Williams 1987; Williams et al. 1989).

In vivo studies are often required as a follow-up to in vitro genotoxicity tests for many classes of chemicals, including pharmaceuticals (ICH 2012). Nevertheless, current regulations either encourage the use of alternatives to animal testing (US Congress 2022) or altogether prohibit the use of laboratory animals for certain compounds (i.e., cosmetic ingredients) (EU 2009) in accordance with the 3R principles of Reduction, Refinement, and Replacement. Such regulations necessitate the development and use of New Approach Methodologies (NAMs) for safety assessment of chemicals in systems which are not considered to be an animal.

## B. Fertilized Avian Egg Fetal Liver Assays

Avian egg-based (*in ovo*) assays, in particular those utilizing chicken embryos, have been used for advancement of research in various fields, notably cancer and tumor immunology (Brunnemann et al. 2002; Enzmann et al. 2013; Enzmann and Brunnemann 1997; Enzmann et al. 2014; Kain et al. 2014; Leupold et al. 2021; Miebach et al. 2022; Ribatti 2021). The avian embryo is a complex, intact, metabolically active organism, with morphogenesis, genotype and phenotype closely resembling that of mammals (Zaefarian et al. 2019), which makes it superior to in vitro or invertebrate in vivo models.

Developed as an alternative model to the cancer bioassay, *in ovo* carcinogenicity assessment assay (IOCA), investigates chemically induced preneoplastic lesions indicative of hepatocarcinogenesis in the developing turkey livers (Brunnemann et al. 2002; Enzmann et al. 2013; Enzmann and Brunnemann 1997; Enzmann et al. 1992; Enzmann et al. 1995).

The Fertilized Avian Egg Fetal Liver DNA Damage Assay (FAEFL/DDA) is an extension of IOCA which measures nuclear DNA damage produced by either direct or indirect mechanisms using intact fertilized eggs of any avian species, most usually chicken or turkey (Kobets et al. 2019a; Kobets et al. 2016; Kobets et al. 2018a; Kobets et al. 2019b; Williams et al. 2014). Two examples of the FAEFL/DDA, namely Chicken and Turkey Egg Genotoxicity Assays (CEGA and TEGA, respectively), have been extensively utilized to evaluate genotoxic potential of various classes of chemicals, including naturally occurring compounds, pharmaceuticals, and prototype carcinogens (Iatropoulos et al. 2017; Kobets et al. 2019a; Kobets et al. 2016; Kobets et al. 2018a; Kobets et al. 2019b; Kobets et al. 2024; Williams et al. 2014). Both models have proven to be reliable tools, not yielding false positive or false negative results confirming their utility for the assessment of potential genotoxicity.

Developed as a screening tool, these NAMs are intended to replace short-term in vivo genotoxicity assays, particularly as a follow-up testing for compounds that yielded positive outcomes in in vitro genotoxicity tests. The rapid growth and development of avian embryo-fetuses (i.e., from 21 to 28 days) results in acceleration of carcinogenic processes, thereby facilitating monitoring of the effects of chemical carcinogens over a shorter time compared to traditional rodent models.

For evaluation of endpoints indicative of chemical-induced DNA damage, TEGA and CEGA utilize 32P-nucleotide postlabeling (NPL) assay (Phillips and Arlt 2014; Phillips and Arlt 2020; Reddy and Randerath 1987) for detection of DNA adducts and the single cell gel electrophoresis (comet) assay (Brendler-Schwaab et al. 2005; OECD 2016; Tice et al. 2000) for measurement of DNA strand breaks. The NPL assay is extremely sensitive and is capable of detecting DNA adducts formed at frequencies as low as 1 in 1010 nucleotides (Gupta 1985; Hwa Yun et al. 2020; Reddy and Randerath 1987). In FAEFL/DDA studies, NPL analysis is usually performed exclusively on the samples from control group and the highest tested dosage of compound of interest. This approach is based on the likelihood that the highest doses will most effectively reveal the presence of adducts, which, as noted by Phillips and colleagues, is sufficient for detecting a positive response (Phillips et al. 2000). Comet assay can be used for the detection of single and double strand DNA breaks as well as alkali-labile sites, in any type of cell or tissue (Glei et al. 2016). For the purpose of FAEFL/DDA, the comet assay is used for the detection of single strand breaks in the liver of each avian fetus. The comet assay can be modified to detect DNA breaks resulting from oxidative DNA damage (Collins et al. 2020; Shukla et al. 2011), thus providing additional mechanistic information about the tested compound.

The *in ovo* assays provide chemical biotransformation activity of a highly proficient intact, but non-animal, organism (Kobets et al. 2018b; Perrone et al. 2004; Rifkind et al. 1979). The average incubation period until hatching is 21 days for the chicken egg and 28 days for the turkey egg. According to the Public Health Service Policy on Humane Care and Use of Laboratory Animals (OLAW 2015), the term "live vertebrate animal" does not apply to avian species until their hatching. Therefore, since termination of avian embryos in FAEFL/DDA is carried out at least 72 hours before hatching, these experiments are not considered to be “animal studies”. Experimental procedures which are performed up to embryonic day 11 are not considered to inflict pain on the chicken embryos, as their nervous system is not completely formed (Bellairs 1959). In addition, the injection procedures conducted into the air sac allow to avoid direct contact of the needle with the embryo-fetus, thus, potential discomfort during the procedure is avoided. Hence, *in ovo* assays can be used for studies where use of animals is undesirable or precluded.

Utilizing the whole organism, CEGA and TEGA are capable of simulating toxicokinetic parameters of administration, distribution, metabolism, excretion (ADME) of that in animals and humans. Test compounds are injected into the air sac from which they are distributed to the fetus via chorioallantoic veins (**Figure 1**). These veins are analogous to umbilical veins in humans and are responsible for gas exchange (Duncker 1978; Van Golde et al. 1996). Another circulatory arc which is composed of the vitelline veins (**Figure 1**), absorbs nutrients from the yolk sac. The intra-embryonic circulation then brings nutrients and oxygen to tissues and collects waste materials. The majority of metabolism occurs in the liver, which develops metabolic capacities from embryonic day 6 (O’connor 1953), the activity of which is comparable to that in postnatal rodents (Perrone et al. 2004). Waste products are then eliminated through the cloaca, a common chamber into which urine and feces are combined for discharge. The cloaca begins to form on embryonic day six (Bakst 1986). Since avian fetal livers contain a full complement of xenobiotic metabolizing enzymes, *in ovo* models can detect genotoxic potential of a wider array of compounds than in vitro assays dependent on supplementation with enzyme preparations (e.g., induced rat or hamster liver S9 fractions). Additionally, similar to in vivo models, the avian embryo-fetus is capable of detoxication and elimination of xenobiotics, in contrast to many in vitro systems which, unless specifically engineered, lack phase II enzyme activity. The results of testing in the *in ovo* models indicate activation of a wide structural variety of activation-dependent genotoxins, e.g., polycyclic aromatic hydrocarbons, aromatic amines, alkenylbenzenes, etc. (Kobets et al. 2019a; Kobets et al. 2016; Kobets et al. 2018a; Kobets et al. 2019b; Williams et al. 2014).

A diagram of the human body

Description automatically generated

Figure 1. Schematic representation of a cross section of fertilized chicken egg. Created with BioRender.com

*In ovo* models can be utilized for an extensive evaluation of other endpoints, including histopathology (Enzmann et al. 2013; Iatropoulos et al. 2017), whereas in vitro systems do not allow such extensive assessment. Thus, in addition to detecting genotoxicity of diverse DNA-reactive carcinogens, FAEFL/DDA can reveal developmental abnormalities (e.g., gallbladder agenesis caused by genotoxic carcinogen diethylnitrosamine (Iatropoulos et al. 2017; Williams G. et al. 2011)) and distinguish adverse and adaptive morphological changes. The chicken genome has been sequenced (Antin et al. 2014; Huang et al. 2023; International Chicken Genome Sequencing Consortium, 2004; McCarthy et al. 2019; Wu et al. 2024) and conservation of many fundamental biological processes between chickens and mammals has been described (Alaiz Noya et al. 2022; International Chicken Genome Sequencing Consortium, 2004; Nie et al. 2010). This allows the enhancement of the model by evaluating changes in tissue-specific gene expression profiles produced by exposures to various chemicals.

*In ovo* assays utilize Specific Pathogen Free (SPF) eggs. This is important because certain pathogens may have the potential to metabolize chemicals thus modulating their adverse effects. Moreover, the eggs are incubated under rigorous conditions (see Methodology section below), and accordingly, the models investigate the direct effects of chemicals, which are not confounded by variables such as diet or light cycle.

The current report summarizes findings for all of the compounds assessed in the CEGA and TEGA models to date and evaluates the predictivity and reliability of the *in ovo* models compared to established in vitro and in vivo genotoxicity and carcinogenicity assays.

# 2. Methodology

## A. Fertilized Chicken and Turkey Egg Fetal Liver Assays

The protocols for the CEGA and TEGA models share many similarities, with exception of the timeline (**Figure 2**) (Kobets et al. 2016; Kobets et al. 2019b; Williams et al. 2014).

A diagram of a diagram of a medical procedure

Description automatically generated with medium confidenceFigure 2. Protocol of fertilized chicken and turkey egg fetal liver assays. Created with BioRender.com

The fertilized white leghorn chicken (*Gallus gallus*) eggs (obtained from Charles River Laboratories (North Franklin, CT)) or broad-breasted white turkey (*Meleagris gallopavo*) eggs (purchased from Aviagen Turkeys, Inc. (Lewisburg, WV)) of undetermined sex were weighed, randomly divided into control and dosed groups and stored at room temperature for no more than 3 days prior to incubation. Incubation commenced on incubation day 0 in 2GIF Styrofoam incubators with automatic egg turners (Murray McMurray Hatchery, Webster City, IA) at 37 ± 0.5°C and 60 ± 5% humidity. The development of eggs was monitored on day eight (8) for chicken eggs and day nineteen (19) for turkeys, by transillumination. Eggs with no or partial development were eliminated. Control and dosed eggs (N ≥ 10 eggs per group) were separated prior to dosing to avoid any possible airborne cross contamination.

Dosing with the test compounds (complete list of tested chemicals is provided in **Tables 1** and **2**) and vehicle was conducted in three (3) consecutive daily injections into the air sac (**Figure 1**). Vehicle was selected based on the solubility of the compound: hydrophilic compounds were dissolved in deionized water, while for lipophilic compounds a 20% aqueous solution of either Kolliphor Oil (Solutol HS15) or Tween 20 was used. An established genotoxic carcinogen, quinoline (Matsumoto et al. 2018; Nagao et al. 1977) was used as a positive control. Tested dosages for test compounds were selected based on the available oral LD50 in rodents, solubility, or toxicity to the avian embryos. The application volume was 50 μl/egg/day (0.15 ml/egg total volume) in CEGA and 0.2 ml/egg/day (0.6 ml/egg total volume) in TEGA. Theoretically, it is most appropriate to conduct dosing on the last 3 days of maturation because the liver would be most developed. Accordingly, injections in the TEGA occur on embryonic days 22-24. However, there is a general agreement that avian embryos after approximately two-thirds of their development may be sufficiently mature to experience pain and discomfort (Bellairs 1959). In consideration of this, injections in CEGA take place on days 9 through 11.

Three hours after the last injection (11 days for chickens and 24 days for turkeys), eggs were terminated, and fetal livers were removed and analyzed for nuclear DNA damage using the NPL (Phillips and Arlt 2020; Reddy and Randerath 1987) and comet (Brendler-Schwaab et al. 2005; OECD 2016; Tice et al. 2000) assays. Groups chosen for analysis had viability levels higher than 50% (at least half of the fetuses in the group were viable upon termination) to avoid false-positive results due to cytotoxicity. In each assay three (3) liver samples (biological replicates) per group were analyzed, owing to the limitation in the number of samples which can be analyzed simultaneously.

For histopathologic evaluation, the termination and collection of liver tissues occur 24 hours and 7 days after the last injection, in order to allow for morphologic changes to develop.

## B. 32P-Nucleotide Postlabeling (NPL) Assay for Nuclear DNA Adducts

Nuclear DNA was isolated from the frozen liver samples using QIAGEN (Valencia, CA) G100 columns following the manufacturer's protocol. DNA samples (10 µg each) with adequate purity (260/280 and 260/230 nm absorbance ratios) were further processed for NPL assay (Phillips and Arlt 2014; Phillips and Arlt 2020; Reddy and Randerath 1987) with nuclease P1 (NP1) digestion (Reddy and Randerath 1986; Reddy and Randerath 1987) and/or OASIS hydrophilic-lipophilic balance (HLB) column enrichment (Gupta et al. 1985) as previously described in detail (Kobets et al. 2019a; Williams et al. 2014) (**Figure 3**).

Quantification of adducts was achieved by integrating individual spots, using the Molecular Dynamics Imager software. DNA adducts in 108 of normal nucleotides were ascertained (threshold for NPL assay in the laboratory is one adduct per 109 of normal nucleotides), and a mean value per group was calculated. Values obtained were considered comparative rather than absolute since the NPL signal is a function of the radiolabeling efficiency of adducts which may differ between adducts, and no internal standard was included.

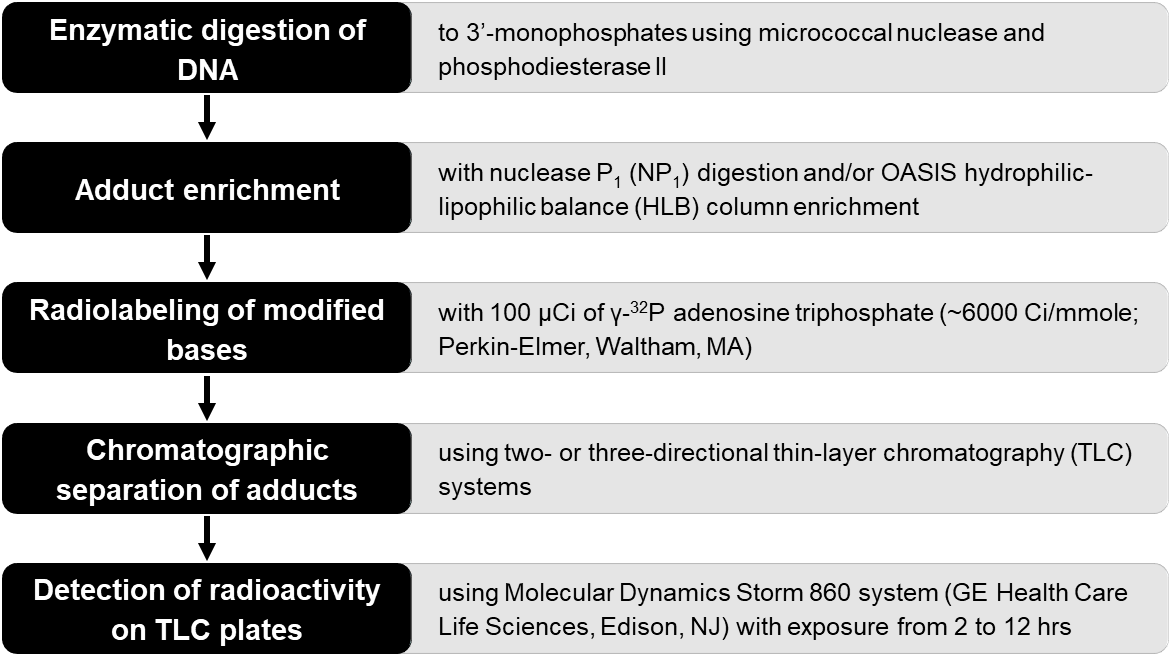


Figure 3. Methodology of the 32P-poslabeling (NPL) assay.

## C. Alkaline Single Cell Gel Electrophoresis (Comet) and Modified Comet Assays for Nuclear DNA Strand Breaks

Liver tissues (200 mg of each liver sample) were minced into fine pieces in 1 ml of ice-cold Hank’s balanced salt solution (HBSS, Gibco, Grand Island, NY) containing 20 mM EDTA and 10% dimethylsulfoxide (DMSO). Aliquots of the cell suspensions were then processed in the standard comet assay (Tice et al. 2000) according to OECD guidelines (OECD 2016), as previously described in detail (Kobets et al. 2018a; Williams et al. 2014) (**Figure 4**). The assay was performed under dim light to prevent ultraviolet light-induced DNA damage.

For the modified comet assay (Collins et al. 2020; Shukla et al. 2011), following lysis, slides were incubated with 50 μl of reaction enzyme buffer (REB) only or 0.5 units of either formamidopyrimidine DNA glycosylase (FPG) or 0.8 units of human 8-oxoguanine-DNA glycosylase (hOGG1) (New England Biolabs-NEB, Inc., Ipswich, MA) for 30 min at 37 °C, as previously described (Iatropoulos et al. 2017). Additional samples from control group incubated with 50 μl of 30μm of hydrogen peroxide (H2O2) on ice for 5 min before lysis serve as a positive control.

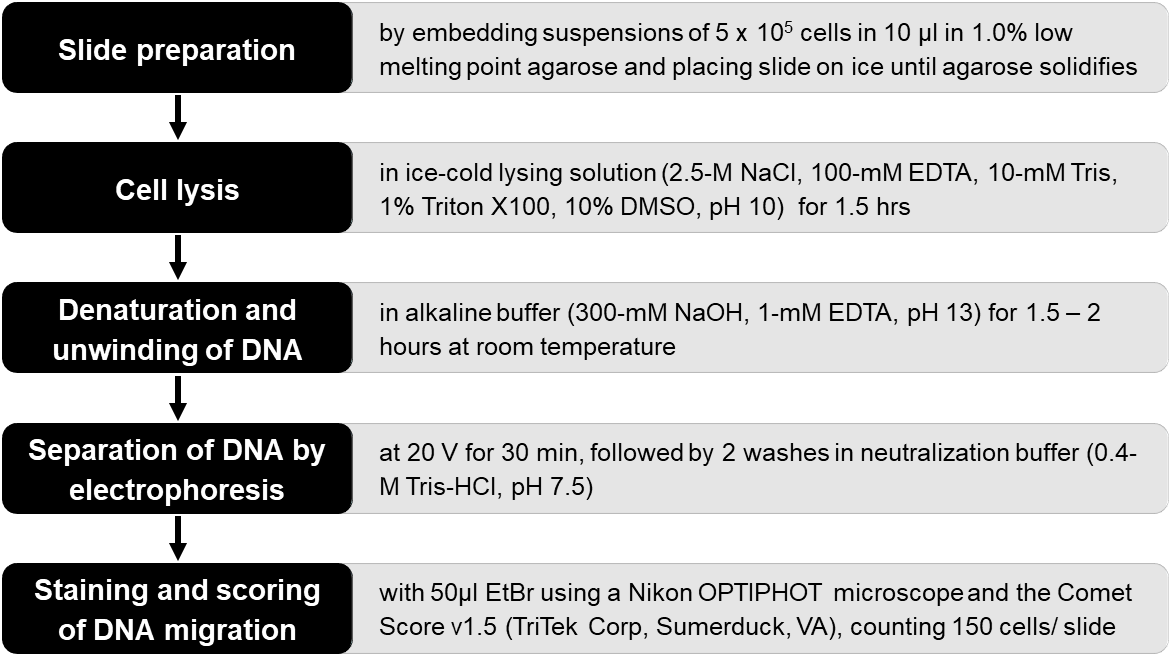


Figure 4. Methodology of the comet assay.

## D. Data Analyses

### i. Statistical Analysis

Results are presented as mean ± SD. Statistical analyses were performed using SigmaStat software v. 3.11.0 (Systat Software Inc., Chicago, IL), with the most appropriate statistical analysis of the data sets, which included the one-way analysis of variance with the pairwise multiple comparison being made by Tukey's method. Linear regression analysis was used to determine dose-related trends. P values < 0.05 were considered significant.

### ii. Interpretation of the Results

Interpretation of the results of NPL assay was done by visual comparison of the chromatograms from dosed groups with corresponding negative control groups, and, if available, positive control groups, which were run simultaneously. A positive result was recorded if the chromatogram from exposed samples displayed any extra spot(s) compared to the negative control group. A negative result was recorded if there were no other spot(s) than the naturally occurring ones in the controls. A positive outcome in the NPL assay provides robust evidence for chemical-induced DNA damage since, owing to the multiple enzyme steps involved in the digestion of the purified DNA and kinase reaction of the resulting deoxyribonucleoside 3′-phosphates, few false positives occur. Thus, a compound positive in the NPL assay is considered positive in the FAEFL/DDA. However, certain DNA adducts do not kinase well or are difficult to separate from the normal nucleotides present. For compounds negative in the NPL assay, a subsequent comet assay is taken into consideration.

The evaluation criteria for determining positive results in the comet assay includes the following (Tug et al. 2024; van der Leede et al. 2014): a statistically significant increase in the percentage of DNA-in-tail in dose group(s) compared with corresponding vehicle control group; a dose-related increase in DNA damage determined by linear regression analysis; the percentage of DNA-in-tail in dose group(s) should be outside of the historical vehicle control range established in the laboratory (for 20% HS, a 95% CI is 7.3 – 8.2 in CEGA and 9.86 – 11.63 in TEGA). Compounds that produce positive comet results are assumed to be positive in FAEFL/DDA, with the caveat that a positive comet outcome may indicate either direct or indirect (e.g., oxidative stress) genotoxic potential of the compound. An equivocal call is made when a clear positive or negative outcome cannot be concluded, or responses are inconsistent between experiments.

### iii. Concordance Analysis

To compare the outcomes observed in *in ovo* models with those from corresponding in vitro and in vivo genotoxicity and carcinogenicity assays, results were categorized as follows based on their alignment:

* overall positive of negative call in the *in ovo* assay which was consistent with results from another endpoint in vitro or in vivo was considered respectively as either true positive (TP) or true negative (TN)
* negative outcome *in ovo* for a compound that produces a positive outcome in another assay was considered as false negative (FN); accordingly, positive outcome *in ovo* for a compound that was negative in another assay was considered as false positive (FP).

A conservative approach was applied when comparing *in ovo* outcomes with available in vitro and in vivo data, treating equivocal results as positive.

The concordance was quantified using the following metrics:

Sensitivity = TP / (TP + FN)

Specificity = TN / (TN + FP)

Positive predictive value (PPV) = TP / (TP + FP)

Negative predictive value (NPV) = TN / (TN + FP)

Accuracy = (TP + TN) / (TP + TN + FP + FN)

False Discovery Rate (FDR) = FP / (FP + TP)

# 4. Results

Currently, the genotoxic potentials of 125 compounds were evaluated *in ovo* in the chicken or turkey FAEFL/DDA. Sixty-six (66) compounds tested exclusively in the chicken embryo-fetal livers, thirty-eight (38) only in turkey fetal livers and twenty-one (21) in both species. The results for these evaluations are summarized in **Tables 1-3**.

## A. Outcomes in the Fertilized Chicken Egg Fetal Liver Assay

Out of eighty-seven (87) compounds tested in CEGA, twenty (20) chemicals (23%) were positive in both the NPL and comet assays and thirty (30) compounds (34.5%) were either positive or equivocal in either NPL or comet assay in chicken embryo-fetal livers. Of these, forty-eight (48) compounds were also assessed for genotoxicity in vitro and forty-two (42) in vivo. Approximately 90% of these compounds (42/48 in vitro and 37/42 in vivo) produced positive results in at least one of the assays (**Table 1**). Out of forty (40) compounds with available carcinogenicity data, thirty-seven (37) compounds (92.5%) were established rodent carcinogens, and two (5%) produced equivocal carcinogenic outcomes (**Table 1**). Out of thirty-seven (37) rodent carcinogens that produced positive results in CEGA, approximately thirty-two (32) (86.5%) have demonstrated the potential to produce tumors in rodent livers. These included 2-acetylaminfluorene, 4-aminobiphenyl, aflatoxin B1 and B2, N-methyl-N'-nitro-N-nitrosoguanidine, *o*-toluidine, safrole, riddelliine, tamoxifen.

The remaining thirty-seven (37) compounds (42.5%) were negative in both the NPL and comet assays in CEGA. Data from in vitro and in vivo genotoxicity assays were available for thirty-six (36) and thirty-one (31) of these compounds, respectively (**Table 1**). While 86% (31/36) of these compounds were positive in at least one of the in vitro assays, only 39% (12/31) compounds produced positive outcomes in vivo. With regard to carcinogenicity, twelve (12) out of twenty-six (26) compounds (46%) were negative in rodent bioassays (**Table 1**).

## B. Outcomes in the Fertilized Turkey Egg Fetal Liver Assay

Out of fifty-nine (59) compounds tested in TEGA, eight (8) compounds (13.5%) were positive in both the NPL and comet assays and twenty-three (23) compounds (39%) were either positive or equivocal in either NPL or comet assay in turkey fetal livers. Among these, thirty (30) compounds had available in vitro genotoxicity data and twenty-five (25) were also evaluated in vivo. In vitro, 76% of the compounds (23/30) were positive in at least one of the assays, and 72% (18/25) produced genotoxic responses in vivo (**Table 2**). Carcinogenicity data were available for twenty (20) of the compounds, with 80% (16/20) being positive and 5% (1/20) equivocal (**Table 2**). Out of sixteen (16) rodent carcinogens which tested positive in TEGA, fourteen (14) (87.5%) (e.g., estragole, safrole, methyl eugenol, 2-acetylaminofluorene, aflatoxin B1) were demonstrated to produce tumors in the livers of rodents.

Nutmeg and pimento berry oils are complex mixtures which, among other constituents, contain genotoxic carcinogens such as myristicin, elemicin, safrole, methyl eugenol, and estragole (Eisenreich et al. 2021). The oils were positive in TEGA, as were the constituent alkenylbenzenes.

The remaining twenty-eight (28) compounds (47.5%) were negative in both the NPL and comet assays in TEGA. All of these were also previously assessed in vitro, with eighteen (18) compounds (64%) producing positive outcomes in at least one of the assays. Among twenty-seven (27) compounds tested in vivo, only nine (9) (33%) were positive (**Table 2**). Finally, out of eighteen (18) compounds assessed for carcinogenic potential, twelve (12) (66.5%) were positive and one (1) (5.5%) equivocal.

Table 1. Outcomes in the Chicken Egg Genotoxicity Assay (CEGA) compared to selected in vitro and vivo genotoxicity data and carcinogenicity in rodents.

| **Compound** | **CAS #** | **CEGA** | | | | **DNA adducts** | **In vitro genotoxicity c, d** | | | | | |  | **In vivo genotoxicity c** | | | | | **CARC c, e** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Tested dosages, mg/egg** | **NPL** | **Comet** | **Ref** | **Ames** | **MCGM** | **MN** | **CA** | **Comet** | **Rat HPC/DRA f** |  | **UDS** | **TGR** | **MN** | **CA** | **Comet** |
| α-Asarone | 2883-98-9 | 2 - 8 | Pos | Pos | [1] | Pos in vitro | Eqv |  | Neg |  | Pos |  |  | |  |  |  |  | Pos |
| β-Asarone | 5273-86-9 | 0.12 - 0.5 ª | Pos | Neg | [1] | Pos in vitro | Eqv |  | Pos |  | Pos |  |  | |  |  |  |  | Pos |
| 1-Aminofluorene | 6344-63-4 | 0.6 - 2.4 b | Pos | Neg | [2] |  | Pos |  |  |  |  |  |  | |  |  |  |  |  |
| 1-Naphthylamine | 134-32-7 | 0.6 - 2.4 b | Neg | Neg | [2] | Pos | Pos | Pos | Pos | Pos |  |  | Pos | |  | Eqv |  | Pos | Neg |
| 2,4-Diaminotoluene | 95-80-7 | 5 -15 | Pos | Pos | [2] | Pos | Pos | Pos | Pos | Pos | Pos |  | Pos | | Pos | Neg |  | Pos | Pos |
| 2,4-Dinitrotoluene | 121-14-2 | 1 - 5 | Pos | Neg | [2] | Pos | Pos | Eqv |  | Neg |  |  | Pos | |  | Neg |  | Neg | Pos |
| 2,6-Diaminotoluene | 823-40-5 | 5 - 15 | Pos | Neg | [2] | Pos | Pos |  | Pos | Pos | Neg |  | Eqv | | Neg | Pos | Neg | Pos | Neg |
| 2,6-Dinitrotoluene | 606-20-2 | 1 - 5 | Pos | Eqv | [2] | Pos | Pos |  |  |  |  |  | Pos | |  | Neg |  | Pos | Pos |
| 2,6-Xylidine | 87-62-7 | 5 - 10 ª | Pos | Pos | [2] | Pos | Neg | Eqv |  | Pos |  |  | Neg | | Pos | Neg |  | Pos | Pos |
| 2-Acetylaminofluorene | 53-96-3 | 0.02 - 0.6 b | Pos | Pos | [3] | Pos | Pos | Pos | Pos | Pos | Pos | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| 2-Aminofluorene | 153-78-6 | 0.3 - 1.2 | Pos | Pos | [2] | Pos | Pos | Pos | Pos |  |  | Pos |  | |  | Neg | Pos |  | Pos |
| 2-Methyl-2-pentanal | 623-36-9 | 7 - 10 ª | Neg | Neg | [4] |  | Neg |  | Pos |  |  |  |  | |  | Neg |  | Neg |  |
| 2-Naphthylamine | 91-59-8 | 0.3 - 1.2 ª | Pos | Pos | [2] | Pos | Pos |  |  | Pos | Pos | Neg | Pos | |  | Eqv |  | Pos | Pos |
| 2-Nitrofluorene | 607-57-8 | 0.3 - 1.2 | Pos | Pos | [2] | Pos | Pos | Pos | Pos | Pos |  |  | Pos | |  |  |  |  | Pos |
| 2-Phenyl-2-butenal | 4411-89-6 | 1 - 5 ª | Neg | Neg | [4] |  | Pos |  |  |  |  |  |  | |  | Neg |  | Neg |  |
| 3,2’-Dimethyl-4-aminobiphenyl | 58109-32-3 | 0.3 - 7 | Pos | Neg | [2] | Pos | Pos |  |  |  |  | Pos | Pos | |  |  |  |  | Pos |
| 3,4-Dimethylaniline | 95-64-7 | 5 - 10 ª | Pos | Pos | [2] | Pos in vitro | Pos |  |  | Neg |  |  |  | |  | Neg |  | Pos |  |
| 4(5)-Methylimidazole | 822-36-6 | 1 - 5 | Neg | Neg |  |  | Neg |  | Pos |  |  |  |  | |  | Neg |  |  | Pos |
| 4-(Dimethylamino)benzene-1-carbohydrazide | 19353-92-5 | 0.15 - 1.25 b | Neg | Neg |  |  |  |  |  |  |  |  |  | |  |  |  |  |  |
| 4-Aminobiphenyl | 92-67-1 | 0.15 - 0.3 ª | Pos | Pos | [2] | Pos | Pos | Pos |  | Pos | Pos | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| 4-Chloro-ortho-toluidine | 95-69-2 | 0.63 - 5 ª | Pos | Eqv | [2] | Pos | Eqv |  |  | Pos | Pos |  |  | |  | Pos |  | Pos | Pos |
| 4-Dimethylaminoazobenzene | 60-11-7 | 0.63 - 2.5 b | Neg | Pos | [2] |  | Pos |  |  | Pos |  | Neg | Pos | |  | Neg |  | Pos | Pos |
| 6-Nitrochrysene | 7496-02-8 | 0.3 - 0.6 | Neg | Neg |  | Pos | Pos | Pos |  |  |  |  |  | | Pos |  |  |  | Pos |
| 6-Thioguanine | 154-42-7 | 1 - 6 | Neg | Neg | [5] |  |  |  |  | Pos | Pos |  |  | |  |  |  |  |  |
| 8-Hydroxyquinoline | 148-24-3 | 0.25 - 1 | Neg | Neg |  |  | Pos |  |  | Pos | Pos | Neg |  | |  | Neg |  |  | Neg |
| Acetaminophen | 103-90-2 | 2 - 8 b | Neg | Neg | [5] | Neg | Neg | Pos | Pos | Pos |  | Neg | Neg | | Neg | Pos | Eqv | Pos | Neg |
| Aflatoxin B1 | 1162-65-8 | 0.0002 - 0.003 ª | Neg | Pos | [3] | Pos | Pos |  | Pos | Pos |  | Pos |  | | Pos | Pos | Pos | Neg | Pos |
| Aflatoxin B2 | 7220-81-7 | 0.0016 - 0.006 | Neg | Pos | [3] | Pos | Neg |  |  |  |  | Pos |  | |  | Pos | Pos |  | Pos |
| Aniline | 62-53-3 | 5 - 20 ª | Neg | Neg | [2] |  | Neg | Pos | Pos | Pos |  | Neg |  | |  | Pos | Pos | Pos | Pos |
| Benzene | 71-43-2 | 1.25 - 15 | Neg | Neg | [2] | Pos | Neg | Eqv | Pos | Pos |  | Neg |  | | Pos | Pos | Pos |  | Pos |
| Benzidine | 92-87-5 | 1.25 - 5 | Pos | Pos | [2] | Pos | Pos | Pos | Pos | Pos | Pos | Pos | Pos | |  | Pos | Pos |  | Pos |
| Benzo[a]pyrene | 50-32-8 | 0.03 - 0.5 | Pos | Pos | [3] | Pos | Pos | Pos | Pos | Pos | Pos | Pos | Eqv | | Pos | Pos | Pos | Eqv | Pos |
| Benzo[e]pyrene | 192-97-2 | 0.25 - 0.5 | Neg | Neg | [3] |  | Pos | Eqv | Pos |  |  | Neg |  | |  |  |  |  | Neg |
| Biphenyl | 92-52-4 | 0.625 - 1.25 b | Neg | Neg | [2] | Neg | Neg | Pos |  | Pos | Pos | Neg |  | |  | Neg | Neg | Pos | Pos |
| Caffeine | 58-08-2 | 2.5 - 5 | Neg | Neg |  |  | Neg | Pos | Pos | Pos |  |  |  | |  | Eqv | Neg |  | Neg |
| Chrysene | 218-01-9 | 0.6 - 2.4 | Neg | Pos |  |  | Pos | Pos | Pos |  |  | Neg |  | | Pos |  | Neg |  | Pos |
| Ciprofloxacin | 85721-33-1 | 1.25 - 5 b | Neg | Neg | [5] |  | Pos | Pos | Pos | Pos |  |  | Neg | |  | Neg | Eqv |  | Neg |
| Clofibrate | 637-07-0 | 5 - 10 | Neg | Neg | [5] | Neg | Neg | Neg | Neg | Eqv |  | Neg | Neg | | Neg | Neg |  | Neg | Pos |
| Clofibric acid | 882-09-7 | 0.1 - 1.5 | Neg | Pos | [6] |  |  |  |  |  | Pos |  |  | |  |  |  |  | Pos |
| 0.4 - 1.5 |  | Pos  + FPG, hOGG1 |
| Colchicine | 64-86-8 | 0.025 ª | Neg | Neg | [5] |  | Neg | Pos | Pos | Pos |  |  |  | |  | Pos |  |  |  |
| Cyclophosphamide | 6055-19-2 | 0.5 - 3 | Neg | Pos | [5] | Pos | Pos | Pos | Pos | Pos | Pos | Neg |  | | Pos | Pos |  | Pos | Pos |
| Dibutylamine | 111-92-2 | 1 - 2 | Neg | Pos |  |  | Neg | Neg |  | Pos | Eqv |  |  | |  | Neg |  |  |  |
| Diethylnitrosamine | 55-18-5 | 0.25 - 4 | Neg | Pos | [3] | Pos | Pos | Pos | Pos | Pos |  | Pos | Pos | | Pos | Neg |  | Pos | Pos |
| Diethylstilbestrol | 56-53-1 | 0.25 - 0.5 b | Pos | Neg | [5] | Pos | Neg |  | Pos | Pos |  | Neg |  | |  | Eqv | Pos | Neg | Pos |
| Diphenhydramine hcl | 147-24-0 | 0.63 - 2.5 ª | Neg | Pos | [5] |  | Neg | Neg |  | Eqv |  |  |  | |  |  |  |  | Eqv |
| Diphenylamine | 122-39-4 | 4 - 8 ª | Neg | Neg |  |  | Eqv | Neg | Pos |  | Neg |  |  | |  | Neg |  |  | Pos |
| Dipropylamine | 142-84-7 | 2 - 4 ª | Neg | Neg |  |  | Neg |  |  |  |  |  |  | |  |  |  |  |  |
| D-Mannitol | 69-65-8 | 5.6 - 45 | Neg | Neg | [6] |  | Neg |  |  | Neg | Neg |  |  | |  | Neg | Neg |  | Neg |
| Doxylamine | 562-10-7 | 2.5 -10 | Neg | Pos | [5] |  | Neg |  |  |  | Eqv |  |  | |  | Neg | Pos |  | Pos |
| Ethylbenzene | 100-41-4 | 2.5 - 5 ª | Neg | Neg |  | Neg | Neg | Pos | Neg |  | Neg | Pos | Neg | |  | Neg |  |  | Pos |
| Etoposide | 33419-42-0 | 1 - 2 | Neg | Pos | [5] |  | Pos | Pos | Pos | Pos | Pos |  |  | | Neg | Pos | Pos | Pos |  |
| Eugenol | 97-53-0 | 1.5 - 3 ª | Neg | Neg | [1] | Neg | Neg | Pos | Pos | Pos | Neg |  | Neg | | Neg | Neg |  |  | Neg |
| Fluorene | 86-73-7 | 0.2 - 1.4 b | Neg | Neg | [2,3] |  | Neg | Pos | Eqv | Eqv |  | Neg |  | |  |  |  |  | Neg |
| Griseofulvin | 126-07-8 | 0.1 - 0.4 | Neg | Pos | [5] |  | Neg | Neg | Pos | Pos |  | Neg |  | |  | Neg | Pos |  | Pos |
| Heliotrine | 303-33-3 | 1 - 3 ª | Neg | Neg |  | Pos | Pos |  |  | Pos | Pos |  |  | |  | Pos |  |  |  |
| Hexanal | 66-25-1 | 1 - 5 ª | Neg | Neg |  |  | Pos | Pos |  | Pos |  |  |  | |  |  |  | Neg |  |
| Hydralazine hcl | 304-20-1 | 1.5 - 6 b | Neg | Eqv | [5] | Pos in vitro | Pos |  |  |  | Pos | Pos |  | |  | Neg |  |  | Eqv |
| Ibuprofen | 15687-27-1 | 3 - 5 b | Neg | Neg | [5] |  | Neg |  |  |  |  |  |  | |  |  | Pos |  | Neg |
| Isoquinoline | 119-65-3 | 2 - 4 ª | Pos | Pos |  |  | Neg |  |  |  |  | Neg |  | |  |  |  |  |  |
| Lasiocarpine | 303-34-4 | 0.25 - 0.5 ª | Neg | Neg |  | Pos | Pos |  |  | Pos | Pos | Pos |  | |  | Pos |  |  | Pos |
| Lidocaine | 6108-05-0 | 5 - 15 ª | Pos | Pos | [5] | Pos | Neg |  |  | Neg |  |  |  | |  |  | Neg |  |  |
| L-Proline | 145-85-3 | 8 - 16 | Neg | Pos |  |  |  |  |  |  |  |  |  | |  |  |  |  |  |
| Methyl eugenol | 93-15-2 | 1 - 2 | Pos | Neg | [1] | Pos | Neg | Neg | Neg | Neg |  |  |  | | Pos | Neg |  | Pos | Pos |
| Methyl methane sulfonate | 66-27-3 | 2.5 ª | Neg | Pos |  | Pos | Pos | Pos | Pos | Pos | Pos | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| Mitomycin C | 50-07-7 | 0.07 - 0.125 ª | Pos | Eqv | [5] | Pos | Pos | Pos | Pos | Pos | Pos | Pos |  | | Pos | Pos | Pos | Pos | Pos |
| Naphthalene | 91-20-3 | 0.63 - 1.25 b | Neg | Neg | [2] | Pos | Neg | Eqv | Pos | Pos |  |  | Neg | |  | Neg |  |  | Pos |
| N-methyl-N'-nitro-N-nitrosoguanidine | 70-25-7 | 0.003 |  | Pos g |  | Pos | Pos | Pos | Pos | Pos |  | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| N-nitrosodiethanolamine | 1116-54-7 | 1 - 4 | Neg | Neg | [3] | Pos | Pos |  | Pos | Pos | Pos |  | Eqv | |  | Neg | Neg | Pos | Pos |
| Nona-2 trans- 6-cis-dienal | 557-48-2 | 0.25 - 0.5 ª | Neg | Neg | [4] |  |  |  | Pos |  |  |  |  | |  | Neg |  | Neg |  |
| o-Toluidine | 95-53-4 | 5 - 20 ª | Pos | Pos | [2] | Pos | Pos | Neg | Pos | Eqv | Pos | Neg |  | |  | Neg | Neg | Pos | Pos |
| p-Cresidine | 120-71-8 | 2 - 4 b | Neg | Pos | [2] |  | Pos |  |  | Eqv |  | Neg | Neg | | Pos | Neg |  | Pos | Pos |
| Phenacetin | 62-44-2 | 1 - 1.5 b | Neg | Neg | [5] |  | Pos |  | Neg | Pos | Pos |  |  | |  | Pos |  | Pos | Pos |
| Phenobarbital | 57-30-7 | 2.5 - 30 ª | Neg | Neg | [5,6] | Neg | Neg | Pos |  | Pos | Neg | Neg |  | |  | Neg | Neg |  | Pos |
| p-Methoxycinnamaldehyde | 1963-36-6 | 1.5 - 2.5 | Pos | Pos | [4] |  | Pos |  | Pos |  |  |  |  | |  | Neg |  | Neg |  |
| p-Phenylenediamine | 106-50-3 | 1.2 - 5 ª | Neg | Neg | [2] |  | Pos | Pos | Pos | Pos | Pos | Neg | Neg | |  | Neg |  | Neg | Neg |
| Prilocaine | 1786-81-8 | 5 - 15 | Pos | Pos | [5] | Pos |  |  |  |  |  |  |  | |  |  |  |  |  |
| Pulegone | 89-82-7 | 0.5 - 1 ª | Neg | Eqv |  |  | Neg |  |  |  |  |  |  | |  | Neg |  |  | Pos |
| Quinoline | 91-22-5 | 2 - 5 ª | Pos | Pos |  | Pos | Pos | Pos |  | Pos |  | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| Resorcinol | 108-46-3 | 2 - 8 | Neg | Neg |  |  | Neg | Pos | Pos | Pos | Pos |  |  | |  | Neg | Neg |  | Pos |
| Riddelliine | 23246-96-0 | 0.5 - 2 | Pos | Pos |  | Pos | Pos |  |  | Pos | Pos |  | Pos | | Pos | Pos |  |  | Pos |
| Safrole | 94-59-7 | 1 - 6 | Pos | Pos | [1] | Pos | Neg | Pos | Pos | Pos |  | Neg | Neg | |  |  | Pos |  | Pos |
| Senecionine | 130-01-8 | 0.75 - 1 b | Neg | Neg |  | Pos | Neg |  |  |  | Pos |  |  | |  |  |  |  |  |
| Streptozotocin | 18883-66-4 | 2.5 - 10 | Neg | Eqv | [5] | Pos | Pos | Pos |  |  | Pos |  |  | | Pos | Pos |  | Pos | Pos |
| Tamoxifen | 54965-24-1 | 0.25 - 0.5 b | Pos | Pos | [5] | Pos | Neg |  | Pos |  | Pos |  |  | |  | Pos | Pos |  | Pos |
| Teniposide | 29767-20-2 | 0.5 - 0.75 b | Neg | Pos | [5] |  | Pos | Pos | Pos | Pos | Pos |  |  | |  | Pos | Pos |  |  |
| Trans-2-Hexenal | 6728-26-3 | 0.25 - 1 ª | Neg | Neg |  | Pos in vitro | Pos | Pos | Pos | Neg |  |  | Neg | | Neg | Neg |  | Neg |  |
| Xylitol | 87-99-0 | 2 - 4 | Neg | Neg |  |  | Neg |  |  |  |  |  |  | |  | Neg |  |  | Neg |

a, next dose level reduced viability by over 50%;

b, higher dosage could not be achieved due to low solubility;

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e, data from Lhasa carcinogenicity database (<https://lcdb.lhasacloud.org/study-information/44482914>), last accessed 11/27/2024.

f, data from: (Williams et al. 1989)

g, dosing was conducted under a different protocol

CEGA References (Ref): [1] (Kobets et al. 2019a); [2] (Kobets et al. 2019b); [3] (Williams et al. 2014); [4] (Thakkar et al. 2024); [5] (Kobets et al. 2022); [6] (Iatropoulos et al. 2017).

*Abbreviations*: Ames, the bacterial reverse mutation test; CA, chromosome aberration assay; CARC, carcinogenicity (mainly in rodents); Comet, alkaline single cell gel electrophoresis assay; Eqv, equivocal (a clear positive or negative outcome cannot be concluded or response is inconsistent between experiments); hcl, hydrochloride; HPC/DRA, hepatocyte DNA-repair test; MCGM, mammalian cell gene mutation assay (mouse lymphoma Tk+/− mutation assay and Hprt mutation assay); MN, micronucleus assay; Neg, negative; NPL, 32P-nucleotide postlabeling assay; Pos, positive; TGR, transgenic rodent gene mutation assays; UDS, unscheduled DNA synthesis

Table 2. Outcomes in the Turkey Egg Genotoxicity Assay (TEGA) compared to selected in vitro and vivo genotoxicity data and carcinogenicity in rodents.

| **Compound** | **CAS #** |  | **TEGA** | |  | **DNA Adducts** | **In vitro genotoxicity c, d** | | | | |  |  | **In vivo genotoxicity c** | | | | | **CARC c, e** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Tested dosages, mg/egg** | **NPL** | **Comet** | **Ref** | **Ames** | **MCGM** | **MN** | **CA** | **Comet** | **Rat HPC/DRA f** |  | **UDS** | **TGR** | **MN** | **CA** | **Comet** |
| α-Asarone | 2883-98-9 | 5 - 10 ª | Pos | Pos | [1] | Pos in vitro | Eqv |  | Neg |  | Pos |  |  | |  |  |  |  | Pos |
| β-Asarone | 5273-86-9 | 2.5 - 5 ª | Pos | Pos | [1] | Pos in vitro | Eqv |  | Pos |  | Pos |  |  | |  |  |  |  | Pos |
| 2,3-Dihydro-1,1-dimethyl-1H-indene-arpropanal | 300371-33-9 | 20 - 40 ª | Neg | Neg | [2] |  | Neg |  | Eqv |  |  |  |  | |  |  |  |  |  |
| 2-Acetylaminofluorene | 53-96-3 | 0.0005 - 0.05 | Pos |  | [3] | Pos | Pos | Pos | Pos | Pos | Pos | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| 4-Hydroxy-2.5-dimethyl-3(2H) furanone | 3658-77-3 | 7.5 - 120 a | Neg | Neg | [2] |  | Pos | Neg | Pos |  |  |  | Pos | |  | Neg |  |  | Neg |
| 100 - 120 a |  | Pos  + FPG |
| Aflatoxin B1 | 1162-65-8 | 0.062 μg - 0.0062 |  | Pos g | [4] | Pos | Pos |  | Pos | Pos |  | Pos |  | | Pos | Pos | Pos | Neg | Pos |
| Allyl isothiocyanate | 57-06-7 | 1.2 - 3 ª | Neg | Neg | [2] |  | Eqv | Pos | Neg | Pos | Pos |  | Neg | | Neg | Neg |  |  | Pos |
| Anethole | 4180-23-8 | 5 - 10 ª | Pos | Neg | [5] | Pos | Eqv | Pos |  | Neg |  |  | Neg | |  | Neg |  |  | Eqv |
| Aristolochic acid I | 313-67-7 | 1 - 2 | Pos | Eqv |  | Pos | Pos | Pos | Pos | Pos | Neg |  | Neg | | Pos | Eqv |  | Pos | Pos |
| Azaserine | 115-02-6 | 1.25 - 2.5 | Neg | Pos |  | Pos | Pos |  |  |  |  |  | Pos | |  | Pos |  |  | Pos |
| Benzo[a]pyrene | 50-32-8 | 0.05 - 0.3 | Pos |  | [3] | Pos | Pos | Pos | Pos | Pos | Pos | Pos | Eqv | | Pos | Pos | Pos | Eqv | Pos |
| Caffeine | 58-08-2 | 5 - 10 | Neg | Neg |  |  | Neg | Pos | Pos | Pos |  |  |  | |  | Eqv | Neg |  | Neg |
| Cinnamaldehyde | 104-55-2 | 10 - 20 a | Neg | Neg | [2] | Pos | Neg | Eqv | Pos | Pos |  |  | Eqv | | Neg |  |  | Neg | Neg |
| Curcumin | 458-37-7 | 5 - 10 ª | Neg | Eqv |  | Pos | Neg |  | Pos | Eqv | Pos |  |  | |  | Neg | Eqv |  | Neg |
| Diacetyl | 431-03-8 | 6.3 - 25 ª | Neg | Neg | [2] | Pos in vitro | Pos | Pos |  |  |  |  | Pos | |  | Neg |  |  | Eqv |
| D-Limonene | 5989-27-5 | 80 - 100 | Neg | Neg | [2] |  | Neg |  |  | Neg |  |  |  | | Neg | Neg |  | Neg | Pos |
| Elemicin | 487-11-6 | 0.2 - 50 a | Pos | Pos | [5] | Pos |  |  |  |  |  |  |  | |  |  |  |  |  |
| Estragole | 140-67-0 | 0.25 - 40 | Pos | Pos | [5] | Pos | Neg |  |  | Neg |  |  | Pos | |  | Eqv |  | Pos | Pos |
| Eugenol | 97-53-0 | 1 - 2.5 ª | Neg | Neg | [2] | Neg | Neg | Pos | Pos | Pos | Neg |  | Neg | | Neg | Neg |  |  | Neg |
| Geraniol | 106-24-1 | 10 - 25 a | Neg | Neg | [2] |  | Neg | Neg | Neg | Neg | Neg |  |  | |  | Neg |  |  |  |
| Geranyl nitrile | 5146-66-7 | 6.3 - 25 a | Neg | Neg | [2] |  | Neg | Neg |  | Pos |  |  | Pos | |  | Pos | Pos |  |  |
| Griseofulvin | 126-07-8 | 1 - 1.5 | Neg | Neg |  |  | Neg | Neg | Pos | Pos |  | Neg |  | |  | Neg | Pos |  | Pos |
| Hydroquinone | 123-31-9 | 5 - 15 | Neg | Pos |  | Neg in vivo | Neg | Pos | Pos | Eqv | Pos |  |  | | Neg | Pos | Pos | Neg | Pos |
| iso-Eugenol | 97-54-1 | 1 - 4 ª | Neg | Neg | [5] |  | Neg |  |  | Neg |  |  |  | |  | Neg |  |  | Pos |
| Isophorone | 78-59-1 | 17 - 70 ª | Neg | Neg | [2] | Neg | Neg | Pos |  | Pos |  |  | Neg | |  | Neg |  |  | Pos |
| Isopropyl alcohol | 67-63-0 | 50 - 100 | Neg | Neg |  |  |  | Neg |  |  |  |  |  | |  | Neg |  |  | Neg |
| Isoquinoline | 119-65-3 | 3.8 - 15 | Neg | Pos |  |  | Neg |  |  | Pos |  | Neg |  | |  | Neg |  |  |  |
| Lasiocarpine | 303-34-4 | 0.3 - 1.2 | Neg | Neg |  | Pos | Pos |  |  | Pos | Pos | Pos |  | |  | Pos |  |  | Pos |
| Lauric aldehyde | 112-54-9 | 40 - 100 b | Neg | Neg | [2] |  | Neg i |  | Pos i |  |  |  |  | |  | Neg i |  |  |  |
| Maltol | 118-71-8 | 5 - 20 | Neg | Pos | [2] |  | Pos |  | Pos | Pos |  |  | Eqv | | Neg | Pos |  | Neg |  |
| Menthol | 89-78-1 | 20 - 60 b | Neg | Neg | [2] |  | Neg | Neg | Neg | Pos |  |  |  | | Neg | Neg |  | Neg | Neg |
| Methyl dihydrojasmonate | 24851-98-7 | 7.5 - 60 a | Neg | Neg | [2] |  | Neg | Neg |  |  |  |  | Neg | |  | Neg |  |  |  |
| Methyl eugenol | 93-15-2 | 0.005 - 4 | Pos | Neg | [2, 5] | Pos | Neg | Neg | Neg | Neg |  |  |  | | Pos | Neg |  | Pos | Pos |
| Methyl isoeugenol | 93-16-3 | 40 - 80 a | Neg | Neg | [5] |  | Neg |  | Neg i |  |  |  |  | |  | Neg i |  |  |  |
| Methylenedioxyphenyl-2-methyl propanal | 1205-17-0 | 25 - 75 | Pos | Neg |  |  | Neg |  | Neg | Pos |  |  |  | |  | Neg |  |  |  |
| Mitomycin C | 50-07-7 | 0.25 - 1 | Pos | Neg h |  | Pos | Pos | Pos | Pos | Pos | Pos | Pos |  | | Pos | Pos | Pos | Pos | Pos |
| Monocrotaline | 315-22-0 | 3 b | Neg | Neg h |  | Pos | Neg | Pos |  | Pos |  |  |  | |  | Pos |  | Neg | Pos |
| Myristicin | 607-91-0 | 0.08 - 50 | Pos | Pos | [5] | Pos | Neg |  |  |  | Neg |  |  | |  | Pos |  |  |  |
| N-methyl-N'-nitro-N-nitrosoguanidine | 70-25-7 | 0.003 |  | Pos g |  | Pos | Pos | Pos | Pos | Pos |  | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| Nutmeg oil | 8008-45-5 | 0.6 - 100 b | Pos | Neg |  |  | Neg |  |  | Neg |  |  |  | |  |  |  |  |  |
| Ochratoxin A | 303-47-9 | 0.05 - 0.1 a | Neg | Pos |  | Pos | Neg | Pos | Pos | Neg |  | Neg |  | |  |  | Pos | Pos | Pos |
| Pent-1-en-3-one | 1629-58-9 | 0.5 - 1 a | Neg | Neg | [2] | Pos in vitro | Pos | Eqv | Neg |  |  |  |  | |  | Neg | Neg | Neg |  |
| Perillaldehyde | 2111-75-3 | 2.5 - 10 | Neg | Neg |  |  | Neg | Neg | Neg | Eqv |  |  |  | |  | Neg |  | Neg |  |
| Pimento Oil | 8006-77-7 | 0.25 - 40 | Pos |  |  |  |  |  |  |  |  |  |  | |  |  |  |  |  |
| Podophylotoxin | 518-28-5 | 0.02 - 0.1 a | Neg | Eqv |  |  | Eqv | Neg | Pos | Pos |  |  |  | |  | Pos |  |  |  |
| p-t-Butyl-α-methylhydro-cinnamic aldehyde | 80-54-6 | 5 - 20 a | Neg | Pos | [2] |  | Neg | Neg | Neg | Pos | Neg |  |  | |  | Eqv |  |  | Pos |
| p-tert-Butyldihydrocinnamaldehyde | 18127-01-0 | 5 - 20 a | Pos | Pos | [2] |  | Neg | Neg | Neg |  |  |  |  | |  |  |  |  |  |
| Quercetin | 117-39-5 | 1 - 2.4 b | Neg | Neg | [2] |  | Pos | Pos | Pos | Pos |  | Neg | Neg | |  | Neg | Neg |  | Pos |
| Quinine | 130-95-0 | 2.5 - 10 b | Neg | Neg |  |  | Neg |  | Neg |  |  |  |  | |  | Neg | Neg |  |  |
| Quinoline | 91-22-5 | 0.04 - 15 a | Pos | Pos | [2, 3] | Pos | Pos | Pos |  | Pos |  | Pos | Pos | | Pos | Pos | Pos | Pos | Pos |
| Resorcinol | 108-46-3 | 2.5 - 5 a | Neg | Neg |  |  | Neg | Pos | Pos | Pos | Pos |  |  | |  | Neg | Neg |  | Pos |
| Riddelliine | 23246-96-0 | 0.5 - 7.5 | Neg | Neg |  | Pos | Pos |  |  | Pos | Pos |  | Pos | | Pos | Pos |  |  | Pos |
| Safrole | 94-59-7 | 0.13 - 2 | Pos | Neg | [5] | Pos | Neg | Pos | Pos | Pos |  | Neg | Neg | |  |  | Pos |  | Pos |
| Streptozotocin | 18883-66-4 | 1 - 5 | Neg | Neg |  | Pos | Pos | Pos |  |  | Pos |  |  | | Pos | Pos |  | Pos | Pos |
| Teniposide | 29767-20-2 | 0.5 - 1.5 | Neg | Eqv |  |  | Pos | Pos | Pos | Pos | Pos |  |  | |  | Pos | Pos |  |  |
| Theobromine | 83-67-0 | 1 - 10 | Neg | Neg |  |  | Neg | Eqv |  | Neg |  |  |  | |  | Pos | Neg |  |  |
| Trans-2-Hexenal | 6728-26-3 | 2.5 - 10 a | Neg | Pos | [2] | Pos in vitro | Pos | Pos | Pos | Neg |  |  | Neg | | Neg | Neg |  | Neg |  |
| Xylitol | 87-99-0 | 2.5 - 5 a | Neg | Pos |  |  | Neg |  |  |  |  |  |  | |  | Neg |  |  | Neg |
| Zearalenone | 17924-92-4 | 0.5 - 1 | Neg | Neg |  | Pos | Neg |  | Pos | Pos | Pos | Neg |  | |  | Pos | Pos | Neg | Pos |

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e, data from Lhasa carcinogenicity database (<https://lcdb.lhasacloud.org/study-information/44482914>), last accessed 11/27/2024.

f, data from: (Williams et al. 1989)

g, dosing was conducted under a different protocol

h, significant decrease in comet tail

i, read-across data

TEGA References (Ref): [1] (Kobets et al. 2019a); [2] (Kobets et al. 2018a); [3] (Kobets et al. 2024); [4] (Williams J. et al. 2011); [5] (Kobets et al. 2016)

*Abbreviations*: Ames, the bacterial reverse mutation test; CA, chromosome aberration assay; CARC, carcinogenicity (mainly in rodents); Comet, alkaline single cell gel electrophoresis assay; Eqv, equivocal (a clear positive or negative outcome cannot be concluded or response is inconsistent between experiments); HPC/DRA, hepatocyte DNA-repair test; MCGM, mammalian cell gene mutation assay (mouse lymphoma Tk+/− mutation assay and Hprt mutation assay); MN, micronucleus assay; Neg, negative; NPL, 32P-nucleotide postlabeling assay; Pos, positive; TGR, transgenic rodent gene mutation assays; UDS, unscheduled DNA synthesis.

## C. Comparison of Outcomes in the Chicken and Turkey Egg Fetal Liver Assays

When comparing overall outcomes for twenty-one (21) compounds tested in both CEGA and TEGA, sixteen (16) compounds (76%) were concordant in both assays, including eleven (11) compounds which were positive and four (4) which were negative (**Table 3**). Teniposide was positive in the comet assay in CEGA, and equivocal in TEGA comet. Five (5) compounds (24%) showed discordant results between CEGA and TEGA (**Table 3**).

Table 3. **Comparison of outcomes in the Chicken (CEGA) and Turkey (TEGA) Egg Genotoxicity Assays.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Compound** | **CEGA** | | |  | **TEGA** | | |
| **Tested dosages, mg/egg** | **NPL** | **Comet** |  | **Tested dosages, mg/egg** | **NPL** | **Comet** |
| α-Asarone | 2 - 8 | Pos | Pos | 5 - 10 ª | | Pos | Pos |
| β-Asarone | 0.12 - 0.5 ª | Pos | Neg | 2.5 - 5 ª | | Pos | Pos |
| 2-Acetylaminofluorene | 0.02 - 0.6 b | Pos | Pos | 0.0005 - 0.05 | | Pos |  |
| Aflatoxin B1 | 0.0002 - 0.003 ª | Neg | Pos | 0.062 μg - 0.0062 | |  | Pos c |
| Benzo[a]pyrene | 0.03 - 0.5 | Pos | Pos | 0.05 - 0.3 | | Pos |  |
| Caffeine | 2.5 - 5 | Neg | Neg | 5 - 10 | | Neg | Neg |
| Eugenol | 1.5 - 3 ª | Neg | Neg | 1 - 2.5 ª | | Neg | Neg |
| Griseofulvin | 0.1 - 0.4 | Neg | Pos | 1 - 1.5 | | Neg | Neg |
| Isoquinoline | 2 - 4 ª | Pos | Pos | 3.8 - 15 | | Neg | Pos |
| Lasiocarpine | 0.25 - 0.5 ª | Neg | Neg | 0.3 - 1.2 | | Neg | Neg |
| Methyl eugenol | 1 - 2 | Pos | Neg | 0.005 - 4 | | Pos | Neg |
| Mitomycin C | 0.07 - 0.125 ª | Pos | Eqv | 0.25 - 1 | | Pos | Neg d |
| N-methyl-N'-nitro-N-nitrosoguanidine | 0.003 |  | Pos c | 0.003 | |  | Pos c |
| Quinoline | 2 - 5 ª | Pos | Pos | 0.04 - 15 a | | Pos | Pos |
| Resorcinol | 2 - 8 | Neg | Neg | 2.5 - 5 a | | Neg | Neg |
| Riddelliine | 0.5 - 2 | Pos | Pos | 0.5 - 7.5 | | Neg | Neg |
| Safrole | 1 - 6 | Pos | Pos | 0.13 - 2 | | Pos | Neg |
| Streptozotocin | 2.5 - 10 | Neg | Eqv | 1 - 5 | | Neg | Neg |
| Teniposide | 0.5 - 0.75 b | Neg | Pos | 0.5 - 1.5 | | Neg | Eqv |
| Trans-2-Hexenal | 0.25 - 1 ª | Neg | Neg | 2.5 - 10 a | | Neg | Pos |
| Xylitol | 2 - 4 | Neg | Neg | 2.5 - 5 a | | Neg | Pos |

a, next dose level reduced viability by over 50%;

b, higher dosage could not be achieved due to low solubility;

c, dosing was conducted under a different protocol;

d, significant decrease in comet tail

*Abbreviations*: Comet, alkaline single cell gel electrophoresis assay; Eqv, equivocal; Neg, negative; NPL, 32P-nucleotide postlabeling assay; Pos, positive.

## D. Concordance Analysis of the *In Ovo*, In Vitro and In Vivo Data

The concordance analysis revealed closer correlation of CEGA and TEGA results with in vivo outcomes compared to in vitro data (**Figures 5 and 6**).

### i. Concordance with In Vitro Data

The overall sensitivity of the CEGA model ranged from 51% to 88% when compared to in vitro assays, including Ames, mammalian cell gene mutation (MCGM), micronucleus (MN), chromosome aberrations (CA), and rat HPC/DRA assays. Specificity was lower, ranging from 29% to 61% (**Figure 5A**). Similarly, sensitivity of TEGA raged from 56% to 80%, and specificity varied from 40% to 61% depending on the in vitro assay (**Figure 5B**). The predictivity of CEGA and TEGA was the highest for Ames and HPC/DRA assays. Notably, FDR were generally low for both *in ovo* models, while accuracy values for most assays exceeded 50% (**Figure 5**).

### ii. Concordance with In Vivo Data

Sensitivity of CEGA for in vivo genotoxicity assays, including unscheduled DNA synthesis (UDS), transgenic rodent assays (TGR), MN and CA assays, ranged from 61% to 89% (**Figure 6A**). Specificity was also high, ranging from 60% to 100%. Overall accuracy was around 80% for most endpoints (**Figure 6A**). Similarly, sensitivity of TEGA varied from 57% to 80%, and specificity varied from 56% to 100% (**Figure 6B**).

CEGA demonstrated a very robust predictivity for carcinogenicity outcomes, with sensitivity of 73%, specificity of 92% and accuracy of 77% (**Figure 6A**). These metrics were slightly lower for TEGA, with sensitivity reaching 57%, specificity being 63%, and accuracy of 58% (**Figure 6B**).

|  |
| --- |
| **A. CEGA vs in vitro genotoxicity assays** |
|  |
| |  |  |  |  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | | **CEGA/Ames** | | **CEGA/MCGM** | | **CEGA/MN** | | **CEGA/CA** | | **CEGA/rat HPC/DRA** | | | TP: 34 | FP: 13 | TP: 20 | FP: 5 | TP: 23 | FP: 2 | TP: 28 | FP: 4 | TP: 23 | FP: 2 | | FN: 14 | TN: 20 | FN: 19 | TN: 2 | FN: 19 | TN: 3 | FN: 22 | TN: 2 | FN: 19 | TN: 3 | |
| **B.** **TEGA vs in vitro genotoxicity assays** |
|  |
| |  |  |  |  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | | **TEGA/Ames** | | **TEGA/MCGM** | | **TEGA/MN** | | **TEGA/CA** | | **TEGA/rat HPC/DRA** | | | TP: 16 | FP: 13 | TP: 12 | FP: 5 | TP: 16 | FP: 5 | TP: 16 | FP: 6 | TP: 6 | FP: 3 | | FN: 7 | TN: 20 | FN: 12 | TN: 7 | FN: 9 | TN: 7 | FN: 15 | TN: 4 | FN: 1 | TN: 3 | |

Figure 5. **Comparison of the outcomes in the Chicken (CEGA) (A) and Turkey (TEGA) (B) egg genotoxicity assays with available in vitro data.** Tables underneath the bar charts represent confusion matrices with numbers of chemicals that produced either positive or negative outcomes for each respective endpoint. Equivocal results were considered to be positive.

*Abbreviations*: CA, chromosome aberration assay; HPC/DRA, hepatocyte DNA-repair test; FDR, false discovery rate; FN, false negative; FP, false positive; MCGM, mammalian cell gene mutation assay; MN, micronucleus assay; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.

|  |
| --- |
| **A. CEGA vs in vivo genotoxicity assays** |
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| |  |  |  |  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | | **CEGA/UDS** | | **CEGA/TGR** | | **CEGA/MN** | | **CEGA/CA** | | **CEGA/CARC** | | | TP: 17 | FP: 3 | TP: 17 | FP: 2 | TP: 20 | FP: 17 | TP: 18 | FP: 4 | TP: 38 | FP: 1 | | FN: 2 | TN: 8 | FN: 2 | TN: 4 | FN: 9 | TN: 19 | FN: 5 | TN: 6 | FN: 14 | TN: 12 | |
| **B. TEGA vs in vivo genotoxicity assays** |
|  |
| |  |  |  |  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | | **TEGA/UDS** | | **TEGA/TGR** | | **TEGA/MN** | | **TEGA/CA** | | **TEGA/CARC** | | | TP: 8 | FP: 4 | TP: 8 | FP: 3 | TP: 15 | FP: 8 | TP: 11 | FP: 0 | TP: 17 | FP: 3 | | FN: 4 | TN: 5 | FN: 2 | TN: 5 | FN: 8 | TN: 18 | FN: 3 | TN: 6 | FN: 13 | TN: 5 | |

Figure 6. Comparison of the outcomes in the Chicken (CEGA) (A) and Turkey (TEGA) (B) egg genotoxicity assays with available in vivo data. Tables underneath the bar charts represent confusion matrices with numbers of chemicals that produced either positive or negative outcomes for each respective endpoint. Equivocal results were considered to be positive.

*Abbreviations*: CA, chromosome aberration assay; CARC, carcinogenicity; FDR, false discovery rate; FN, false negative; FP, false positive; MN, micronucleus assay; NPV, negative predictive value; PPV, positive predictive value; TGR, transgenic rodent gene mutation assays; TN, true negative; TP, true positive; UDS, unscheduled DNA synthesis.

### iii. Performance of NPL and Comet Assays

Thirty (30) compounds were positive in the NPL assay in CEGA and seventeen in TEGA. Most of these chemicals are capable of binding to DNA in vitro and in vivo (**Tables 1** and **2**). In the comet assay, thirty-seven (37) were positive and six (6) equivocal in CEGA and eighteen (18) were positive and four (4) equivocal in TEGA.

The concordance analysis for outcomes in the NPL or comet assays with respective endpoints tested in other systems is provided in **Figure 7**. The NPL assay in CEGA and TEGA strongly correlated with the ability of a chemical to form DNA adducts in vitro and/or in vivo. The specificity of the assay for DNA adduct detection was 100% in both *in ovo* models, while sensitivity was 61% in CEGA and 45% in TEGA (**Figure 7**). For comet assay outcomes, CEGA sensitivity was 66% and 71% and specificity was 100% and 82% for in vitro and in vivo outcomes, respectively (**Figure 7A**). The comet assay in TEGA exhibited similar pattern but lower sensitivity (42% for in vitro and 63% for in vivo comet assay) and specificity (40% for in vitro and 64% for in vivo comet assay) (**Figure 7B**). Most accuracy values for NPL and comet assay outcomes in CEGA and TEGA exceeded 50%.

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| --- |
| A. CEGA vs in vitro and vivo DNA damage endpoints |
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| |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | | **CEGA NPL/DNA adducts** | | **CEGA comet /In vitro comet** | | **CEGA comet /In vivo comet** | | | TP: 27 | FP: 0 | TP: 21 | FP: 0 | TP: 20 | FP: 2 | | FN: 17 | TN: 6 | FN: 11 | TN: 6 | FN: 8 | TN: 9 | |
| **B. TEGA vs in vitro and vivo DNA damage endpoints** |
|  |
| |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | | **TEGA NPL/DNA adducts** | | **TEGA comet /In vitro comet** | | **TEGA comet /In vivo comet** | | | TP: 13 | FP: 0 | TP: 5 | FP: 3 | TP: 5 | FP: 4 | | FN: 16 | TN: 3 | FN: 7 | TN: 2 | FN: 3 | TN: 7 | |

Figure 7. Comparison of the outcomes in the 32P-nucleotide postlabeling (NPL) and comet assays in the Chicken (CEGA) (A) and Turkey (TEGA) (B) egg genotoxicity assays with respective in vitro and in vivo endpoints. Tables underneath the bar charts represent confusion matrices with numbers of chemicals that produced either positive or negative outcomes for each respective endpoint. Equivocal results were considered to be positive.

*Abbreviations*: FDR, false discovery rate; FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.

# 5. Discussion

The concordance analysis of outcomes for 125 compounds tested in the *in ovo* assays (**Figures 5 and 6**) documented a reasonably high reliability of CEGA and TEGA in detecting genotoxic potential of chemical entities. Comparison with genotoxicity and carcinogenicity endpoints tested in other systems in vitro and in vivo revealed that CEGA and TEGA outcomes align more closely with in vivo genotoxicity data than with in vitro results. Thus, for CEGA, approximately 90% of the compounds positive in the system demonstrated genotoxicity in at least one of in vivo assays, while alignment for TEGA was slightly lower at 72%. Notably, specificity and sensitivity of CEGA and TEGA varied depending on the compared endpoint (**Figures 5 and 6**) suggesting that the *in ovo* assays are particularly robust for detecting certain types of DNA damage.

CEGA also demonstrated strong predictivity, with a sensitivity of 73% and specificity of 92%, for carcinogenicity outcomes, in particular for genotoxic carcinogens (**Table 1** and **Figure 6A**). In comparison, TEGA had slightly lower predictive power with a sensitivity of 57% and specificity of 63%. Nevertheless, TEGA still showed strong alignment with rodent carcinogenicity findings, notably, for activation-dependent carcinogens (e.g., estragole, methyl eugenol, and safrole) (**Table 2**). The high concordance of CEGA and TEGA with in vivo data, coupled with their ability to detect a wide range of genotoxic carcinogens, positions these *in ovo* models as valuable NAMs for regulatory testing.

One of the key strengths of CEGA and TEGA lies in their metabolic competence which simulates mammalian biotransformation processes more accurately than traditional in vitro systems and enables detection of genotoxic potential for compounds requiring metabolic bioactivation. Thus, the majority of polycyclic aromatic hydrocarbons, aromatic amines, and alkenylbenzenes were consistently identified as genotoxic *in ovo* (**Tables 1 and 2**), aligning with their genotoxic potential in vivo.

The comparative analysis of CEGA and TEGA demonstrated high degree of agreement between results in both models for the majority (76%) of tested compounds (**Table 3**). Nevertheless, five (5) compounds, griseofulvin, riddelliine, streptozotocin, trans-2-hexenal, and xylitol, exhibited discordant outcomes between CEGA and TEGA (**Table 3**), highlighting potential differences in sensitivity or the biological responses of the two avian systems. Such discrepancies may reflect species-specific variations in metabolic enzyme expression or differences in assay conditions, such as dose levels and timing of dosing. For example, dosing in TEGA occurs later (incubation days 22 to 24) than in CEGA (incubation days 9 to 11), as such, metabolism in the turkey fetal liver is likely to be at more advanced stages of development.

In order to analyze both CEGA and TEGA effectively, it is essential to take into account temporal and spatial differences that exist in the morphogenetic development of the turkey and the chicken fetuses (Bakst 1997). The eggs of these two avian species are also physically different; the white leghorn chicken egg is roughly 60 g, while the medium white turkey egg is approximately 90 g. The fetuses inside the chicken and turkey eggs develop at different rates. Specifically, the cells within the turkey egg multiply more rapidly compared to those of the chicken egg, thereby resulting in a larger egg (Bakst 1997). In addition, compared to the chicken egg, turkey eggs have a lower hatchability rate. These differences might play a role when comparing responses of the two eggs to the chemicals assessed. This also suggests that while CEGA and TEGA can complement each other, they may differ in their capacity to respond to certain genotoxic mechanisms. Combining data from both models could enhance the overall predictivity for in vivo outcomes. Further investigation may be warranted for discordant compounds to determine whether differences are due to assay-specific factors or intrinsic variability within the test systems.

# 6. Conclusions

The findings from the current study support the utility of CEGA and TEGA as reliable NAMs for genotoxicity assessment. FAEFL/DDA models provide a metabolically competent alternative to in vivo genotoxicity assays, demonstrating strong concordance with in vivo outcomes and high predictivity for carcinogenic potential.

# 7. Statement of Author Contributions

Dr. Williams and Dr. Kobets contributed to the study design and data analyses. Both authors have complete access to the data. Dr. Kobets prepared the manuscript draft, figures, and tables. Dr. Williams provided important intellectual input and revisions. Both authors approved the final manuscript.

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