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Assessment of the genotoxic and mutagenic effects induced by T-2 mycotoxin in HepG2 cells

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ABSTRACT

The T-2 toxin is a mycotoxin produced by molds belonging to *Fusarium*. Among the *Fusarium* mycotoxins, trichothecenes are frequently reported in food and feed, being the T-2 toxin (T-2) the mycotoxin which possesses the highest toxicity. According to EFSA, T-2 is found in various cereal grains used in food and feed products, mainly in oats, and it has a high environmental impact due to its mechanisms of toxicity. However, recent information on its genotoxic and mutagenic effects is lacking. This work aimed to evaluate the genotoxic and mutagenic potential of T-2 in vitro. For this purpose, HepG2 cells were exposed to 15, 30, and 60 nM T-2 for 24 h, then the DNA damage was evaluated by the micronucleus and the comet assays. In addition, point mutation analysis was performed by the bacterial reverse mutation test using 0.15–60 nM of T-2 concentrations. The results showed chromosomal damage at 60 nM T-2 since significantly more MN appeared at this concentration than in the control samples. Regarding the comet assay, DNA double helix breaks appeared at all concentrations tested and, in a concentration-dependent manner. However, no mutagenic effects were observed at any of the concentrations tested for the *Salmonella typhimurium* (*S. Typhimurium*) strains TA98, TA100, TA1535, TA1537, or the *Escherichia coli (E. Coli)* WP2 strain in the absence or presence of a metabolic activation system. Therefore, these results showed that T-2 mycotoxin produced genotoxic effects by MN and comet assay, while no mutagenicity was observed. However, further research simulating different metabolic activation pathways and the combined exposure of this mycotoxin with other mutagenic chemicals that could be present in the diet is necessary to discard the mutagenic potential of T-2 fully. These results highlight the carcinogenic potential and danger associated with T-2 exposure and should be considered to prevent associated food risks for the human population.

1. Introduction

The increasing incidence of cancer is a public health concern and may be attributed to changes in the environment and lifestyle exposures, including diet (Lécuyer et al., 2022). During the last decades, several investigations have been carried out to study how the whole diet may cause point mutations, thus, increasing the risk of developing cancer (Agudo et al., 2018; Shivappa et al., 2014; Van Woudenbergh et al., 2013). Several dietary factors such as nitrites, coffee, tea, processed meat, or alcohol have been associated with cancer risk (IARC, 2023). Although the role of these factors in carcinogenesis is not clearly understood, evidence suggests that chronic diseases and carcinogenesis are promoted by inflammatory processes (Lécuyer et al., 2022; Mantovani et al., 2008). Some food contaminants, such as mycotoxins, to which we could be exposed through our daily diet have been classified by the International Agency for Research on Cancer (IARC) as carcinogenic.

Abbreviations: 2-AA, 2-aminoanthracene; 9-AC, 9-aminoacridine; 2-NF, 2-Nitrofluorene; AFB1, Aflatoxin B1; BEA, Beauvericin; CECT, Spanish Collection of Culture Strains; CRL, control; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, Dimethyl sulfoxide; DON, Deoxynivalenol; EDTA, Ethylenediaminetetraacetic acid; EFSA, European Food Safety Agency; EMA, nucleic acid dye A; EtOH, ethanol; IARC, International Agency for Research on Cancer; IC_{50}, Mean inhibition concentration; LMA, Low Melting point Agarose; MeOH, methanol; MN, micronucleus; MTT, methylthiazol tetrazolium salt; NADP, Nicotinamide Adenine Dinucleotide Phosphate; NaN_3, sodium azide; NBCS, Newborn Calf Serum; OECD, Organisation for Economic Cooperation and Development; OTA, Ochratoxin A; PBS, Phosphate Buffered Saline; PI, Propidium Iodide; RNase A, ribonuclease A; S9, metabolic activation system fraction; SEM, Standard Error of the Mean; Trp, tryptophan.

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This is the case of aflatoxins that have been classified as carcinogenic to humans (Group 1) and fumonisin B1 and B2, fusarin C, and ochratoxin A (OTA), which have been classified as possibly carcinogenic to humans (Group 2B) (IARC, 2023).

The T-2 toxin (T-2) belongs to the large group of mycotoxins called trichothecenes, which represent the main group of *Fusarium* toxins, and the trichothecene that possesses the highest toxicity. The T-2 is mainly found in grain cereals (EFSA, 2011a). The T-2 may cause adverse effects at the cellular and organ levels, such as hepatotoxicity, neurotoxicity, immunodepression, and bone marrow injury (Dai et al., 2019). Regarding its carcinogenicity potential, the IARC classifies toxins derived from *Fusarium sporotrichioides*, including T-2, as "not classifiable as to its carcinogenicity to humans" (Group 3) due to the available data do not support an evaluation of sufficient evidence in experimental animals or mechanistic studies (IARC, 2023). However, no recent information on in vitro mutagenic properties of T-2 has been published and no further conclusions have been formulated yet (EFSA, 2011a).

Considering that humans and animals could be continuously exposed to T-2 through diet, the analysis of the mutagenic and genotoxic potential of this mycotoxin results in great interest. According to the European Food Safety Agency (EFSA), a standard in vitro battery is recommended for the genotoxicity testing of food contaminants (EFSA, 2011b). This battery includes in vitro tests for detecting chromosomal aberrations, such as the micronucleus (MN) test (TG OECD 487) and the comet assay (TG OECD 489), together with a bacterial mutation assay for detecting gene mutations, such as the Ames test (TG OECD 471).

Microscopic chromatin fragments detached from the main cell nucleus, called micronuclei, are indicative of chromosome breakage or mitotic spindle failure. Exposure to genotoxic substances induces the formation of MN in interphase cells. In the MN test, dyes that bind to DNA through photoactivation are used in interphase cells that are analyzed microscopically for the presence of micronuclei (Jakšić et al., 2012). Similarly, the comet assay is a sensitive method to assess DNA damage in single cells. This method determines the frequency of DNA breakage by measuring the electrophoretic migration of DNA from nucleoids obtained after cell lysis in a thin layer of agarose (Afanasieva and Sivolob, 2018). In contrast to the MN test and the comet assay, which are both tests for genotoxicity testing, the bacterial reverse mutation assay is a mutagenicity test that allows the detection of point mutations using different bacterial strains of *Salmonella typhimurium* and/or *Escherichia coli* (Mortelmans and Zeiger, 2000). This assay detects late DNA lesions, such as mutations, which are the result of a balance between induced DNA damage and DNA repair. However, unlike the comet assay that determines premutagenic lesions, providing very important information on the mechanism of action of chemical compounds; the bacterial reverse mutation assay does not provide information on the mechanism of action of the compounds, which is crucial for a correct characterization of risk assessment (Hansen et al., 2009). Importantly, many chemicals are biologically inactive unless they are biotransformed to more reactive intermediate metabolites. Cytochromes P450 is a superfamily of cysteine thiolate-ligated heme-containing monooxygenase enzymes that catalyze the oxidation and metabolism of a large number of xenobiotics and endogenous compounds. Cytochrome P450 enzymes are mainly present in liver cells and also to a lesser extent in other parts of the body such as the lung and kidney cells (Cook et al., 2016). Since the bacterial reverse mutation assay employs prokaryotic cells, it requires the addition of an exogenous metabolic activation system that contains the necessary enzymes for biotransformation and mimics mammalian in vivo conditions (Tejs, 2008).

Some studies have evaluated the genotoxicity by the MN and comet assays after mycotoxin exposure in different cell lines (Horvatovich et al., 2013; Rakkestad et al., 2010; Zhang et al., 2017). However, in human hepatocarcinoma (HepG2) cells, there is no literature regarding the evaluation of the genotoxicity of T-2. Accordingly, the objective of this study was to evaluate the in vitro genotoxicity and mutagenic potential of T-2 mycotoxin. For this purpose, we developed an in vitro genotoxicity strategy, according to EFSA recommendations, in which we integrated validated assays that detect different premutagenic DNA lesions in HepG2 cells (comet assay and MN determination), and point mutations in five selected bacterial strains (bacterial reverse mutation assay). The HepG2 cells were selected because the liver is the main T-2 metabolizing organ, but also because the liver is the main target organ for T-2. The mutagenic effect of T-2 in the different bacterial strains was performed in the presence and absence of a commercial cofactor-supplemented post-mitochondrial fraction (S9) prepared from rodent livers, according to OECD guidelines. The S9 fraction is responsible for carrying out the T-2 metabolization, in case it occurs via CYP450.

2. Materials and methods

2.1. Reagents, media, and test strains

The reagent grade chemicals and cell culture compounds used, namely Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, trypsin/EDTA solutions, Phosphate Buffered Saline (PBS), newborn calf serum (NBCS), dimethyl sulfoxide (DMSO), agarose, low melting temperature agarose (LMA), disodium ethylenediaminetetraacetate dihydrate (Na_2-EDTA), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), ribonuclease A (RNase), paraformaldehyde, histidine, biotin, tryptophan, D-glucose 6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP), 2-aminoanthracene (2-AA), mitomycin C, sodium azide (NaN_3), 2-nitrofluorene (2-NF), 9-aminoacridine (9-AC), Nutrient Broth No. 2, KCl and $MgCl_2$, were acquired from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH), ethanol (EtOH), sodium chloride (NaCl), propidium iodide (PI), and sodium hydroxide (NaOH) were purchased from Merck Life Science S.L. (Madrid, Spain). The Litron In Vitro Microflow Kit for the MN assay by flow cytometry was purchased from Litron Laboratories (Litron Laboratories, Rochester, NY). Rat liver (Sprague Dawley) S9 fraction was acquired from Fisher Scientific (Waltham, MA, USA). Tryptone Soy Agar and bacteriological agar were purchased from Scharlab (Barcelona, Spain). The S9 mix for the bacterial reverse mutation assay consisted of 100 mM phosphate buffer (pH 7.4), 4 mM NADP, 5 mM D-glucose-6-phosphate, 33 mM KCl, 8 mM $MgCl_2$ and 10% (v/v) S9 liver fraction. Deionized water (resistivity < 18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The standard of T-2 (MW: 466.52 g/mol) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the mycotoxin were prepared in MeOH at the appropriate working solution and maintained in the darkness at −20 °C.

The *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2 were supplied by the Spanish Collection of Culture Strains (CECT, Valencia, Spain).

2.2. Cell culture and treatment

The HepG2 cells (ATCC: HB-8065) were cultured in DMEM medium supplemented with 10% NBCS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The incubation conditions were pH 7.4, 5% CO_2 at 37 °C, and 95% air atmosphere at constant humidity. The HepG2 cells were subcultured after trypsinization in a 1:3 split ratio twice a week. The T-2 concentrations selected in the present study were IC_{50} (60 nM), $IC_{50}/2$ (30 nM), and $IC_{50}/4$ (15 nM). The IC_{50} value was obtained according to previous assays carried out after 24 h of T-2 exposure in HepG2 cells by the tetrazolium salt (MTT) (Taroncher et al., 2020). Final mycotoxin concentrations tested were achieved by adding T-2 mycotoxin to the culture medium with a final MeOH concentration $\leq$ 1% (v/v). Appropriate controls containing the same quantity of solvents were included in each experiment.

2.3. Alkaline comet assay

The DNA strand break induction was determined by alkaline comet assay (pH > 13), as described by Mallebrera et al. (2016). Briefly, HepG2 cells were seeded in six-well plates. Once the cells reached 80% confluence, cells were exposed to 15, 30, and 60 nM T-2 for 24 h. Then, cells were suspended in LMA (1% PBS, 37 °C), and 70 μL of the suspension were transferred to agarose pre-coated slides (1% H_2O) and covered with a coverslip (24 ×24 cm). After gelling for 10 min at 4 ºC, the coverslip was gently removed and the slides were immersed in lysis solution for at least 1 h at 4 °C in the darkness. After lysis, the slides were covered by electrophoresis buffer for 40 min at room temperature to allow the DNA to unroll. Electrophoresis was run in the same solution for 20 min at 1 V/cm and 300 mA. When an electric field was applied, intact DNA strands remained in the head, while the broken pieces of DNA migrated toward the anode forming a typical comet tail. After electrophoresis, the slides were dried in 96% EtOH (−20 ºC, 5 min) and left to dry completely at room temperature overnight. Cells were stained with 500 μL of PI (20 μg/mL) and analyzed using a Leica DM-4500B microscope (Leica Microsystems, Wetzlar, Germany) at 20x magnification. Two slides were prepared for each treatment condition and three independent replicates were performed. Fifty randomly selected individual cells per slide were analyzed by using Comet Assay Software Project Lab (CaspLab) 1.2.3b1 version. The results were expressed in terms of three comet assay parameters, namely the percentage of DNA in the tail (% tail DNA), tail length, and olive tail moment.

2.4. Micronucleus assay

Micronucleus assay was carried out using the Litron In Vitro Microflow kit (Litron Laboratories, Rochester, NY). This assay should be performed by exposing logarithmically dividing cells to the toxicant for 1.5–2 normal cell cycles. At this time, cells must be harvested and processed for MN scoring. The assay was performed according to the manufacturer's instructions based on previous reports (Bryce et al., 2008). Briefly, 47.2×10^4 cells/well were seeded in six-well plates and exposed to 15, 30, and 60 nM T-2 for 48 h, which is equal to 1.5–2 doubling times for this cell line. On the day of the experiment, cells were stained with the nucleic acid dye A (EMA) solution and placed on the ice near a light source for 30 min, to induce its photoactivation. The EMA fluorochrome dye is a reagent that crosses the compromised outer membrane of apoptotic and necrotic cells and binds to DNA through photoactivation. Then, cells were washed, lysed with the Litron Lysis kit solution, and preserved from light for 60 min. During the lysis step, cytoplasmic membranes were digested to liberate nuclei and MN. Finally, a lysis solution containing SYTOX fluorochrome, which labels all chromatin, was added and cells were incubated for 30 min at room temperature in darkness. The differential staining allows the distinction between healthy chromatin and dead/dying cells. Analysis was performed by using a BD FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometry, following the instructions and templates provided by the Litron In Vitro Microflow Kit manual and as described by Bryce et al. (2008). Three independent experiments were performed. The results were expressed as a percentage of MN per 20,000 cells.

2.5. Bacterial reverse mutation assay

The mutagenic effect of T-2 mycotoxin was evaluated by the bacterial reverse mutation assay according to the preincubation method described by the Organisation for Economic Cooperation and Development (OECD) test guideline (TG) 471 (OECD, 2020). The tester strains utilized were *S. Typhimurium* TA98, TA100, TA1535 and TA1537, and *E. coli WP2*. A total of 7 concentrations of T-2 toxin were analyzed (from 0.15 to 60 nM). Preliminary experiments with each strain were performed to confirm that the T-2 mycotoxin concentrations used in this study were not toxic for the bacteria. The test was conducted in the absence, or presence, of a metabolic activation system derived from a rat liver extract (S9 fraction). In the experiments, the bacterial cultures were grown in 20 mL of Nutrient Broth nº 2 for 10–12 h at 37 °C to an approximate density of 10^9 cells/mL. Then, 100 μL of the tester strains were mixed with 100 μL of each concentration of the test solution and 500 μL of buffer or S9 mix, and preincubated at 37 °C for 20 min. After the incubation period, 2 mL of melted top agar containing 0.05 mM of biotin/histidine or tryptophan was added, and the entire mixture was rapidly poured onto minimal agar plates. The plates were incubated at 37 °C for 72 h and then the number of the revertant colonies were counted. Negative (1% DMSO) and positive mutagenesis controls were included in each experiment. In the absence of S9 fraction, positive controls were 1 μg/plate 2-NF for TA98, 1 μg/plate NaN_3 for TA100 and TA1535, 50 μg/plate 9-aminoacridine for TA1537, and 1 μg/plate mitomycin C for *E. coli WP2*. For the metabolic activation experiments, 2.5 and 20 μg/plate 2-AA were used for *S. typhimurium* strains and *E. coli WP2*, respectively. Three replicate plates were performed in each treatment condition. Results were expressed as the mean number of revertants per plate. According to this assay, a substance is considered mutagenic if the number of mean revertant colonies at two consecutive concentrations is at least two-fold higher than the number of revertants in the corresponding negative control (Dolan et al., 2021).

2.6. Statistical analysis

Data were expressed as mean ± SEM of different independent experiments. The statistical analysis of the results was performed by Students t-test for paired samples. The difference level of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Alkaline comet assay

In this study, the DNA strand breakage was analyzed by the comet assay. The comet tails after the exposure of all T-2 concentrations tested (15, 30, and 60 nM) in HepG2 evidenced the DNA strand breakage (Fig. 1b-d). No comet tail was observed in the control group, indicating no DNA damage in cells not exposed to the mycotoxin (Fig. 1a).

For the T-2 concentrations tested, an increase in tail DNA %, tail length, and olive tail moment in a concentration-dependent manner was observed (Table 1). According to these results, the increase in tail DNA% ranged from 13.94 to 52.57%, for tail length from 9.98 to 80.83%, and for olive tail moment from 1.90 to 23.22%.

3.2. Micronucleus assay

Fig. 2 shows the MN detected in HepG2 cells after T-2 exposure for 48 h. The results demonstrated that only at 60 nM T-2, a significant increase in MN (12.1 ± 1.2%) was produced, compared to the control (6.9 ± 0.9%). So, T-2 exhibits genotoxicity at the highest concentration tested in HepG2 cells.

3.3. Bacterial reverse mutation assay

The results for the bacterial reverse mutation assay in the five selected bacterial strains after T-2 exposure are shown in Table 2. The different positive controls used in the absence or presence of the S9 fraction significantly increased the mutant frequencies for all the strains over the negative controls, indicating that experimental conditions were correct. Moreover, the mutant frequencies in the positive and negative controls were similar to those found in other studies in all the five strains used (Levy et al., 2019). As can be observed in Table 2, when bacteria were treated with the different T-2 concentrations, no significant differences were observed in the number of revertant colonies with those obtained in the negative controls for all the strains. Therefore, our

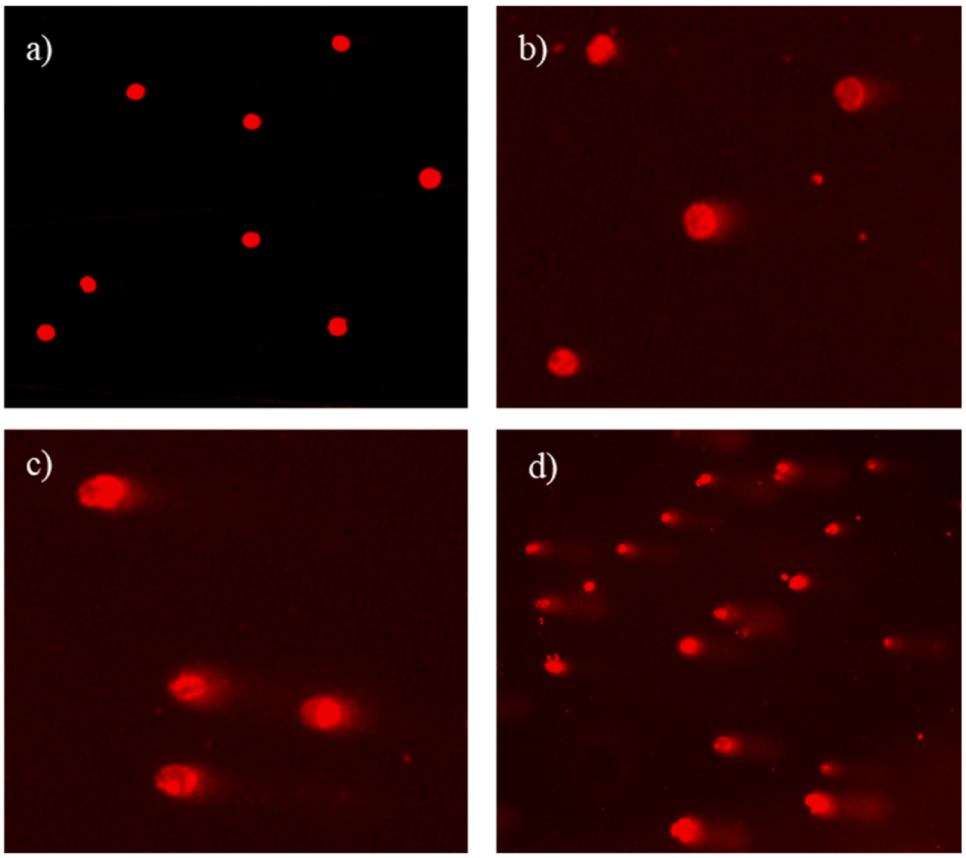

Fig. 1. Effects of T-2 on DNA damage in HepG2 cells measured by alkaline comet assay. The cells were treated with 0 (control) (a), 15 (b), 30 (c), and 60 (d) nM T-2 for 24 h. Cells were observed under a fluorescent microscope with 20 x magnification and quantified.

Table 1
The tail DNA %, tail length, and olive tail moment in HepG2 cells exposed to 15, 30, and 60 nM T-2 for 24 h. Data represent the mean ± SEM. (*) $p \leq 0.05$ indicates a significant difference compared to the control.

Samples	Tail DNA (%)	Tail length	Olive tail moment
Control	3.74 ± 3.21	4.27 ± 1.21	0.53 ± 0.44
15 nM T-2	13.94 ± 7.98 *	9.98 ± 3.46 *	1.90 ± 1.05 *
30 nM T-2	19.52 ± 7.33 *	30.91 ± 14.38 *	5.14 ± 3.85 *
60 nM T-2	52.57 ± 12.08 *	80.83 ± 19.94 *	23.22 ± 9.36 *

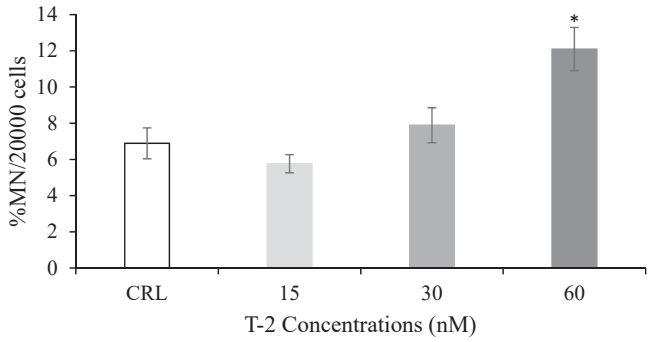

Fig. 2. Genotoxicity assessment by micronucleus assay in HepG2 cells exposed to 15, 30, and 60 nM T-2 for 48 h. Data are expressed as a percentage of MN per 20,000 cells ± SEM (n = 3). (*) $p \leq 0.05$ indicates a significant difference compared to the control (CRL).

results show that T-2 does not induce point mutations by base pair substitutions or frameshifts in either the genome of *S. Typhimurium* or *E. coli* under the experimental conditions used. These results were observed in both, in the absence or presence of the S9 metabolic system, indicating that either T-2 or its metabolites do not induce a mutagenic response in the bacterial reverse mutation assay at the tested concentrations.

4. Discussion

The genotoxicity induced by T-2 is one mechanism of action that has not been widely understood yet. Moreover, there is scarce information about the effects produced by the T-2. Thus, more information would be useful to food safety agencies to facilitate the establishment of a more accurate T-2 concentration so that the population can be safely exposed.

The genotoxic effect of T-2 was evaluated by the comet and MN assays according to the methods described in TG OECD 489 and 487, respectively. Regarding the comet assay, our results showed that after 15, 30, and 60 nM T-2 exposure, DNA strand breakage was observed in a concentration-dependent manner. Similarly, Zingales et al. analyzed the genotoxic potential of sterigmatocystin produced by *Aspergillus* fungi in SH-SY5Y cells and reported increasing values of tail DNA %, tail length, and olive tail moment with increasing concentrations ($IC_{50,}$ $IC_{50}/2$, and $IC_{50}/4$) of sterigmatocystin. However, they obtained lower values respect those obtained in this study regarding the tail DNA % (10.09% vs 52.57%), tail length (32.94% vs 80.83%), and olive tail moment (4.69% vs 23.22%) (Zingales et al., 2021). Mamur et al. reported that fusaric acid, which is a mycotoxin less toxic than T-2, induced comet tail intensity at 3.12 (7.18), 6.25 (6.40), and 12.50 (6.69) μg/mL in a concentration-dependent manner, respect to control (4.22) in isolated human lymphocytes after 1 h of exposure. Nevertheless, comet tail

Table 2
Results in the bacterial reverse mutation assay after the exposure of T-2 (0.15–60 nM) to *Salmonella typhimurium* and *Escherichia coli WP2* strains, in the absence or presence of S9 fraction in the growth medium.

Concentration (nM)	TA98		TA100		TA1535		TA1537		WP2	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.15	34	34	118	104	24	31	9	8	18	18
0.3	29	32	127	116	34	30	10	8	14	18
1.5	30	33	121	122	28	29	9	6	14	18
3	30	32	125	124	30	30	7	5	16	8
15	35	29	146	98	26	28	10	7	12	20
30	26	32	117	125	34	28	7	7	12	12
60	28	22	124	118	33	28	7	6	10	12
Negative control[a]	30	32	118	112	34	30	8	6	14	15
Positive control	161[b]	1275[c]	799[d]	2827[c]	760[d]	158[c]	341[e]	170[c]	135[f]	40[c]

Values are the mean number of revertants per plate of three replicates.
[a] 1% DMSO;
[b] 2-Nitrofluorene (2-NF);
[c] 2-Aminoanthracene (2-AA);
[d] NaN_3;
[e] 9-aminoacridine (9-AC); mitomycin C

length was not affected (Mamur et al., 2020). Also, these authors described a significant increase in comet tail intensity at 0.14, 0.29, and 1.15 μM Enniatin A regarding the control after 1 h of exposure in isolated human lymphocytes (Mamur et al., 2018). On the other hand, significant DNA damage was detected by comet assay at 1 and 12 μM beauvericin (BEA) in CHO-K1 and Caco-2 cells after 24 h of exposure (Mallebrera et al., 2016; Prosperini et al., 2013). However, Takakura *et al.* studied the genotoxicity of deoxynivalenol (DON), the main type B trichothecene mycotoxin produced by *Fusarium* fungi, in TK6 and HepaRG cells; and they evidenced that this mycotoxin did not induce primary DNA damage by the comet assay (Takakura et al., 2014). In addition, the DNA damage observed in HepG2 cells after T-2 exposure may lead to genomic instability and the development of chronic alterations. Therefore, it is crucial to reach more mechanisms of genotoxicity. In our work, we decided to continue by evaluating the possible generation of MN after T-2 exposure, to gauge the severity of cell damage and to achieve a better understanding of the molecular mechanisms of toxicity at the cellular level.

Assessing MN is a useful tool to measure the rate of chromatin damage. The results that we obtained in this work at the concentrations tested, evidenced that only at the IC_{50} of T-2 an increase of MN was observed compared to the control. Limited literature about the induction of micronuclei produced by T-2 is available. Only Somoskői et al. investigated the genotoxic response of T-2 exposure in blastocysts of mouse embryos. These authors reported that 0.75 ng/mL (1.6 nM) T-2 increased MN up to 33.3% after 96 h of exposure (Somoskoi et al., 2016). However, no significant differences were found between control and treated blastocysts at 0.5 ng/mL (1.1 nM) T-2 (Somoskői et al., 2018). The MN induction after exposure to other mycotoxins has been studied in different cell lines. Takakura *et al.* reported that a short treatment (3 h) of 25 μM DON and other trichothecene from *Fusarium* genera with a similar mechanism of action to T-2, produced 9.8% more MN than the solvent control in human TK6 cells. Moreover, after 24 h of exposure, 3.2 μM DON produced an increase of up to 17.4% MN compared to the control (Takakura et al., 2014). Juan-García et al. evaluated the induction of MN by the main metabolite of DON, the 3-ADON, in HepG2 cells. These authors evidenced that 3 μM of 3-ADON produced an increase of 13.1% MN in HepG2 cells after 48 h of exposure compared to the control (Juan-García et al., 2018). Similarly, an increase of 14% of MN compared to the solvent was found after 0.5 μg/mL (637.8 nM) BEA exposure in PK15 cells (Klarić et al., 2008). On the other hand, Juan-García *et al.* showed that BEA and OTA caused a strong induction of MN at 1.25 and 25 μM, respectively, compared to control in HepG2 cells (Juan-García et al., 2019). Induced MN frequencies above the solvent control of citrinin (50 to 100 μM) and OTA (20 to 30 μM) were reported after 24 h in V79 cells (Föllmann et al., 2014). Regarding aflatoxin B_1 (AFB_1), one of the most toxic mycotoxins, Singto *et al.* reported that HepG2 treatment with 3 μg/mL (9.6 μM) of AFB1, significantly increased (1.8-fold respect to control) the number of micronucleated cells (Singto et al., 2020). Accordingly, Tadee et al. also showed that 5 μg/mL (16 μM) AFB_1 induced genotoxicity in HepG2 cells increasing MN after 24 h of exposure (Tadee et al., 2020).

Undoubtedly, scientific research has demonstrated that a high proportion of MN is associated with apoptosis. In the last decade, important research has been carried out on apoptosis as one of the main mechanisms of action of this mycotoxin (Agrawal et al., 2015; Lei et al., 2017; Wu et al., 2019). Apoptosis could also be related to cell cycle arrest, especially when it is produced by a genotoxicant, to prevent the transmission of damaged genetic information and the promotion of DNA repair. Also, the apoptosis could be related to the generation of reactive oxygen species, or different signaling pathways, such as the MAP Kinase pathway (Wada and Penninger, 2004). Thus, it is necessary to continue studying this signaling pathway, such as the expression of C-Jun N-terminal kinase (JNK), ERK, or p38, to confirm whether this mycotoxin affects this path in hepatic cells and how it regulates the expression of apoptotic genes cell survival.

The mutagenic effect of T-2 was evaluated by the bacterial reverse mutation assay. This test is the most widely used method to assess the potential of chemical agents to produce gene mutations such as substitution, addition, or deletion of one or a few DNA base pairs (Press et al., 1983). The method is based on the ability of different strains of *S. typhimurium* and *E. coli* to reverse mutations in the histidine (*his*-) and tryptophan (*trp*-) operon genes in the presence of mutagenic agents, respectively.

Our results showed that T-2 exposure at the range of concentrations tested in this study does not produce point mutations in the absence or presence of the S9 metabolic system. The absence of mutagenicity of T-2 has been previously described on the *S. typhimurium* histidine-requiring strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 and *Saccharomyces cerevisiae* with and without metabolic activation system (EFSA, 2011a). In our study, an *E. coli* WP2 tryptophan-dependent strain was included. The use of this strain detects trp− to trp+ reversion at a site blocking a step in the biosynthesis of tryptophan before the formation of anthranilic acid. The target site for a specific back mutation consists of an ochre (UAA) nonsense mutation located in the *trpE* gene. This can be reverted to wild type by any type of base change (base pair substitution mutation, i.e., transversions and transitions) in the original alteration site or elsewhere in the chromosome, suppressing the original defect (Mortelmans and Zeiger, 2000). The use of *E. coli* WP2 allows the detection of certain oxidizing and cross-linking mutagens not observable

by using the above-mentioned *S. Typhimurium strains* (OECD, 2020). Our results confirmed that T-2 does not induce mutations according to these mechanisms at the concentrations tested, supporting the lack of mutagenic activity of this mycotoxin observed for *S. Typhimurium* strains.

As previously mentioned, a rat liver S9 fraction was employed as a source of metabolic activation in this study. Liver S9 fraction is standardly used in mutagenic in vitro assays such as the bacterial reverse mutation assay. However, some mutagenic compounds may need another type of bioactivation since different metabolic transformations occur in the cells of different tissues (Jeong, 2017). Moreover, variability in genotoxic responses can occur due to differences in the metabolic fraction properties, including enzyme concentration, induction treatment or rat strain, and even production batches and suppliers (Brendt et al., 2021; Stott et al., 2004). Consequently, the inclusion of alternative in vitro metabolic activation systems in genotoxicity and carcinogenicity testing has been proposed (Ku et al., 2007). In this context, Alonso-Jauregui and colleagues performed the genotoxicity assessment of 12 mycotoxins, including the T-2, with the SOS/umu test, a method that shows a strong correlation with the bacterial reverse mutation assay (Alonso-Jauregui et al., 2022). These authors compared the results obtained for each mycotoxin with a liver S9 fraction and an alternative S9 fraction from the kidneys. They found that T-2 displayed a negative mutagenic response without metabolic activation and with liver S9. However, in the presence of the kidney S9 activation system, a positive mutagenic response was observed in three out of four experiments at concentrations higher than 31 μg/mL, even if not a clear concentration–response was obtained in any of the experiments. The results obtained by these authors confirm that the organ used for the S9 fraction preparation influences the genotoxic activity of some mycotoxins, including the T-2. Moreover, the same authors, detected the presence of an epoxide alert in the common scaffold of trichothecenes such as T-2, by using the expert knowledge-based platform DEREK Nexus® (Alonso-Jauregui et al., 2022) which predicts a plausible mutagenicity and chromosomal damage in vitro and in vivo. Hence, this information suggests that further mutagenic studies employing different types of S9 fractions should be performed to discard the mutagenic potential of this mycotoxin.

Several mycotoxins, either from the same or from different fungal species, may occur simultaneously in feed and food products. For this reason, Smerák et al. (2001) studied the mutagenicity of the combination of selected trichothecenes such as T-2 and the well-known mutagen AFB1, using the Ames test on the strains TA98 and TA100. These authors found that 0.1, 0.25, and 1 μg/dish T-2 concentrations did not produce any mutagenic effect when administered alone. However, when bacteria were exposed to T-2 (0.1 μg/dish) in combination with AFB1, the mixture produced a significantly stronger mutagenic effect than AFB1 alone (Smerák et al., 2001). Similarly, Garofalo et al. (2023) studied the capability of trichothecenes to increase the genotoxicity induced by various genotoxins in proliferating intestinal IEC-6 cells using DNA damage quantification by in-cell-western. The T-2 showed the strongest DNA damage exacerbation effect of all the trichothecenes studied, higher than diacetoxyscirpenol, nivalenol, fusarenon-X, and NX toxin. The authors reported that T-2 exacerbated the genotoxicity induced by the radiomimetic drug, phleomycin, which causes the oxidation of bases and strand breaks; the dietary fungicide captan that induces oxidative DNA damage and strand breaks; and colibactin, a bacterial genotoxin produced in the gut, that induces DNA inter-strand crosslinks. Thus, these results demonstrated that T-2 causes DNA damage exacerbating the phenotype of genotoxins with diverse mechanisms of action. In addition, the absence of a mutagenic response in the bacterial reverse mutation assay observed for T-2 in this study is consistent with the results previously reported by other authors. However, further research simulating different metabolic activation pathways and the combined exposure of this mycotoxin with other mutagenic chemicals that can be present in the diet is necessary, to prevent food risks to the human population.

5. Conclusion

This research analyzed the genotoxicity and mutagenicity potential of T-2 toxin in vitro. Our results suggest that this mycotoxin is genotoxic but not mutagenic based on the determination of DNA damage formation, single-stranded DNA breaks, MN formation, and non-growth of revertant bacterial colonies. These findings contribute to defining the toxicological profile of the mycotoxin by in vitro methods and should be considered for evaluating the impact of T-2 during risk assessment. Nonetheless, studies simulating different metabolic activation pathways, chemical mixtures, and in vivo conditions should be performed to corroborate these results and confirm food safety.

CRediT authorship contribution statement

Cristina Fuentes: Formal analysis, Investigation, Methodology, Writing – original draft. **Mercedes Taroncher:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **María-José Ruiz:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Yelko Rodríguez-Carrasco:** Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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