Comet assay study of the genotoxic effect of T-2 and HT-2 toxins in chicken hepatocytes

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Background and aims: The aim of this study was to verify that the comet assay can be used to investigate the DNA damaging effects of T-2 and HT-2 toxins in the liver of broiler chickens. The comet assay is a favorable genotoxic analysis because it is cheap, simple, and can be used in many organisms and different tissues. *Materials and methods:* Male broiler chickens were fed with T-2/HT-2 toxins-contaminated diet for 14 days. The comet assay was successfully adapted to chicken liver cells, and the DNA damage was determined by a decrease in the comet parameter (DNA % in the tail) in the experimental groups. *Results:* The method of evaluation was found to be critical because DNA damage could not be detected exactly using the CometScore software, due to inaccurate separation of head and comet. However, this problem can be solved by visual evaluation. *Conclusion:* In the case of the visual evaluation, each toxin-treated group differed significantly from the control group, indicating that the assay can be useful for the assessment of primary DNA damage caused by T-2/HT-2 toxins.

INTRODUCTION

T-2 and HT-2 toxins belong to the type A trichothecenes (Shokri et al., 2000; Yang et al., 2016), which are apolar compounds, therefore easily pass through the cell membrane. Mostly, the necrotrophic polyphagous phytopathogenic *Fusarium* moulds, such as *Fusarium sporotrichoides, Fusarium poae*, and *Fusarium graminearum*, are present in the temperate zone, and these produce T-2 toxin (Somoskői et al., 2012).

T-2 toxin is known to be the most toxic mycotoxin with a trichothecene structure (Escrivá et al., 2015; Osselaere et al., 2013). HT-2 toxin has been identified as the major metabolite of T-2 toxin *in vivo* and *in vitro* with similar toxicity (Yang et al., 2016). Many undesirable effects of the T-2 mycotoxin were already shown: inducing apoptosis, obstructing cell growth, restraining protein synthesis, immunosuppression, and fertility disorders and enhancing the formation of reactive oxygen species (Chen et al., 2008; Horvatovich et al., 2013). Vomiting, diarrhea, and mucosal irritations were noticed by both animals and humans after consuming cereal grains contaminated with T-2 toxin (Horvatovich et al., 2013). The International Agency for Research on Cancer (IARC) ranks the T-2 toxin among category 3, which means that it is a non-classified human carcinogenic (IARC, 2015).

T-2 toxin shows high affinity for transpeptidases, an important subunit of the ribosome; therefore, it blocks protein synthesis and indirectly DNA and RNA syntheses (Escrivá et al., 2015). However, its genotoxic nature and the process of DNA damage have not been fully uncovered, yet (Franki et al., 2006; Hafner et al., 2012; Horvatovich et al., 2013). According to the aforementioned studies, nuclear damage could manifest in different ways: chromosome aberrations, micronucleus interchanged or sister chromatid exchange, non-planned DNA synthesis, chromosome fracture, or formation of DNA adducts (Escrivá et al., 2015; Hafner et al., 2012).

The study of Hafner et al. (2012) showed the migration of DNA in the spleen of roosters after 17-day-long T-2 toxin exposure. Less than 1.5 mg of T-2 toxin/kg feed DNA breaking was not visible by the comet assay in leukocytes isolated from the spleen. However, measurable DNA damage was found when the contamination level was much higher, i.e., 13.5 mg T-2 toxin/kg feed. These results suggest the possibility of single- or double-strand breaking of DNA as the genotoxic effect of T-2 toxin (Rezar et al., 2007).

Sokolovic et al. (2007) used the comet assay to investigate the T-2 toxin-induced DNA damage in chicken lymphocytes. T-2 toxin was applied by gavage directly into the craw of chicken in a dose of 0.5 mg/kg body weight. Significant DNA breakage was found as compared to the control group after 24 hr. The generally accepted No Observed Adverse Effect Level in poultry is 0.5 mg T-2 toxin/feed kg (Osselaere et al., 2013), while the LD_{50} value is 4.97 mg/kg in chicken (Sokolovic et al., 2008).

DNA damage can be measured by different methods, such as the PCR-based ones, flow cytometry, and Halo assay. The comet assay can detect DNA damage in a single cell – also known as single-cell gel electrophoresis – so the frequency of the damage in a cell population can be predicted in a relatively simple and sensitive way (Augustyniak et al., 2014; Kent et al., 1995). The presence of genotoxic materials in the environment can also be detected using this method (Azqueta et al., 2009). It is suitable for quantitative measurements of direct DNA damage, and is one of the best methods for the analysis of single- or double-strand DNA breaks or oxidative DNA damage (Dhawan et al., 2009; Kent et al., 1995). The comet assay was also used for the analysis of DNA damage of ionizing radiations, freezing, and thawing in chicken liver cells (Duarte et al., 2009; Faullimel et al., 2005) during storage, and also effects of different carcinogens (Williams et al., 2014), such as aflatoxin B_1 (Williams et al., 2011) in chicken embryo liver.

The aim of this study was to evaluate the effect of T-2 toxin on DNA damage in chicken liver cells. The effect of T-2 toxin on DNA damage has not been analyzed in chicken liver up to date; yet, the liver is the major site of the biotransformation of T-2 toxin and some of its bioactive metabolites, such as HT-2 toxin, which is equally toxic as T-2 toxin (Eriksen & Alexander, 1998).

MATERIALS AND METHODS

Ethical issues

Feeding experiments were carried out according to the general rules of animal experiments and followed the 3R criteria (replacement/reduction/refinement). The experiment was authorized by the Directorate of Food Safety and Animal Health of Governmental Office of Pest County (license number: XIV-I-001/1880-5/2012).

Animal design, mycotoxin production, and experimental contamination

A total of 20 Cobb 540 male broiler chickens at 14 days of age (body weight: 395.85 ± 47.72 g) were investigated.

Four (experimental: Eg1, Eg2, Eg3 and control) groups were formed, with five chickens in each. The animals were fed with commercial broiler starter feed (13.4 MJ/kg AME, 20% crude protein, 10% ether extract, 3.5% crude fiber, 35 mg/kg vitamin E, and 0.25 mg/kg selenium). The nutrient content of this feed met the requirements for broiler chickens (Hungarian Feed Code, 2004). The experiment lasted 14 days and started at 14 days of age. The animals fed the mycotoxin-contaminated diets continuously during the trial.

T-2 toxin was produced under controlled laboratory conditions on corn grits by *F. sporotrichoides* NRRL 3229 strain (Agricultural Research Service Culture Collections, National Centre for Agricultural Research, Peroria, IL, USA) by the method of Fodor et al. (2006). T-2 toxin-contaminated corn grit was mixed with the feed before the trial. Measured mycotoxin content of the experimental diets is presented in Table 1.

The feed sampling was immediately after the preparation of the diets. T-2 and HT-2 toxin concentrations of the feed were measured with three replicates by the method of Trebstein et al. (2008) with high-performance liquid chromatography (HPLC) method with immunoaffinity cleanup. The detection limit of T-2 and HT-2 toxin was 0.02 mg/kg feed.

Chickens were euthanized by cervical dislocation, and liver samples were collected *post mortem*, after the necropsy. The samples were placed in Eppendorf tubes and were stored for not more than 20 min on ice before analysis.

Comet assay

To demonstrate the effect of T-2/HT-2 toxin on the chicken liver cells, the comet assay was performed under alkaline conditions according to Singh et al. (1988) and Devaux et al. (1997) with minor modifications. All the chemicals used were obtained from Sigma (St. Louis, MO, USA). Single-cell suspensions were prepared by diluting the liver samples with phosphate buffer solution (PBS; 1:100, v/v; 10 mM; pH: 7.3–7.5), then the samples were homogenized (Cordless Microtube Homogenizer, Fisher Scientific Co., Pittsburgh, PA, USA). Hydrogen peroxide (10 μl, 3% v/v) was used to create the positive control samples (group C+). First, the slides were covered with 0.8% (w/v) normal melting point agarose. When the first layer had solidified 1% (w/v), low melting point agarose was spread on the slides, diluted with the same volume of cell suspension. This layer was allowed to solidify onto the slides by placing them on melting ice for 5 min.

Table 1. Measured mycotoxin content of the experimental diets

Experimental group	Mycotoxin content of the feed (mean $\pm$ SD)	
	T-2 toxin (mg/kg)	HT-2 toxin (mg/kg)
Control	<0.02	<0.02
Eg1	0.127 ± 0.033	0.088 ± 0.023
Eg2	0.235 ± 0.055	0.149 ± 0.010
Eg3	0.989 ± 0.168	0.570 ± 0.061

Note. SD: standard deviation; Eg1: Experimental group 1; Eg2: Experimental group 2; Eg3: Experimental group 3.

Finally, the third layer of 0.5% (w/v) low melting point agarose gel was added to cover the second layer and again, allowed to solidify for 5 min. Slides were immersed to a freshly prepared lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, and pH: 10) at 4 °C for at least 1 hr. After the lysis of the cells, the slides were transferred to an electrophoresis tank filled with electrophoresis buffer (0.3 M NaOH and 1 mM EDTA). After the electrophoresis (20 V, 300 mA, and 24 min), the slides were rinsed with a neutralizing solution (0.4 M Tris and pH: 7.5). This step was repeated after 5 min. Up to this point, all activities were carried out in the dark. Each slide was stained with 50 µl of ethidium bromide (0.05 mM) and was covered with a coverslip. Digital images of the slides were made by a QCapture MicroPublisher 3.3 RTV camera attached to a fluorescent microscope (Nikon Eclipse E600, Nikon Instruments, Melville, NY, USA) equipped with a 20× magnification objective. At least 150 cells were analyzed in each control and treated group. DNA damage was evaluated by CometScore software (TriTek, Tritek Corporation, VA, USA), and percent DNA in the tail was used as the principal parameter.

The results were also analyzed visually, as in case of high-scale damage visual analysis is able to detect the degree of damage. Visual analysis was performed according to the method of Singh et al. (1988) and Collins et al. (1997). Comets were evaluated individually on a 0–4 scale during the analysis, based on the quantity of DNA fragments in the tail of the comet. At least 100 cells in each group were evaluated as was proposed by Horvatovich et al. (2013).

Statistical analysis

The statistical analysis was carried out using the R software package (R Core Team, 2013). Normal distribution of the groups was checked by Shapiro–Wilk test. The DNA% in tail values of the treated, positive control, and control groups was compared with non-parametric Kruskal–Wallis test with $p \leq .05$ level of significance.

RESULTS

There are many comet assay parameters as Olive tail moment, tail length, lenght/height, tail extent, tail optical intensity, and head DNA in the literature (Kumaravel et al., 2009); however, the percentage of DNA in the tail of comet is the most informative and frequently used parameter (Collins, 2004; Tice et al., 2000). Based on the method of the evaluation, different tendencies were found. The evaluation of CometScore showed a significant variation in the toxin-treated groups. T-2/HT-2 toxin treatment had a significant effect ($p < .001$) on the DNA damage. DNA damage was the lowest in the control group (C), whereas T-2 and HT-2 toxins caused an increase in DNA damage at the dose of 0.215 mg/kg; thus, Eg1 significantly differed from all the other groups (Table 2). T-2 and HT-2 toxins had a negative effect on DNA damage at the dose of 0.384 mg/kg (Eg2); however, the mean value of DNA% in the tail was lower as compared to the Eg1 group. The Eg2 group significantly differed from both the Eg1 and Eg3 groups ($p < .001$). In the case of the highest dose, the toxin treatment did not affect the DNA migration. However, the Eg3 group had the greatest range regarding the comet parameter (Fig. 1).

In parallel with the evaluation by the CometScore software, visual evaluation was also performed on the 0–4 scale (Table 2). The visual evaluation also proved that the mycotoxin treatment has a significant effect on DNA damage ($p < .001$) by Kruskal–Wallis test. T2 and HT-2 toxins induced a significant increase in the DNA damage in the Eg1 group. A high extent of DNA damage was also detected in the samples of the Eg2 group but the average score was lower than in the Eg1 group. These directions of the difference were similar to the results of the Comet Score evaluation. The Eg2 group revealed a significant difference from the Eg1 group ($p = .006$) and also from Eg3 groups ($p = .001$). High DNA fragmentation was found in the Eg3 group by visual evaluation. The average score in the Eg3 group was higher than in Eg1 or Eg2 group. This result was supported by the image analyses of comets, which proved that the head and the tail were not clearly distinct.

DISCUSSION

The comet assay is a frequently used method in human cells, laboratory, and non-laboratory animals for the evaluation of DNA damage. The application of this method is wide also in poultry species, for instance in quail meat, chicken embryo liver, spleen, and blood. The comet assay has not been used previously for the evaluation of the DNA damaging effects of mycotoxins in chicken liver. According to our experiences with this method, dilution rate is an important factor with the

Table 2. Results of the comet assay parameter (DNA% in the tail) and the visual evaluation

Experimental group	DNA% in the tail (%)		Visual evaluation (point)	
	Mean	SD	Mean	SD
Control (C)	0.11[a]	0.0002	0.13[a]	0.3
Positive control (C+)	0.946[b]	1.591	3.67[c]	0.7
Eg1	4.319[c]	5.396	3.50[c]	0.8
Eg2	1.142[b]	1.194	3.10[b]	0.9
Eg3	0.445[a]	1.709	3.60[c]	0.8

Note. Different superscript letters in the same column represent significant difference ($p < .05$). *SD*: standard deviation; Positive control (C+): control samples treated with hydrogen peroxide (3% v/v); Eg1: $0.127 + 0.088$ mg HT-2 kg^{-1}; Eg2: $0.235 + 0.149$ mg HT-2 kg^{-1}; Eg3: $0.989 + 0.57$ mg HT-2 kg^{-1}.
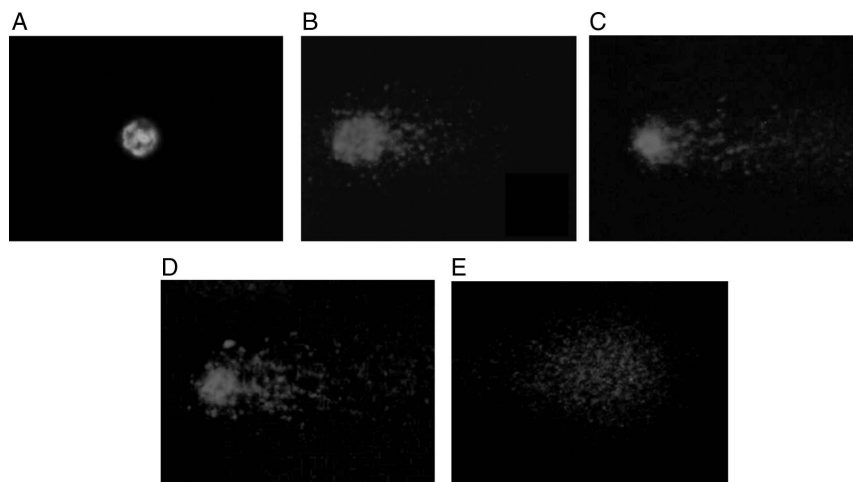
Fig. 1. Representative pictures of comet assay on the effect of different dosage of T-2 + HT-2 mycotoxins (anode to the right, ethidium bromide staining, 20× magnification). (A) Control (undamaged), (B) positive control treated with hydrogen peroxide (10 μl, 3% v/v), (C) Experimental group 1 (treated with 0.127 ± 0.033 mg/kg T-2 + 0.088 ± 0.023 mg/kg HT-2 toxins), (D) Experimental group 2 (0.235 ± 0.055 mg/kg T-2 + 0.149 ± 0.010 mg/kg HT-2 toxins), and (E) Experimental group 3 (0.989 ± 0.168 mg/kg T-2 + 0.570 ± 0.061 mg/kg HT-2 toxins)

right density of the comets, the prohibition of lysis, and the overlap of the comets can be avoided. Overlapping may cause incorrect scoring (Collins, 2014). In preliminary studies, we had defined the optimal cell density, which makes the comet method applicable in chicken liver cells that made possible the following interpretation of our results (unpublished data). Dilution rates (1:1 or 1:10) were proposed by the standard protocol of Devaux et al. (1997). In the case of chicken blood, a 1:200 dilution rate in PBS was proposed (Sokolovic et al., 2007). However, based on our preliminary experiment, 1:100 dilution rate was applied to investigate the damage in the chicken liver cells.

T-2 toxin is harmful (LD_{50}: 4.97 mg/kg) in poultry species. However, toxicity is lower after deacetylation, which produces the first bioactive metabolite, HT-2 toxin (LD_{50}: 7.22 mg/kg). T-2 toxin or HT-2 toxin exposure can cause adverse effects, such as oxidative stress or inhibition of protein, DNA and RNA syntheses (Leal et al., 1999; Sokolovic et al., 2008; Yang et al., 2016). In previous *in vivo* experiments genotoxic, no significant cytotoxic effects were observed at higher mycotoxin doses in poultry (Rezar et al., 2007). DNA damage in liver was reported in mice and rats (Atroshi et al., 1997; Rizzo et al., 1998) but limited data are available regarding poultry. In this study, we proved that T-2/HT-2 toxin has a DNA damaging effect. This effect was detected at the contamination level of 0.215 mg T-2/HT-2 toxin/kg feed, which was approximately the same as the maximum proposed concentration in the EU (2013/165/EU) and half of that proposed by Eriksen and Pettersson (2004) as a tolerable limit for poultry. In the case of similar dosage, more than 4% of fragmented DNA was found in the liver cells with a great deviation. At higher doses of 0.384 mg T-2/HT-2 toxin/kg feed, the effect was also proved. However, in this case, the expected damage could not be detected by the CometScore software. At the highest dose level, i.e., 1.559 mg T-2/HT-2 toxin/kg feed, it was unexpected that there was no significant DNA damage evaluated by the CometScore software. However, some previous

studies proved that as high as 5 mg T-2 toxin/kg feed has a genotoxic effect in poultry species (Franki et al., 2006; Hafner et al., 2012; Rezar et al., 2007).

The comet assay is a simple method but care must be taken over practical details. This method has its advantages and boundaries as well. Collins (2014) identified some critical factors, such as agarose concentration, the density of comets, incubation time, or electrophoresis parameters. Moreover, the evaluation method is also important to draw the right conclusions. During the manual evaluation, the expert needs to mark the comet head. In case of 1.559 mg T-2/HT-2 toxin/kg feed, there was a problem during evaluation because in most cells only the tails were noticeable and the tail and the head were not distinct. As the head and the tail parts of the comets could not be distinguished, evaluation by the CometScore software was incorrect, and it was not possible to analyze the damaged cells by this method. In such cases, visual evaluation is necessary due to limitations of the manual comet boundary and head position selection (Horvatovich et al., 2013). Automated analysis would also be suitable, but the accuracy of the automatic comet identification is also limited at extreme high damage conditions (Gyori et al., 2014). Therefore, visual evaluation was carried out for measuring the extent of the damage. The visual analysis showed significant damage in case of all of the three mycotoxin-treated groups, and the score was between 3 and 4. Our results also showed that it is beneficial to use the different evaluation methods together to get accurate information about the extent of the DNA damage. The large fan-like tail and small head (hedgehog) of the comet could be the effect of apoptosis, although this characterization may not always be accurate. For example, hedgehog comets are induced by radiation (Tice et al., 2000). We did not identify comets with this character and the applied mycotoxin doses do not cause cytotoxicity (Rezar et al., 2007). Our data add new information about the DNA damaging effect of T-2/HT-2 toxin in chicken hepatocyte cells *in vivo*.

CONCLUSION FOR FUTURE BIOLOGY

According to our best knowledge, this is the first experiment, which used the comet assay to analyze the effect of the T-2 and HT-2 toxins in chicken hepatocytes successfully. The study shows the potential DNA damaging effect of the T-2/HT-2 toxin after 14 days of exposure. The method of evaluation is critical and should be based on the extension and type of the DNA damage. Maximum concentration limits proposed by the EU for T-2/HT-2 toxin (0.25 mg/kg feed) should also be reconsidered because in this study 0.215 mg T-2/HT-2/kg feed dose had a significant DNA damaging effect.

Ethical Statement: All applicable international and national guidelines for the care and use of animals were followed. The experiment was authorized by the Directorate of Food Safety and Animal Health of Governmental Office of Pest County (license number: XIV-I-001/1880-5/2012).

Data Accessibility: The raw generated data are available from the corresponding author (MK-W) on request.

Competing Interests: The authors declare no competing interests.

Authors' Contributions: RTS provided significant contributions to the development of comet assay and analysis of data and interpretation of the results. MK-W and MM contributed to the conceptualization and the revision of the manuscript. ME contributed to the conceptualization and the correction of the manuscript. KB contributed to conceptualization and the preparation the mycotoxin-contaminated feed. NF contributed to drafting and the correction of the manuscript. ÁH contributed to the development of comet assay. BK contributed to drafting and revision of the manuscript. RTS and MK-W contributed equally to this work.

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