Thesis

University of Veterinary Medicine Budapest

Department of Physiology and Biochemistry

The effect of T-2 toxin on cell metabolism, oxidative stress and ER stress in 3D hepatic cell cultures of chicken origin

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List of abbreviations

ANOVA	One way variance analysis
BSA	Bovine serum albumin
CCK-8	Cell-counting kit 8
DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmatic reticulum
ERAD	ER associated degradation
GI	Gastrointestinal
GRP78	Glucose-related protein 78
HBSS	Hank's balanced salt solution
HeLa	Henrietta Lacks
HRP	horseradish peroxidase
HSP27	Heatshock protein 27
MDA	Malondialdehyde
MIO	Magnetic iron oxide
NP	Non-parenchymal
PBS	Phosphate buffer saline
PC	Protein carbonyl
Poly-HEMA	Polyhydroxyethylmethacrylate
ROS	Reactive oxygen species
SEM	Standard error of the mean
TDI	Tolerable daily intake
Tmax	Maximum plasma concentration
TMB	Tetramethylbenzidine
WST-8	Water Soluble Tetrasolium Salt 8

Table of Contents

Introduction

T-2 toxin is a trichothecene mycotoxin produced by different *Fusarium* species, which commonly contaminate cereal grains and their processed food products [1]. In Europe, 41% of cereals and their processed products are contaminated with T-2 toxin, thus contamination is frequent and therefore a relevant concern for animal and human safety [2].

Fusarium fungi can have many adverse effects after consumption, including but not limited to nausea, skin blisters, weight loss, failure to thrive, immunosuppression. Mycotoxins produced by the fungus genus *Fusarium* are collectively called fusariotoxins. This group of toxins includes T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON), and zearalenone. The most toxic of the fusariotoxins, T-2 toxin, causes disease and immunosuppression in poultry. DON and zearalenone are particularly toxic to poultry but may cause disease in pigs.

Literature review

Biochemistry and toxicity

T-2 toxin has a sesquiterpene skeleton connected to a 12,13-epoxy ring structure which determines much of its toxicological activity. The toxin is resilient in different light and temperature conditions, although a strongly acidic or alkaline environment deactivates it easily [3]. The tolerable daily intake (TDI) of the toxin was established as 0,7 μg/kg bw (body weght) by the European Union law in May 2001 [4].

T-2 toxin is the only fusariotoxin that can be absorbed directly through the skin [3]. Elimination is rapid, reducing the risk of toxicity and persistence in tissues. T-2 toxin poisoning has not yet been linked to consuming avian products by humans, presumably because of the limited avian absorption and fast excretion in humans. However, several metabolites (including deacetylated forms of T-2 toxins and 3′-OH T-2) have been identified. These may be responsible to a high percentage of human exposure after consuming

food of avian origin. An estimated 50% of its metabolites have been named and isolated [5]. However, the toxicokinetics and toxicodynamics of the toxin is still mostly unknown, hence further research on the toxicity of these metabolites is necessary.

T-2 toxicosis in the poultry industry

The consequences of T-2 toxin poisoning vary considerably between species, with cats being particularly sensitive to its effects, while birds are generally thought to be more resistant. T-2 toxicosis in poultry results in feed refusal, caustic injury of the oral mucosa and areas of the skin in contact with the mold, acute digestive disease, as well as injury to the bone marrow and immune system. Possible lesions caused by T-2 toxin in poultry include necrosis and ulceration of the oral mucosa, reddening of the gastrointestinal (GI) mucosa, mottling of the liver, atrophy of the spleen and other lymphoid organs, and visceral hemorrhages. Specific symptoms can be noted in laying hens, such as decreased egg production, depression, recumbency, feed refusal and cyanosis visible in the comb and wattles [6].

T-2 toxicosis in chicken is usually diagnosed based on evident moldy ingredients or feed, combined with suggestive symptoms and lesions along with impaired production. A definitive diagnosis requires detection and quantification of the specific toxin, which can be difficult because of the rapid and high-volume use of feed and ingredients in poultry operations. T-2 toxicosis can also lead to intermittent disease or immunosuppression without clear symptoms. Due to all of these factors, it is challenging to determine the actual frequency of T-2 toxicosis in the poultry industry.

There is no specific treatment for T-2 toxicosis in poultry. Nonspecific toxicologic therapies using activated charcoal in the feed have a sparing effect, yet they are not practical in larger production units. The management of feed to prevent mold growth and the production of mycotoxins, as well as regular inspection of feed storage and feeding systems, are the main methods used to avoid mycotoxicoses, including T-2 toxicosis. Temperature extremes and high relative humidity should also be avoided in poultry houses.

Cellular effects of T-2 toxin

The chemical structure of T-2 toxin contains a thiol group, which makes it a potent protein and DNA synthesis inhibitor [7]. *In vitro* studies show that it promotes apoptosis in various cell types. Apoptosis occurred most commonly in the skin, kidney, brain and bone marrow in mouse [8]. This is probably due to oxidative damage to cells, targeted at biomolecules like lipids, proteins and nucleic acids. The main reactive oxygen species (ROS) involved in the oxidation of proteins, lipids and DNA appear to be hydrogen peroxide, hydroxyl radicals and superoxide molecules [9]. In addition, T-2 toxin has been shown to decrease the function of the innate immune system [10].

T-2 toxin is thought to inhibit protein synthesis by binding and inactivating the peptidyl-transferase enzyme. The main molecular target of T-2 toxin is the 60S ribosomal unit, where it prevents polypeptide chain formation. This inhibitory effect is prominent in actively proliferating cells, such as skin, GI tract, bone marrow, thyroid and erythroid cells [8, 11, 12]. The inhibition of protein synthesis in these proliferative tissues is most likely the root of many of the T-2 toxicosis clinical symptoms in poultry, including acute digestive disease, as well as injury to the bone marrow and immune system. T-2 toxin inhibits specific proteins related to the coagulation pathway, leading to visceral hemorrhages often seen in affected poultry. Additionally, it has been shown to interact with subcellular structures, causing the disruption of the mitochondrial morphology and rough endoplasmatic reticulum [8].

One of the major consequences of oxidative stress in cells is the elevated lipid peroxidation. The produced oxidants, such as free radicals induce lipid peroxidation in the cells. Lipids with carbon-carbon double bonds, mainly polyunsaturated fatty acids (PUFAs) are attacked by these oxidants. One of the main final products of the peroxidation of PUFAs is malondialdehyde (MDA) [13] which is a commonly used lipid peroxidation therefore oxidative stress marker [14].

One of the most harmful oxidative protein modification is protein carbonylation, which is considered a major hallmark of oxidative stress-

related disorders. Protein carbonyl (PC) content is often measured to assess the extent of oxidative stress in the context of cellular damage [15].

Oxidative stress is often associated with the development of endoplasmic reticulum (ER) stress [16, 17]. Newly synthesized proteins are folded and altered in the ER. These activities are regulated by various chaperones and folding enzymes, including the heat shock protein 70 (HSP70) family member, glucose regulated protein 78 (GRP78) [18, 19]. In non-stressed cells, GRP78 binds misfolded proteins along with unassembled complexes, and initiates ER associated degradation (ERAD) [20]. Its expression increases as a consequence of ER stressors, such as disrupted calcium storage within the cells, or treatment with reagents that inhibit protein glycosylation.

Oxidative and ER stress also affect the production of certain small heat shock proteins (sHSPs). These proteins have a molecular weight of 12-43 kDa and they are often involved in different cellular stress processes, including oxidative stress, chemical stress, as well as heat shock [17]. Heat shock protein 27 (HSP27) is a member of the sHSP protein family. During oxidative stress, it functions as an antioxidant, reducing the produced ROS concentration by increasing intracellular glutathione and decreasing intracellular iron levels. When exposed to chemical stress, HSP27 acts as an anti-apoptotic agent [21–23]. ER stress also induces the phosphorylation of HSP27 [24].

Cell cultures

History

The first successful attempt to keep harvested cells alive was performed in the late 19th century by Wilhelm Roux. He kept cells from the neural plate of chicken embryos alive for several days in a saline buffer. The first reproductible technique was achieved by Ross Granville Harrison when he cultured frog nerve cells using the hanging lymph drop culture method in 1907. [25] In 1910, Montrose Burrows adapted the hanging drop method for use in warm blood tissue. Cells from many small mammal species were

subsequently cultured by using plasma from the cell donating individual. Cell cultures were yet regarded as difficult to manage and having no practical application. The potential of cell cultures was not recognized until the introduction of synthetic culture media in the early 1940s.

Another significant step in the application of cell cultures in research and medicine was the development of the HeLa cell line in 1951 by George Otto Gey. The cells were harvested from Henrietta Lacks, a cervical cancer patient. The HeLa cells paved the way for the development of the Salk polio vaccine a decade later, and is one of the most valued cell lines for cancer research to this day.

In 1965, the first hybrid mammalian cell cultures were achieved by Harris and Watkins [26], and in 1975, the first monoclonal antibodies were discovered by Kohler and Milstein [25].

Nowadays, cell cultures are used in basic and medical science for fundamental, preclinical and cancer research, genetic manipulations, vaccinations, and more.

Cell culture types

Cell cultures are an *in vitro* method of research, to study what happens *in vivo*. Cell cultures obtained from a living organism are considered primary cell cultures. They usually contain populations of different cell types that represent the source tissue. Primary cell cultures are difficult to isolate and have a short lifespan, but they closely mimic the *in vivo* genetic features of tissues and tumors, making them an important research tool [27]. Monolayer cell lines are typically grown in two dimension (2D) on the bottom of a petri dish [27]. Since their development, 2D cell cultures have proven essential for research, although they have numerous limitations.

The environment of an *in vitro* research model should closely resemble the *in vivo* environment for valuable research. 2D cell cultures are a good approximation for many epithelial systems. Epithelial tissues develop an apical domain that faces the environment, a lateral domain that communicates with adjacent cells and is characterized by specialized attachment areas, and a basal domain that rests on the basal lamina and anchors the cell to the

underlying connective tissue in order to form continuous barriers to protect against the outside environment [28] [29].

However, 2D cell cultures are limited, when the investigated tissue does not naturally have an apical-basal morphology. In these cultures, the cells are forced into an apical-basal orientation, affecting cell function, organization of the cytoskeleton, secretion and cell signaling [27]. Differences in cell orientation lead to a changed response to apoptosis [30] and the continuous interaction with the basal membrane influences cell division [31]. The modified cell morphology also affects gene expression, leading to changes in the biochemistry of the cell [32].

An important scientific advancement over the past 30 years has been the development of three-dimensional (3D) cell cultures. 3D cell cultures were first achieved as a reproducible research model by Petersen and Bissel in 1992 in order to simulate breast tissue [31]. They arose from the necessity to bring *in vitro* cell culture models closer to the *in vivo* reality. Besides the absence of the forced apical-basal polarity, physiological interactions between cells and between cells and the extracellular matrix (ECM) are allowed in these models [33–35]. Because of these qualities of the 3D cell cultures, gene expression, apoptosis and cell division are also more similar to the *in vivo* physiology [36].

In traditional 2D cell cultures, all cells have an unlimited, homogenous access to medium components such as oxygen, nutrients, metabolites, signal molecules, resulting in homogenous growth and proliferation. In native tissues, there is a diffusion gradient in the nutrient supply to cells, i.e. cells inside the tissue have access to fewer nutrients, while those on the outside have access to more. Such a diffusion gradient is also observed in 3D cell cultures (**Figure 1**) [37].

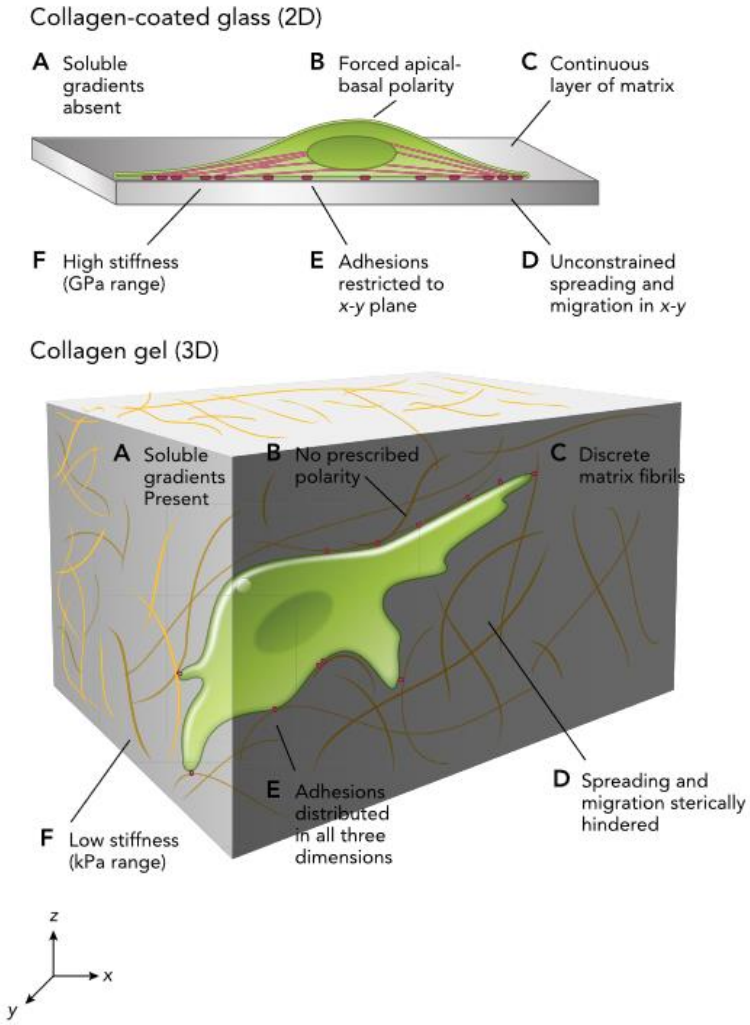

Figure 1: Cells in 2D and 3D microenvironments interact differently with their surroundings due to differences in the mechanical and chemical cues, with a 3D microenvironment more closely mimicking *in vivo* hepatic tissue. (Baker, 2012)

3D cell culture types

3D cell aggregates can be produced using various 3D techniques, and can either be grown in suspension, encapsulated, or on top of a 3D matrix. 3D methods can be divided into the following categories: hanging drop methods, non-adherent surface methods, suspension cultures, scaffold-based hydrogels, magnetic levitation and bio-printing [38].

In the hanging drop method, drops of cell suspension are placed on the underside of a petri dish lid, which is then placed onto a PBS (phosphate buffer saline)-containing petri dish. The cells hang on the lid due to surface tension and accumulate at the tip of the drop, where they spontaneously aggregate, and form spheroids [39, 40].

The non-adherent surface methods use ultra-low attachment plates coated with a substance that prevents the cells from attaching to the surface of the wells (agar or poly-HEMA), forcing them to aggregate and form spheroids [41].

In suspension cultures, a cell suspension is placed inside a container. The cells are kept in suspension either by agitation or by increasing the media viscosity (by adding carboxymethyl cellulose) [42]. When agitation is used, the container is gently stirred or rotated, commonly by spinner flasks and bioreactors. The continuous agitation prevents the cells from adhering to the container walls and promotes cell-cell interactions.

Natural or synthetic hydrogels have been also used to develop 3D culture models. Naturally derived hydrogels include Matrigel, collagen, alginate and fibrin, while polyethylene glycol is a type of synthetic hydrogel [43][38]. When a solidified matrix is used, cells are simply layered on top of it. This allows the cells to adhere to each other and form spheroids that are attached to the hydrogel. When liquid hydrogel is used, the cells are mixed with the before seeding. The cells get embedded within the hydrogel when it jellifies [38].

In the present study, magnetic 3D bioprinting was used to develop 3D cell cultures. In this method, cells are magnetized with biocompatible magnetic nanoparticles that consist of gold, poly-L-lysine and magnetic iron oxide

(MIO). Treated cells are trypsinized and seeded onto an ultra-low attachment plate. A magnetic drive is placed under the plate, resulting in the aggregation of the cells into 3D spheroid structures. The cells start producing ECM proteins including collagen, fibronectin and laminin, and cell-cell as well as cell-ECM interactions are established [44]. There are many advantages of this method compared to other 3D cell cultures. Spheroids are formed easily and quickly, and the culturing procedure is similar to that of 2D cell culturing. In this method, specialized equipment is not needed, and it is compatible with a various detection techniques, such as flow cytometry, Western blot and real time RT-PCR [45].

Aims

T-2 toxin exposure in chickens results in feed refusal, injury of the oral mucosa, acute digestive disease, as well as bone marrow damage and disturbances in the function of the immune system, all leading to a reduced growth rate and production [6, 46]. As chicken has become the most consumed meat product worldwide, it is arguably more crucial than ever to understand of how T-2 toxin exposure affects the chicken liver - the main site of the metabolism of the toxin - to prevent future economic losses. The most important cellular effects of T-2 toxin is the inhibition of protein and nucleic acid synthesis, and the initiation of apoptosis [47, 48]. T-2 toxin can also elevate ROS production, thus induce oxidative stress that results in lipid peroxidation and might cause ER stress in the cells.

The aim of the present study was to assess the effects of T-2 toxin on oxidative and ER stress in 3D chicken hepatocyte-NP cell co-cultures. To investigate different T-2 toxin exposures, the cell cultures were subjected to various T-2 toxin concentrations (100, 500, 1000 nM), for different incubation times (24, 48 hours). In order to evaluate the pathological effects of the toxin, the metabolic rate of the cells, as well as the potential oxidative stress and ER stress were measured.

Materials and Methods

All reagents were purchased from Merck KGaA (Darmstadt, Germany) except when otherwise specified. Animal procedures were performed according to the international and national law, as well as the institutional guidelines. and were authenticated by the Local Animal Welfare Committee of the University of Veterinary Medicine, Budapest and by the Government Office of Zala County, Food Chain Safety, Plant Protection and Soil Conservation Directorate, Budapest, Hungary.

Preparation of the solutions

The first step was the preparation of the solutions needed for the isolation. The following solutions were used for the perfusion:

• 150 ml EGTA (ethylene glycol tetraacetic acid) (0,5 mmol/l) containing HBSS (Hanks Balanced Salt Solution)

• 150 ml EGTA-free HBSS

• 100 ml MgCl2 and CaCl2 (both 7 mmol/l) containing HBSS supplemented with 1mg/ml type IV collagenase

Following the tenfold dilution in sterile distilled water of the HBSS stock solution, 4,7 ml 7,5% $NaHCO_3$ was added to 1000 ml buffer. To prepare the EGTA containing HBSS, 1,052 ml of EGTA was added to 200 ml buffer to set the concentration to 0,5 mmol/l. The collagenase-containing solution was made by mixing 100 ml HBBS with 1 ml MgCl2, 1 ml CaCl2 (both with a concentration of 7 mmol/l) and 250 mg of collagenase.

Isolation of the chicken liver cells and non-parenchymal cells

The hepatic cells were isolated from six week old male Ross-308 broiler chickens (obtained from Gallus Poultry Farming and Hatching Ltd, Devecser, Hungary) as described by Mackei et al [49]. The animals were decapitated under CO_2 narcosis, after which they were fixated in a recumbent position. The feathers were removed in the ventral area and a laparotomy was performed. The gastropancreaticoduodenal vein was canulated to serve as the inflowing branch, while the cardium was used as the draining branch to

collect the buffers during the hepatic perfusion. The liver was perfused using a three-step procedure at a flow rate of 30 ml/minutes. The used buffers were warmed up to 40°C and were freshly oxygenated with Carbogen. To remove the calcium, the liver was first perfused by 150 ml of 0,5 mmol/L EGTA containing HBSS buffer. EGTA is a chelat-forming agent that binds to the magnesium and calcium ions, and in doing so helps by loosening up the cell-cell conjunctions, the disintegration of the tissue and the isolation of the cells. After this, the liver was flushed with 150 ml of EGTA-free HBSS followed by 130 ml of the collagenase solution, which is responsible for the degradation of the extracellular matrix. The effluent collagenase solution was collected in a sterile trypsinization flask and then recirculated until the liver was disintegrated. The liver was placed in a sterile beaker after the excision, and the subsequent steps were carried out on ice, and in a sterile environment.

The capsule was cut up, and the liver was placed in ice-cold HBBS buffer supplemented with 50 ml BSA (bovine serum albumin) (2,5%). The suspension was then filtered through three layers of sterile gauze into a sterile centrifugation tube. To stop the activity of the collagenase enzymes and to prevent cell aggregation, the primary cell suspension was incubated in BSA containing HBBS buffer on ice for 40-50 minutes. The cell suspension was then centrifuged three times for 3 minutes at a low-velocity (100×g) in Williams Medium E supplemented with 0,22% $NaHCO_3$, 50 mg/ml gentamycin, 2mM glutamine, 4 µg/l dexamethasone, 20 IU/l insulin and 5% FBS.

Following these steps, the viability of the cells as well as the total cell number were determined in a Bürker Chamber using trypan blue staining. For this, 200 µl concentrated cell suspension were mixed with 800 µl Williams Medium E. Then the same amount of trypan blue stain was added to 200 µl of the diluted cell suspension. With this staining, the ratio of dead and living cells can be determined. Trypan blue can only enters cells with a damaged membrane, thus the dead cells appear blue. The cell suspension was then diluted to a concentration of 106 cells/ml.

The previously achieved supernatant was centrifuged multiple times in order to isolate the non-parenchymal (NP) cells. First on 350×g for 10 min, then three times on 800×g for 10. The sediment of the supernatant was resuspended in 5 ml Williams Medium E and the viability, as well as the cell number were determined using the previously described procedure. The final concentration of 106 cells/ml was achieved by the dilution of the suspension. 60 ml of hepatocyte suspension was mixed with 10 ml of NP cell suspension to produce 70 ml co-culture suspension. In these co-cultures, the hepatocyte to NP cell ratio was 6:1.

3D cell cultures and treatments

The special equipment and chemicals necessary for the preparation of magnetic three-dimensional (3D) cell cultures were obtained from Greiner Bio-One Hungary Kft. (Mosonmagyaróvár, Hungary). In order to magnetize the cells, 800 µL of magnetic nanoparticles (NanoShuttle^{TM}-PL) were added to 8 ml of cell suspension. NanoShuttle^{TM}-PL consists of gold, iron oxide and poly-L-lysine. These nanoparticles electrostatically bind to the cell membranes of the cells, resulting in a magnetization of the cell. NanoShuttle^{TM}-PL can stay attached to the cell membrane for up to 8 days. They are biocompatible and do not effect the cellular metabolism, proliferation and inflammatory responses.

Following the incubation with the NanoShuttle^{TM}-PL, 100 µL of the cell suspension were transferred into each well of a cell repellent 96-well plate. The plate was then incubated for one hour at 37°C. During this time, the NanoShuttle^{TM}-PL particles bind to the cell surface. Afterwards, the plate was placed on top of a so called Spheroid Drive, that contains small magnets under each well of the plate. The plate was then incubated with the Spheroid Drive at 37°C with the CO_2 concentration set to 5%. After 24 hours, the culture medium was changed to serum-free medium. For this, a so called Holding Drive was used that has bigger magnets between four wells of the 96-well plate. The cells were further incubated for 24 hours, then they were treated with three different (100, 500 and 1000 nM) T-2 toxin concentrations. The cell cultures were incubated at 37°C with the CO_2 concentration set to 5% and the medium was changed every 24 hour throughout the experiment.

Measurements

Metabolic activity

Metabolic activity of the cells was evaluated after each incubation time (24, 48 hours) with Cell Counting Kit-8 (CCK-8) assay. CCK-8 is a colorimetric method used to detect changes in color intensity. CCK-8 reagent contains Water Soluble Tetrasolium Salt 8 (WST-8), which is converted to orange coloured formazan by the coenzymes produced by viable cells (**Figure 2**). The intensity of the produced orange colour can be measured spectrophotometrically and it is directly proportional to the metabolic activity of the cells, thus their viability.

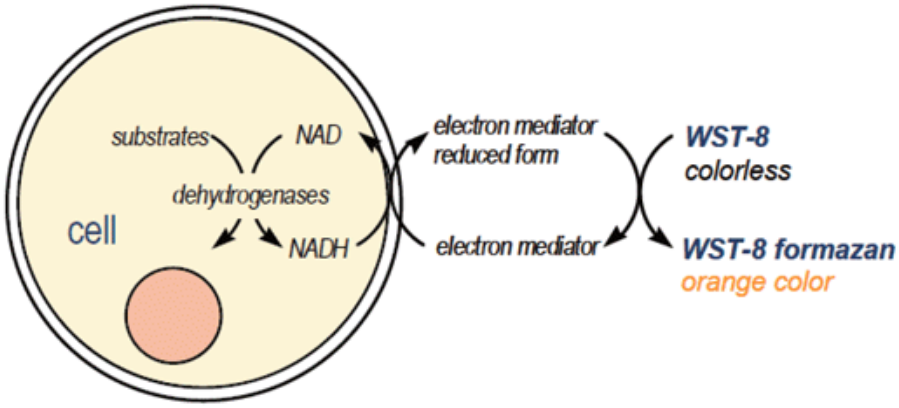

Figure 2: Assay mechanism of WST-8 conversion by dehydrogenases in viable cells, Instruction Manual by PromoCell (PromoCell GmbH, WST-8 Instruction Manual, Heidelberg, Germany)

Following the instructions of the manufacturer's protocol, 100 µl fresh Williams Medium E and 10 µl of the CCK-8 solution was added to each well of the 96 well microplate. The plate was then incubated for two hours at 37°C. The absorbances were measured at 450 nm with a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

MDA

Malondialdehyde (MDA) concentration of the cells was assessed after each incubation time (24, 48 hours) by chicken specific Double Antibody Sandwich ELISA test from the medium of the cell cultures. This method is widely used for a target analyte with more than two potential epitopes. Both the pre-coated *capture* antibody - in this case, an anti-chicken MDA monoclonal antibody - and the *detection* antibody - a biotinylated polyclonal antibody – can recognize the epitopes (**Figure 3**).

First, 100 µl of standard and sample were measured into each well of a 96-well plate pre-coated with antibodies. The plate was then incubated at 37°C for 90 minutes. Thereafter, 100 µl of biotinylated antibody specific for MDA was added to the wells, and the plate was incubated at 37°C for 60 minutes. The next step, 100 µl of horseradish peroxidase (HRP) and avidin containing enzyme conjugate was added to the wells. This compound binds to the biotinylated antibodies, thus the quantity of the reporter enzyme would be positively correlated to the quantity of MDA in the sample. The plate was incubated again for 30 minutes at 37°C. Lastly, 100 µl of TMB (tetramethylbenzidine) containing Colour reagent was added into each well. TMB is a substrate used for colorization. TMB reacts with the HRP to form a blue product. With the substrate, the plate was incubated for 20-30 minutes at 37°C. The plate was also washed between each step to remove any unbound compounds. In the final step, 100 µl of Stop solution was added into each well to stop the enzymatic reaction. The absorbance was then read on 450 nm with a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

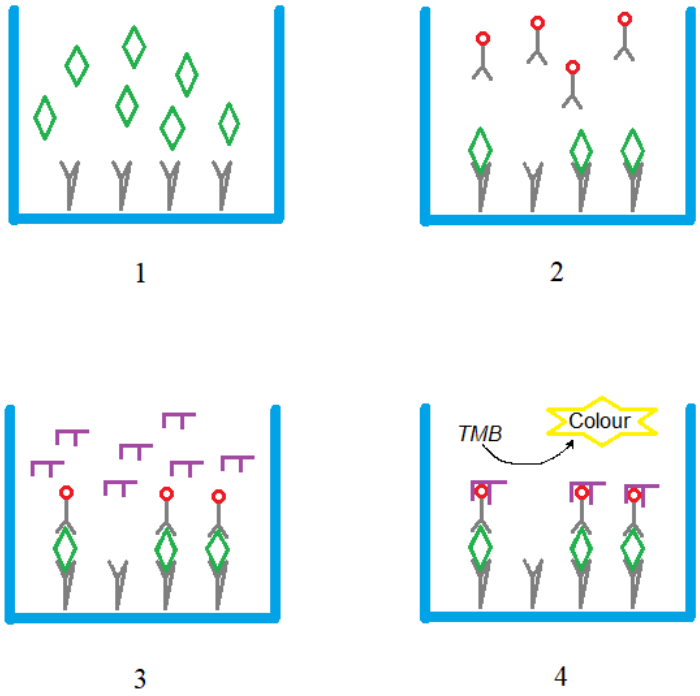

Figure 3: Schematic visualisation of the Double-Antibody Sandwich ELISA method. 1: precoated capture antibodies present in the wells bind to the target analytes. 2: antibody-antigen complex has been formed, and detection antibodies are added. 3: antibody-antigen-antibody complex has been formed, HRP + avidin is added to the wells. 4: TMB is added to the complex and converted into coloured compound.

Protein carbonyl

After 48 hours, protein carbonyl (PC) concentration of the cells was determined from the cell lysates using chicken specific Double Antibody Sandwich ELISA test according to the manufacturer's protocol as previously described.

HSP27

After each incubation period (24, 48 hours), heat shock protein 27 (HSP27) concentration of the cell cultures was assessed from the medium of the cell cultures by chicken specific Double Antibody Sandwich ELISA test according to the manufacturer's protocol as previously described.

Glucose related protein 78

Glucose related protein 78 (GRP78) concentration of the cell cultures was assessed after each incubation time (24, 48 hours) from the medium of the cell cultures by chicken specific competitive ELISA test. Competitive ELISA is an excellent method to detect small analytes in complex mixtures. With this, the concentration of an analyte is measured through its interference with the ELISA assay signal. In this method, the unlabelled primary antibody is incubated with the samples containing the target analytes.

The formed complexes are added to an antigen-coated well. Unbound antibodies are then removed by washing the plate. The more antigen in the sample, the more antigen-antibody complexes are formed, resulting in a decrease in the amount of unbound antibodies that are available to bind to the antigen in the well, thus "competition". In the next step, a secondary antibody is added that is coupled to the enzyme. After washing the plate, with the addition of the substrate, the remaining enzyme produces a chromogenic or fluorescent signal. The intensity of the developed colour indicates the amount of the primary antibody that is bound to the antigen-coated well.

Following the manufacturer's protocol, 100 µL of standard and sample was measured into each well of an antigen-coated plate. 10 µL of Balance Solution was added only to the sample-containing wells, and 50 µL of Enzyme Conjugate was added to every well. The plate was then incubated for 60 minutes at 37°C. After the incubation, the plate was washed to remove any

unbound compounds. Next, 50 μL of Substrate A and 50 μL of Substrate B were measured into each well, followed by an incubation of 15-20 minutes at 37°C. To stop the enzymatic reaction, 50 μL of Stop Solution was measured into each well, and the absorbance was read on 450 nm with a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

Results

Metabolic activity

The metabolic activity of the cell cultures was determined by CCK-8 assay.

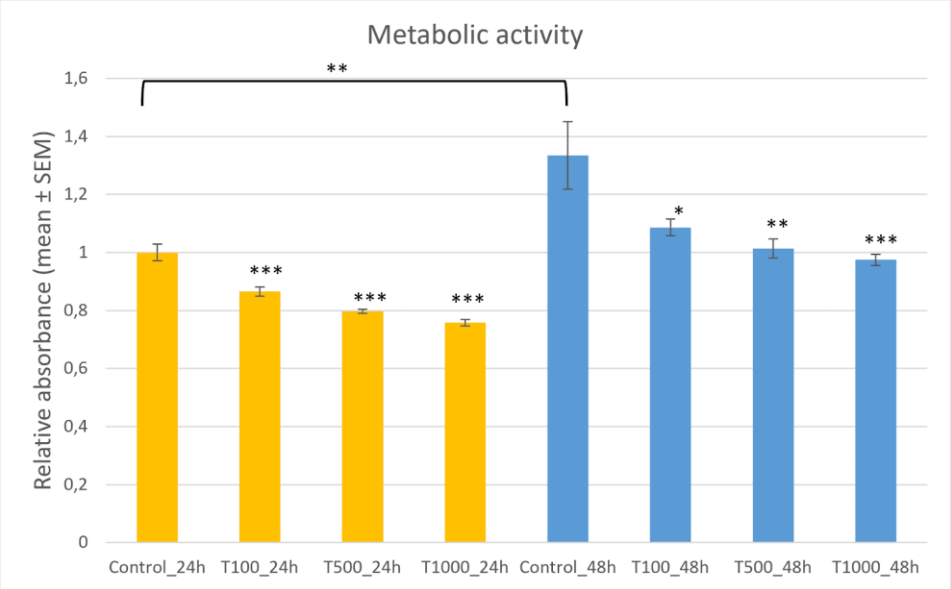

Figure 4: Effects of 24 and 48 h T-2 toxin treatment on the metabolic activity of primary hepatic 3D cell cultures of chicken origin assessed by CCK-8 test. Yellow bars: 24 h, blue bars: 48 h treatment. Control: cells without T-2 toxin exposure; T100: 100 nM, T500: 500 nM, T1000: 1000 nM T-2 toxin treatment .Relative absorbances were calculated by considering the mean value of the Control group of 24 hours incubation as 1. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The effects of T-2 toxin on the metabolic activity of the cells is shown in **Figure 4**. The metabolic activity in each treatment group was significantly decreased ($p = 0.000359$, $p = 0.000000283$, $p = 1.95E-08$, $p = 0.0173$, $p = 0.00358$, 0,000766) after 24 and 48 hours. The metabolic activity of the control group of 48 hours of incubation was significantly higher ($p = 0.00129$) than that of the 24 hours incubated control group.

Malondialdehyde

The MDA release of the cells was measured by chicken specific ELISA test from the cell culture medium.

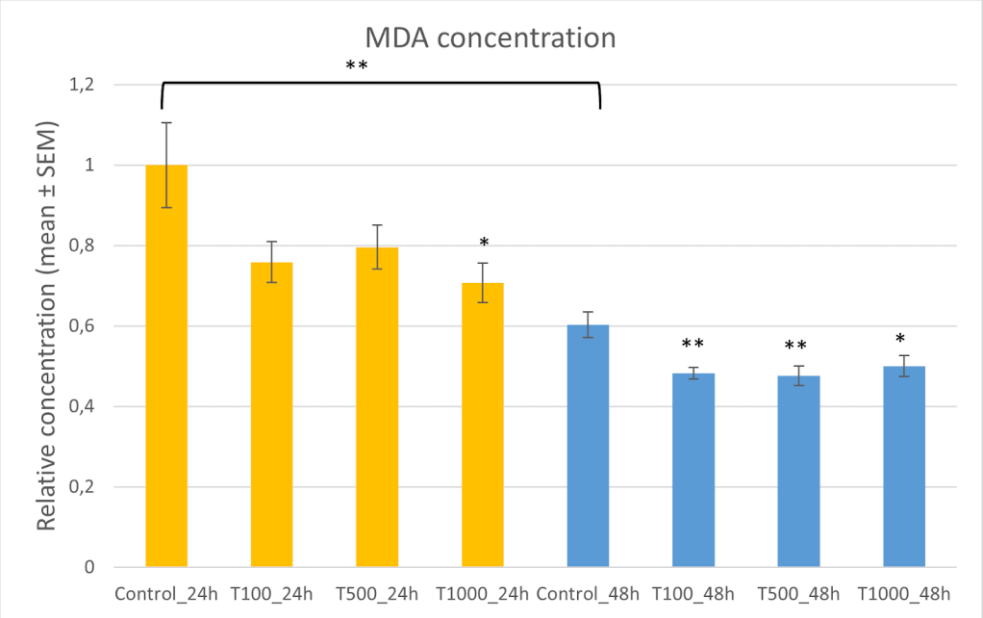

Figure 5: Effects of 24 and 48 h T-2 toxin treatment on the MDA concentration of primary hepatic 3D cell cultures of chicken origin. Yellow bars: 24 h treatment; blue bars: 48 h treatment. Control: without T-2 toxin treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. MDA concentration was measured by chicken specific ELISA test. Relative concentrations were calculated by considering the mean value of the Control group of 24 hours of incubation as 1. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$.

The effects of T-2 toxin on the MDA concentration of the cell cultures is shown in **Figure 5**. After 24 hours, only the 1000 nM toxin concentration reduced significantly ($p = 0.0306$) the MDA release of the cells, whereas after 48 hours, the MDA production of the cell cultures was significantly decreased ($p = 0.00646$, $p = 0.00984$, $p = 0.0329$, respectively) by all three concentrations of T-2 toxin. The MDA concentration of the control group after 48 hours of incubation was significantly lower ($p = 0.00477$) than that of the controls following 24 hours of culturing.

Protein carbonyl

The PC content of the cell cultures was measured by chicken specific ELISA test, from the cell lysates after 48 hours.

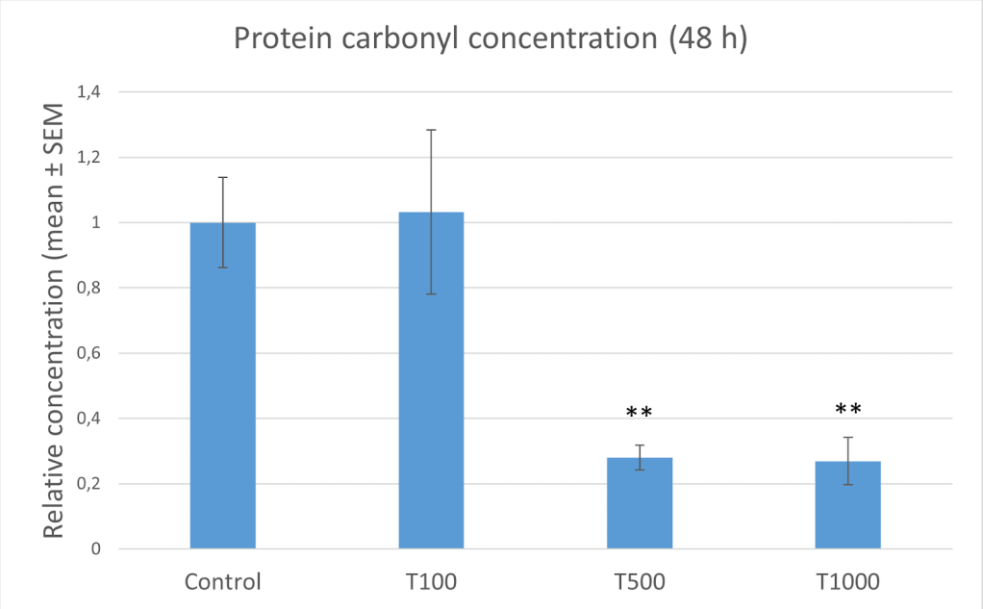

Figure 6: Effects of 48 h T-2 toxin treatment on the PC concentration of the lysate of primary hepatic 3D cell cultures of chicken origin. Control: without T-2 toxin treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. PC concentration was measured by chicken specific ELISA test. Relative concentrations were calculated by considering the mean value of the Control group as 1. Relative absorbances were calculated by considering the mean value of the Control group of 24 hours of incubation as 1. Results are expressed as mean ± SEM. ** $p < 0.01$.

The effects of T-2 toxin on the PC concentration of the cell cultures is shown in **Figure 6**. The higher levels of T-2 toxin (500 nM and 1000 nM) significantly decreased ($p = 0.00286$, $p = 0.00161$, respectively) the PC concentration of the cultured cells.

HSP27

The HSP27 content of the cell cultures was measured by chicken specific ELISA test.

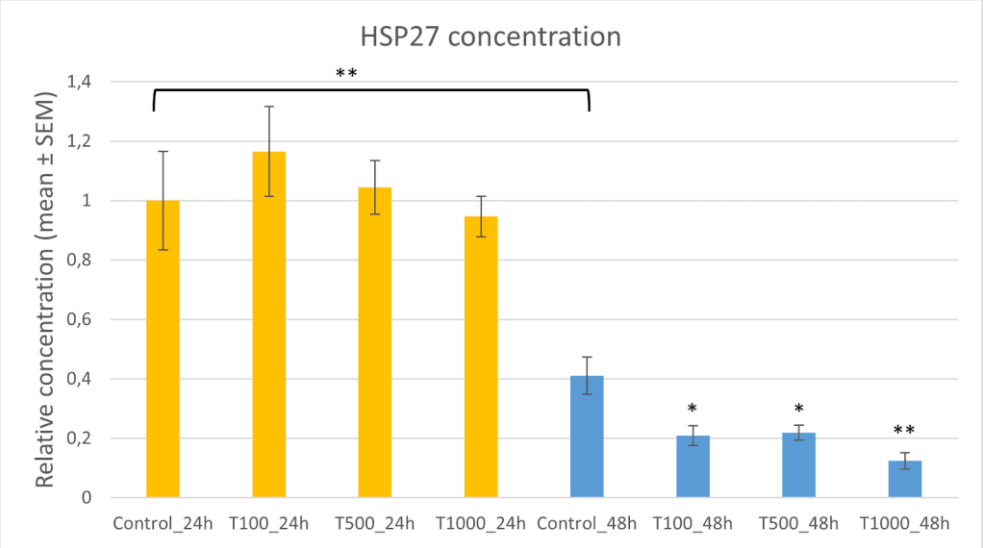

Figure 7: Effects of 24 and 48 h T-2 toxin treatment on the HSP27 concentration of primary hepatic 3D cell cultures of chicken origin. Yellow bars: 24 h treatment; blue bars: 48 h treatment. Control: without T-2 toxin treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. HSP27 concentration was measured by chicken specific ELISA test. Relative concentrations were calculated by considering the mean value of the Control group of 24 hours of incubation as 1. Results are expressed as mean ± SEM. * $p < 0.05$; ** $p < 0.01$.

The effects of T-2 toxin on the HSP27 concentration of the cell cultures is shown in **Figure 7**. After 24 hours, no significant changes were detected in the HSP27 concentration of the cell cultures. The HSP27 production of the 48-hour control group was significantly lower ($p = 0.00767$) than that of the control cells after 24 hours of culturing. All T-2 toxin treatments significantly decreased ($p = 0.0172$, $p = 0.0168$, $p = 0.00178$) the HSP27 concentration of the cell cultures when applied for 48 hours.

GRP78

The GRP78 concentration of the cell cultures was measured by chicken specific ELISA test.

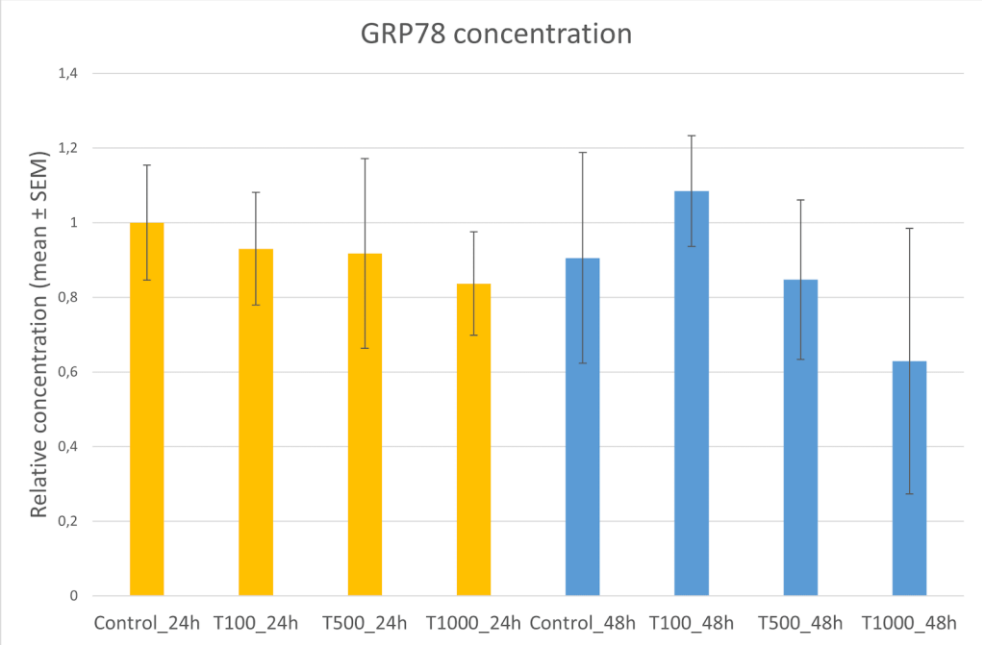

Figure 8: Effects of 24 and 48 h T-2 toxin treatment on the GRP78 concentration of primary hepatic 3D cell cultures of chicken origin. Yellow bars: 24 h treatment; blue bars: 48 h treatment. Control: without T-2 toxin treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. GRP78 concentration was measured by chicken specific ELISA test. Relative concentrations were calculated by considering the mean value of the Control group of 24 hours of incubation as 1. Results are expressed as mean ± SEM.

The effects of T-2 toxin on the GRP78 concentration of the cell cultures is shown in **Figure 8**. No significant changes were observed in the GRP78 concentration of the cell cultures, either after 24 or 48 hours.

Statistics

R 3.5.3. software (GNU General Public License, Free Software Foundation, Boston, MA, USA) were used for the complete data analysis. Regarding the dilution of the T-2 toxin, 15 wells were included to one treatment group on the 96-well plates. Differences between the various groups were assessed using one-way variance analysis (ANOVA) and Dunett's post hoc test was used for pairwise comparisons. The results were evaluated as mean ± standard error of the mean (SEM). Differences were assumed significant at $p < 0.05$.

Discussion

T-2 toxin is a trichothecene mycotoxin produced by different *Fusarium* species that frequently contaminate cereal grains and their processes food products [1]. T-2 toxin is present in 41% of cereals and their processed products in Europe [2]. Since poultry species primarily consume cereals, they are more likely to be exposed to the toxin.

T-2 toxin has a genotoxic, cytotoxic and immunomodulatory effect in poultry that leads to feed refusal, acute digestive disease, reduced productivity, atrophy of the lymphoid organs and lymphoid depletion, as well as decreased egg production [6]. Although there are several decontamination methods, none have been established on industry wide scales [50–52]. Since strategies for preventing contamination and methods of decontamination have not had a reductive impact, understanding the cellular mechanism of action of T-2 toxin has crucial importance.

The main cellular effect of T-2 toxin is the inhibition of protein synthesis through the binding of the peptidyl transferase enzyme [7]. In addition, it is likely to induce oxidative stress in cells, which leads to increased lipid peroxidation, DNA damage and apoptosis [3, 8, 47, 53]. Furthermore, oxidative stress is also often associated with the development of ER stress.

After being absorbed through the GI tract, T-2 toxin enters the liver, where it undergoes extensive metabolization before being excreted through the bile. For this reason, our 3D hepatocyte-NP cell co-cultures are excellent *in vitro* models to study the cellular effect of T-2 toxin. In the present study, the hepatocyte to non-parenchymal cell ratio was set to 6:1. This model represents a state of mild hepatic inflammation with moderate intrahepatic macrophage migration [54, 55]. 3D cell cultures imitate the *in vivo* environment better by allowing the natural morphology of the cells, as well as cell-cell and cell-ECM interactions. In case of treatment solutions and nutrients, the presence of a diffusion gradient is also observed. In this study, 3D cell cultures were developed using magnetic 3D bioprinting. This method is simple, cost effective and requires similar maintenance as the traditional 2D cell cultures.

To study the effect of T-2 toxin *in vitro*, magnetic 3D cell cultures were developed, and the cells were treated with 100, 500 and 1000 nM T-2 toxin for different (24 and 48 hours) incubation periods.

The impact of T-2 toxin on the viability of the cells was evaluated by Cell Counting Kit-8 (CCK-8) assay. Our results showed a significant decrease in the metabolic activity of the cells that was inversely correlated with the applied T-2 toxin concentrations (**Figure 4**). This suggests a clear negative effect of the toxin on the viability of the chicken hepatocytes. Metabolic depression was alleviated after the longer incubation time (48 hours), indicating a metabolic adaption by the liver cells. These results are in correlation with previous findings of other publications indicating a cytotoxic effect of T-2 toxin on different cell lines of various animal species [3, 7]. This cytotoxic effect was concluded to be present when porcine brain capillary endothelial cells were incubated with 10 nM T-2 toxin for 24 h, which lead to decrease of cell viability to 65% [56]. A study investigating the dose-dependent cytotoxicity of T-2 toxin on reproductive cells using TM3 Leydig cells, concluded that T-2 toxin is highly cytotoxic to TM3 leydig cells [57]. Plasma proteins involved in coagulation and fibrinolysis have also been shown to be impacted by the cytotoxic effects of T-2 toxin [58]. Morel-chany et al researched the cytotoxic and cytostatic effects of T2- on cells in culture derived from rat hepatomas and rat livers, and concluded that the effects are dependent on concentration as well as exposure [59], which the results from this present study show as well.

To investigate the possible oxidative stress inducing effect of the toxin, two parameters (MDA and PC) was measured. The 1000 nM T-2 toxin concentration significantly reduced the MDA release of the cells after 24 hours, whereas after 48 hours the MDA release of the cell cultures was significantly decreased by all three concentrations of T-2 toxin. Furthermore, the higher levels of T-2 toxin (500 nM and 1000 nM) significantly decreased the PC concentration of the cultured cells after 48 hours. These results suggest that T-2 induced protective mechanisms in the cells, resulting in the reduction of oxidative stress.

In order evaluate wether ER stress developed in the cell cultures or not, two ER stress marker concentrations (GRP78 and HSP27) were measured. No significant changes were observed in the GRP78 levels after the treatments, which suggests that the used T-2 toxin concentrations and incubation times did not induce ER stress. In case of the HSP27, no significant changes were observed after 24 hours. However, after 48 hours, all T-2 toxin treatments significantly decreased the HSP27 concentration of the cell cultures. This result correlates with previous findings that suggest HSP27 plays a role in early stages of stress response and cytoprotection [17, 24]. Burban et al showed that activation of the HSP27-PI3K-AKT pathway protects against certain types of ER stress [17], while Ito et al showed that ER stress induces the phosphorylation of HSP27 [24].

Based on these findings, T-2 toxin may have caused a mild metabolic depression in the hepatic cell cultures, and might also have induced protective mechanisms resulting in reducing oxidative stress and potential ER stress in the cells when used in higher concentrations for longer periods of time. The induction of these protective mechanisms in cell cultures is probably due to the fact that 3D cell cultures better mimic *in vivo* conditions.

Abstract

T-2 toxin is a trichothecene mycotoxin produced by different *Fusarium* species that often contaminate cereal grains and their processed food products. Since poultry species consume mainly cereals, they are at a greater risk of T-2 toxin exposure. The toxin has genotoxic, cytotoxic, and immunomodulatory effects in poultry, leading to reduced productivity. The main cellular effect of the toxin is the inhibition of protein synthesis. It also induces oxidative stress that could lead to endoplasmic reticulum (ER) stress. Therefore, the investigation of the cellular effects of T-2 toxin is highly important to agriculture and veterinary medicine.

In this study we examined the cellular effects of T-2 toxin in three-dimensional (3D) hepatocyte – non-parenchymal (NP) cell co-cultures of chicken origin. The co-cultures were exposed to three concentrations (100, 500, and 1000 nM) of T-2 toxin for different incubation times (24 and 48 h). The metabolic activity of the cells was determined by CCK-8 (Cell Counting Kit-8) test. To assess the oxidative stress and ER stress, the amount of protein carbonyl (PC) was measured from cell lysates, while malondialdehyde (MDA) as well as glucose regulated protein 78 (GRP78) and heat shock protein 27 (HSP27) were measured from medium using chicken-specific ELISA tests.

Our results showed that T-2 toxin significantly decreased the metabolic activity in every treatment group after both incubations. Metabolic adaptation was indicated by the liver cells as metabolic depression was alleviated after the longer incubation period. The MDA release of the cells was significantly decreased only in the 1000 nM treatment group after 24 h, while after 48 h all three concentrations of T-2 toxin significantly decreased the MDA production of the cell cultures. The higher levels of T-2 toxin significantly decreased the PC concentration of the cultured cells after 48 h. No significant changes were observed in GRP78 concentration of the cell cultures. In the case of HSP27, all T-2 toxin treatments significantly decreased concentration after 48 h.

These findings suggest that T-2 toxin triggered a mild metabolic depression of the hepatic cells, but in our 3D co-culture model that mimics better the *in*

vivo conditions, the toxin could also induce protective mechanisms within the cells resulting in the reduction of oxidative and by chance ER stress when applied in higher concentrations for longer periods of time.

Hungarian abstract

A T-2 toxin *Fusarium* gombafajok által termelt trichotecénvázas mikotoxin. E gombafajok gyakran fertőznek meg különféle gabonanövényeket, és a belőlük előállított élelmiszeripari termékeket. A szárnyasok különösen kitettek a T-2 toxin okozta mikotoxikózisnak, mivel nagyrészt gabonaalapú takarmányt fogyasztanak. A toxin genotoxikus, citotoxikus és immunmoduláló hatást is kifejt az állatokban, ami a termelékenység csökkenéséhez vezethet. A toxin legjelentősebb sejtszintű hatása a fehérjeszintézis gátlása, emellett oxidatív stresszt is kiválthat, amely endoplazmatikus retikulum (ER) stressz kialakulásához vezethet. Mindezek miatt a T-2 toxin sejtszintű hatásainak vizsgálata rendkívül fontos a mezőgazdaság és az állatgyógyászat számára egyaránt.

Munkánk során a T-2 toxin májsejtekre gyakorolt hatását vizsgáltuk csirke eredetű háromdimenziós (3D) hepatocita – nem-parenchimális (NP) sejt ko-kultúrákban. A sejttenyészeteket háromféle (100, 500 és 1000 nM) T-2 toxin koncentrációval történő kezelésnek tettük ki különböző ideig (24 és 48 óra). A sejtek metabolikus aktivitását CCK-8 (Cell Counting Kit-8) teszt segítségével határoztuk meg. Az oxidatív stressz és az ER stressz vizsgálatához a sejtek lizátumából a protein-karbonil (PC), a tápfolyadékból pedig a malondialdehid (MDA), valamint a glükóz-szabályozott fehérje-78 (GRP78) és a hősokkfehérje-27 (HSP27) mennyiségét csirkespecifikus ELISA-tesztekkel mértük.

Eredményeink azt mutatták, hogy a T-2 toxin az összes kezelési csoportban szignifikánsan csökkentette a metabolikus aktivitást mindkét inkubációs időt követően. A hosszabb inkubációs idő alkalmazása során a metabolikus aktivitás csökkenése kisebb mértékű volt, amely a máj-eredetű sejtek metabolikus adaptációjára utalhat. Az MDA termelődést 24 óra után csak az 1000 nM T-2 toxin csökkentette szignifikánsan, 48 óra után azonban már mindhárom kezelési koncentráció kifejtette ezt a hatást. A magasabb koncentrációban alkalmazott toxin 48 órás kezelést követően szignifikánsan csökkentette a sejtek PC koncentrációját. A GRP78 szintje nem változott szignifikánsan a sejttenyészetekben. A HSP27 mennyiségét 48 óra után minden alkalmazott T-2 toxin koncentráció szignifikánsan csökkentette.

Eredményeink alapján elmondható, hogy a T-2 toxin kis mértékben csökkentette a sejtek metabolikus aktivitását, de nagyobb koncentrációban és hosszabb ideig alkalmazva az *in vivo* körülményeket jól tükröző 3D ko-kultúra modellünkben a toxin védekező mechanizmusokat is indukált a sejtekben, amelyek az oxidatív és esetlegesen az ER stressz csökkenését is eredményezhették.

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Appendix 1. Declaration regarding TDK research paper-thesis equivalence

DECLARATION

I hereby declare that the thesis entitled *"The effect of T-2 toxin on cell metabolism, oxidative stress and ER stress in 3D hepatic cell cultures of chicken origin"* is identical in terms of content and formal requirements to the TDK research paper submitted in 2022.

Date: 29/10/2023

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