Thesis

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Effects of T-2 toxin in 3D hepatic cell cultures of chicken origin

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1. List of abbreviations

DNA	Deoxyribonucleic acid
3D	Three dimensional
NP	Non parenchymal
CCK-8	Cell Counting Kit-8
LDH	Lactate Dehydrogenase
ELISA	Enzyme-linked Immunosorbent Assay
2D	Two dimensional
ECM	Extracellular Matrix
DON	Deoxynivalenol
UV	Ultraviolet
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
IL-2	Interleukin-2
IL-5	Interleukin-5
IL-1	Interleukin-1
IL-6	Interleukin-6
EGTA	Ethylene Glycol Tetraacetic acid
HBBS	Hanks Balanced Salt Solution
BSA	Bovine Serum Albumin
WST	Water Soluble Tetasolium Salt
INT	Iodonitrotetrazolium chloride
HE	Hematoxylin and eosin
SEM	Standard error of the mean

2. Introduction

Broiler chickens are commonly exposed to different environmental and dietary factors, such as mycotoxins, which can negatively affect their health and productivity. Mycotoxins are secondary metabolites produced by molds contaminating different crops. The most prominent secondary metabolites are trichothecene mycotoxins, such as T-2 toxin. The main effects of these toxins in poultry are reduced feed intake, body weight and productivity, immunosuppression as well as gastrointestinal, neurological and reproductive disorders. They can also cause increased free radical production, resulting in oxidative stress leading to changes in cell viability, DNA damage and apoptosis. Generally, these changes can be linked to inflammatory processes. In addition, trichothecene mycotoxins may also affect the xenobiotic biotransformation of the liver. In most cases, the dietary uptake of T-2 toxin cannot be avoided, thus investigations on the effects of T-2 toxin on a cellular level is of high importance.

In the present study the effects of T2-toxin were examined in three-dimensional (3D) hepatocyte-non-parenchymal (NP) cell co-cultures of chicken origin. 3D cell cultures have many advantages compared to monolayer cell cultures, because the produced spheroids mimic *in vivo* features of the examined organs better. In 3D cultures cells preserve their original phenotype and function, as well as diverse cell-cell and cell-extracellular matrix interactions are formed. 3D cell cultures were developed by preparing magnetic spheroids: magnetic nanoparticles were added to the isolated cells, which were aggregated into 3D spheroid structures by using a magnetic drive.

To examine the cellular effects of the T-2 toxin on the cellular viability and inflammatory responses, hepatocyte-NP cell co-cultures were exposed to various concentrations (100, 500 and 1000 nM) of T-2 toxin for different incubation times (24, 48 and 72 hours). To determine the extent of the effects of T-2 toxin on the hepatic cells regarding cytotoxicity, apoptosis and immunoregulation, various parameters were measured. The viability of the cells was assessed by CCK-8 (Cell Counting Kit-8) assay. The number of dead cells was determined by the lactate dehydrogenase (LDH) activity. Concentration of interleukin-6 (IL-6), a pro-inflammatory cytokine, was quantified by chicken specific ELISA test.

3. Literature review

3.1 The brief history of cell cultures

Since the beginning of the last century, *in vitro* cell cultures allowed major discoveries in the field of cell biology. The foundation for monolayer cell cultures was laid when Ross Granville Harrison successfully cultured frog nerve cells outside the organism. In the late 1930's, cell cultures were seen as failures, since they were too difficult to handle, not replicable and had no forms of applications. The change came in the early 1940's with the development of synthetic culture media for plant and animal cells. Since then, many important breakthroughs and discoveries were made. In 1973, the basis in development of gene integration into cellular genomes was created by the introduction of DNA into mammalian cell cultures. Furthermore, in 1975, the first monoclonal antibodies were discovered. (Rodríguez-Hernández, et al., 2014)

Cell cultures are mainly used as experimental models in basic and medical science, in the study of physiological requirements for specific cell types and investigation of cell development and differentiation. Further applications of the cell cultures are pathological studies, genetic manipulation and biotechnology. The main fields that profit from the advance of cell cultures nowadays are virology, oncology, pharmaceutical development and many medical routine applications. (Arango, et al., 2013)

3.2 2D cell culture systems

In vitro cell cultures helped understanding basic *in vivo* mechanisms of cell behavior, such as cell differentiation, migration, growth and mechanics. These cell behaviors are influenced by the biochemical characteristics and environment of the cell. To understand the *in vivo* processes that underlie of function and formation of organs, it is important to interpret the mechanics behind these behaviors. (Duval, et al., 2017)

The aim was to create a model that mimics the cellular microenvironment as closely as possible. The main challenge in creating such a model was the construction of the

tissue-tissue interface, the control of the distribution of oxygen, carbon dioxide, nutrients and waste and many other factors that are important to regulate the *in vivo* activities.

Traditional monolayer (two-dimensional, 2D) cell cultures were developed over a century ago to study cellular responses to biophysical and biochemical stimuli and are still widely used nowadays. In a traditional 2D cell culture, the cells adhere to a collagen-coated flat glass or plastic surface of a cell culture dish (e.g., Petri dish) and grow there as a monolayer. (Kapałczyńska, et al., 2016)

In the cell culture dish, a specific culture media is needed by the cultivated cells, which can contain serum, nutrients, carbon source, organic supplements and antimicrobial or antifungal agents that inhibit the contamination of the cell culture. The required temperature, CO_2 and O_2 levels are precisely regulated as well. (Arango, et al., 2013)

3.2.1 Transition into 3D cell culture systems

The main goal of the cell culture systems is to imitate the *in vivo* conditions of the isolated cells as accurate as possible. This includes the microenvironment, the cell-cell interactions, the growth and the multiplication of the cells.

The 2D cell cultures have a limit in fulfilling these conditions. They offer a standardized material for studies, are highly reproducible and simple, thus easy to interpret. However, it cannot provide the same conditions as the *in vivo* environment. 3D cell cultures systems were developed to achieve the *in vivo* conditions as accurate as possible. Since the 1970s, 3D cell cultures are gradually coming into the spotlight for different studies and experiments. (Arango, et al., 2013)

There are numerous difficulties with 2D cell cultures. One of the major problems is that the cells cultivated on a flat surface obtain a different morphology and geometry while growing; they can take up a more flat or elongated shape. This results in a different arrangement of cell components and cytoskeleton. If the nucleus is affected, gene expression and protein synthesis, as well as cell secretion and signaling can be influenced. (C. Jensen & Y. Teng, 2020) (Duval, et al., 2017)

The construction of physiological environment for some cell types (e.g., hepatocytes) was challenging, so alternative methods were developed. In traditional 2D cell cultures,

hepatocytes have a lower amount of transcriptional genes and have a different morphology. To successfully cultivate hepatocyte cell cultures, the sandwich culture method was created. This method places the hepatocytes between two layers of extracellular matrix (ECM), polyacrylamide or collagen (**Figure 1**). These cell cultures imitate the *in vivo* function and morphology more accurately than traditional 2D cell cultures. Development of this method was a big step for drug discovery, where the researchers were keen in understanding pharmacokinetics in relation to the liver as one of the main organs of xenobiotic metabolism in an organism. (Duval, et al., 2017)

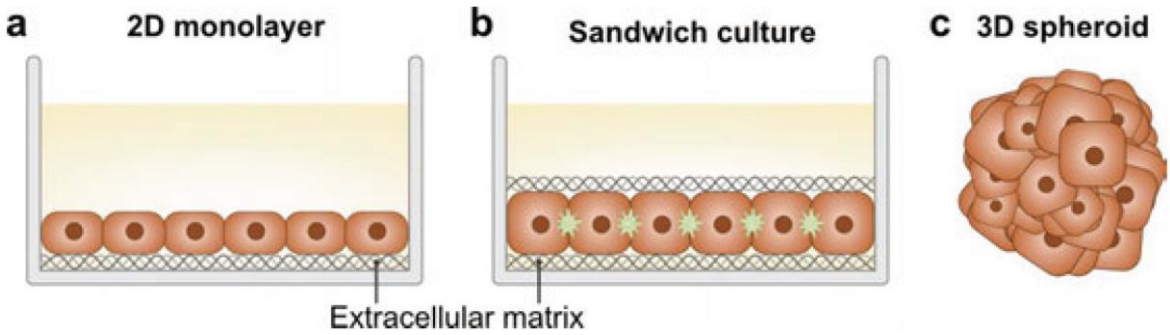

Figure 1: Example of culture configurations for studies of hepatocytes. (a) Traditional 2D monolayer, where hepatocytes are cultured on a surface coated with ECM proteins. (b) Sandwich configuration, in which a second layer of ECM is added on top of the cells. (c) 3D spheroid culture, where hepatocytes aggregate to form a spheroidal microtissue. *(Ölander, 2019)*

Results of different studies regarding 3D cell cultures showed that the increase of dimensionality of the ECM in the direct cell environment can considerably affect the cell's proliferation, differentiation, response to mechanical stimuli and survival. (Duval, et al., 2017)

3.3 Why the 3D cell cultures

3D (three-dimensional) cell cultures have many advantages compared to traditional monolayer cultures, because they imitate the *in vivo* environment better. In these cell cultures, cells can keep their morphology and function, as well as grow and expand in third dimension similarly how they are arranged in the tissue. Due to the 3D arrangement of the cells the access to oxygen, nutrients and metabolites is variable: the cells in the outer layers receive more and the cells in the inner layers receive less of these compounds. (Hoarau-Véchot, et al., 2018) (Kapałczyńska, et al., 2016) Moreover, cell junctions between neighboring cells ensure communication, the transport of small molecules, the diffusion of drugs and the receiving of electrical impulses as shown in **Figure 2**. These signals influence the differentiation, proliferation and gene expression of the recipient cells. (Shri, et al., 2017) (Ravi, et al., 2014) Because of these favorable qualities, 3D cell cultures are promising models for in vitro studies.

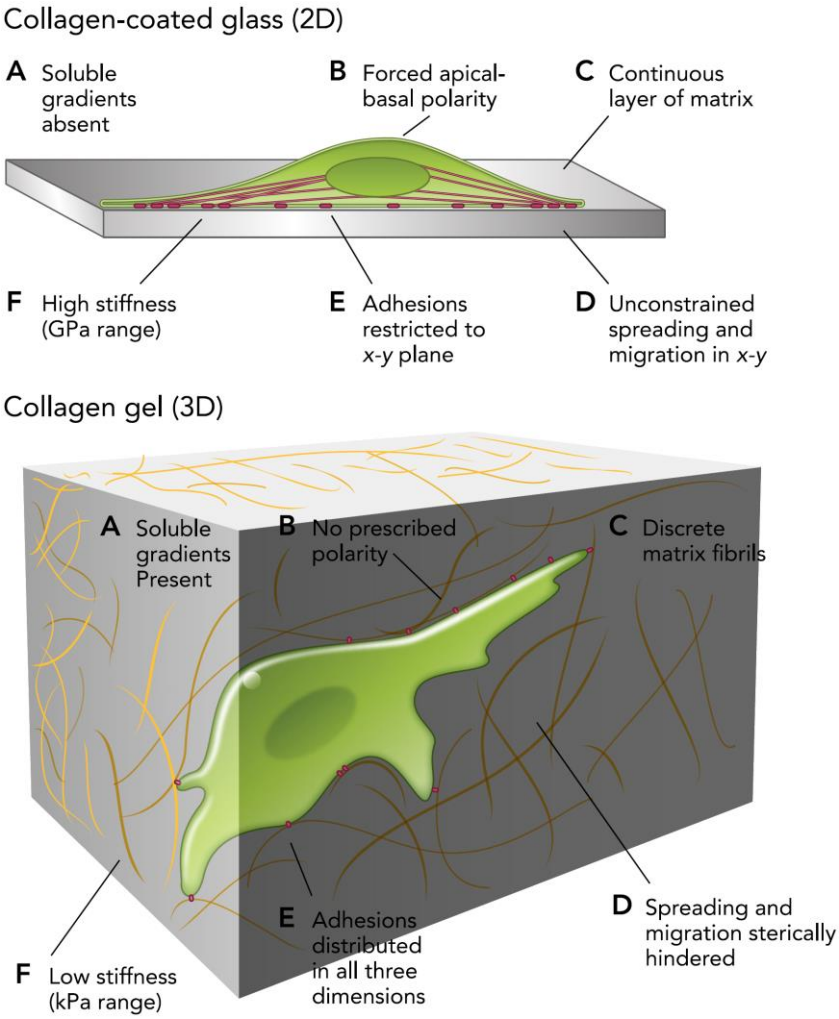

Figure 2 Cells in 2D and 3D microenvironments interact differently with their surroundings due to differences regarding the received mechanical and chemical signals

Drug resistance of the cells in 3D cell cultures is higher, and the metabolism is better than in 2D cell cultures. Because of this, results of studies and experiments can represent the actual *in vivo* effect on cells much better. Animal models are usually used for drug testing, but they are expensive, and there are many concerns regarding animal welfare. Due to the ability of 3D cell cultures to imitate the structure of an organ or tumor, the effect of newly developed drugs can be tested on the cultivated cells from a specific organ instead of animal models. (Hoarau-Véchot, et al., 2018)

The only negative aspect of the 3D cell culture is that the cost and time expense is much higher than the 2D cell cultures. Therefore, the replicability of the 3D cell culture is not nearly as high as the 2D. (C. Jensen & Y. Teng, 2020)

3.4 3D cell culture systems

There are scaffold-free and scaffold-based 3D culture methods. In scaffold-based culturing methods, there are scaffolds made from synthetic or natural material. (Knight & Przyborski, 2014) The are many types of 3D culture methods, for example the hanging drop method, the non-adherent surface method, the hydrogel-based scaffolds, as well as magnetic bioprinting methods. (Hoarau-Véchot, et al., 2018), (C. Jensen & Y. Teng, 2020)

3.4.1 Hanging drop method

In the hanging drop method, a tiny droplet with a volume less than 50 µL is placed on the underside of a Petri dish lid or a hanging drop plate. Because of the surface tension, a hanging drop is formed and in each drop one spheroid can be produced. The medium should not contain any substances that disturb the spontaneous formation of the cells into spheroids. There is a bottom tray with a liquid that keeps the humidity around the drop at the necessary level to counteract evaporation. This method is simple and consistent, the only difficulty is to change of the medium. (Hoarau-Véchot, et al., 2018), (Langhans, 2018)

3.4.2 Non-adherent surface method

Another approach to create 3D cell cultures is the non-adherent surface method. Principle of this method is that the surfaces of the wells or culturing systems are coated with a unique substance, which reduces cell adherence and therefore enhances the spontaneous formation of spheroids. These spheroids then easily can be taken out from the wells for other experiments. This method is simple and easy to handle, but these special coatings can be costly. (Kapałczyńska, et al., 2016), (C. Jensen & Y. Teng, 2020), (Langhans, 2018)

3.4.3 Hydrogel-based scaffolds

In hydrogel-based scaffolds, cells are mixed within hydrogel or seeded on top of a solid hydrogel, where they form spheroids. There are natural hydrogels, that support many physiological cell functions, leading to high cell viability, regulated proliferation and differentiation, as well as synthetic hydrogels, that offer a consistent structure and foreseeable manipulation of substances but lack physiological cell interactions. (Langhans, 2018), (Hoarau-Véchot, et al., 2018), (Cushing & Anseth, 2007)

3.4.4 Magnetic 3D bioprinting

In the present study, magnetic 3D bioprinting was used to create 3D cell cultures. In this method, cells are magnetized with biocompatible magnetic nanoparticles that consist of gold, iron-oxide and poly-L-lysin. These cells are aggregated into 3D spheroid structures by the help of a magnetic drive. Magnetic 3D cell cultures have many advantages: spheroids can be formed easily and quickly, they can be observed under the microscope, the culturing routine is similar to 2D cell culturing, and specialized equipment is not needed. Also, this technique is compatible with various detection methods, such as fluorescent microscopy, Western-blot, qRT-PCR, flow cytometry and different viability assays. (Langhans, 2018), (Hoarau-Véchot, et al., 2018), (Souza, et al., 2010)

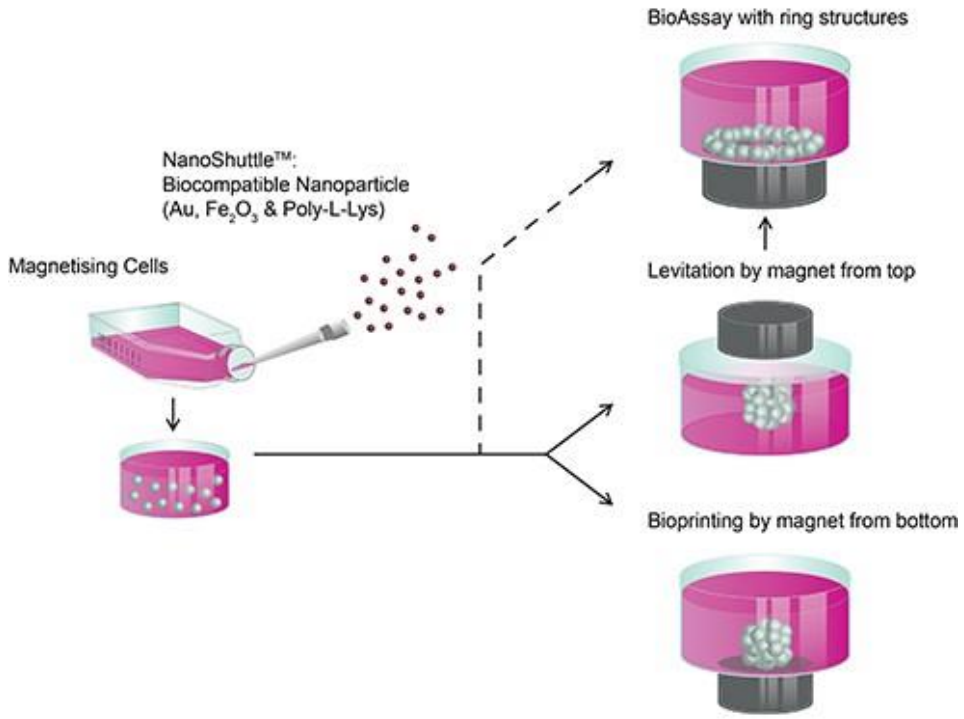

Figure 3: Spheroid formation by printing rings, magnetic levitation or bioprinting by Greiner Bio One 3D cell culture that was used *(*https://3dcellculture.gbo.com/)

4. T-2 toxin

Mycotoxins are secondary metabolites produced by different species of fungi. Mycotoxicosis is the consequence of the ingestion of contaminated food, which plays a highly important role concerning the livestock animal diseases and the resulting economic problems. The most prominent mycotoxins are the trichothecene mycotoxins produced by various *Fusarium* species. Warm weather with high humidity increases the chance of plant infection by the *Fusarium* species which often contaminate different types of grains, such as wheat, corn, barley, oat and rye. (Adhikari, et al., 2017), (Sokolović, et al., 2008)

Trichothecenes are tetracyclic sesquiterpenoids containing a 12,13-epoxytrichothec-9-ene ring. The epoxy ring is responsible for the toxicity. They are classified into 4 groups (A, B, C, D) based on the functional groups or the absence of them in their structure. T-2 toxin is a type A trichothecene. Type A trichothecenes do not have a carbonyl group at C8. In type B trichothecenes, the structure contains a carbonyl group at C8 position. The most important type B trichothecene is deoxynivalenol (DON). In type C trichothecenes (e.g., crotocin and braccharin), a second epoxy ring is found between C7 and C8 or C9 and C10. Type D trichothecenes (e.g., satratoxin and roridin) contain a macrocyclic ring between C4 and C15. The main cytotoxic effects of trichothechene mycotoxins are the inhibition of eukaryotic protein synthesis and immunomodulatory effects. Animal studies have shown that immunomodulatory effects range from suppression to stimulation of the immune system in a dose-dependent manner (Sudakin, 2003), (Hossam El-Din, 2013)

T-2 toxin produced by different *Fusarium* species (such as *F. moniliforme, F. exquisite, F. culmorum, F. solani*) is a type A trichothecene and one of the most toxic mycotoxins. It is nonvolatile, has a low molecular weight and is insoluble in water. The resistance of T-2 toxin is high against heat and UV light; thus, it stays active after the food processing. The average amount of T2-toxin is classified between 0,01 mg/kg and a maximum of 0,71 mg/kg in grain. (Sokolović, et al., 2008), (Adhikari, et al., 2017)

4.1 Toxicity

The uptake of T-2 toxin happens by food or water intake, by inhalation of air or aerosols and by transdermal absorption. All routes of infection can result in systemic toxicity. Most mycotoxins enter the body by absorption in the gastrointestinal tract and then are transported via the *v. portae* to the liver, which is the main organ for the metabolism of T-2 toxin. The extensive metabolism of T-2 toxin is carried out by three major metabolic pathways: hydrolysis, hydroxylation and conjugation. (Park, 2019)

The effects of T-2 toxin in poultry are determined by the amount of the toxin, the method of infection, the time of exposure, the sex and the general condition of the animal, as well as the presence of other diseases. However, poultry is less sensitive than ruminants or swine to T-2 toxin, due to the low absorption and fast excretion of the toxin. The lethal dose of T2-toxin that would kill 50% of a 7-day old broiler population is 4,97 mg/kg. (Sokolović, et al., 2008)

T-2 toxin has a dose-dependent effect on the immune system: in low dosage it can stimulate, in high dosage it can inhibit the immune system. Additionally, in the gastrointestinal tract, T-2 toxin causes necrotic lesions, starting from the mouth. The effects of the toxin on the liver are quite impactful; by inhibiting the protein synthesis, the amount and activity of the detoxifying enzymes are decreased. Furthermore, T2-toxin negatively affects the performance of poultry; it decreases the food intake and weight gain, as well as the quality and number of produced eggs. (Adhikari, et al., 2017), (Sokolović, et al., 2008), (Guerre, 2015) (Wyatt, et al., 1973)

4.2 Effects on cellular level

The primary effect of T-2 toxin on a cellular level is the inhibition of protein synthesis by binding the peptidyl transferase at the large ribosomal subunit (60S). In addition, T-2 toxin can inhibit the DNA and RNA synthesis. Besides, T-2 toxin can elevate the level of reactive oxygen species (ROS) in the cell, thus induces oxidative stress. Oxidative stress occurs when the antioxidants of a cell cannot counterbalance the increase in ROS production. Rising levels of ROS have a negative effect on the cellular homeostasis, structure and function. Chronic high levels of ROS or continuous exposure to oxidative stress results in the

induction of apoptotic pathways as shown in **Figure 4**. (Betteridge, 2000), (Hossam El-Din, 2013) (Mackei, et al., 2020) (Park, 2019) High levels of ROS are also associated with DNA damage, elevated lipid peroxidation and changes in the cell-signaling and inflammatory pathways. (Ayala, et al., 2014) (Reis & Spickett, 2012) (Kinnunen, et al., 2012)

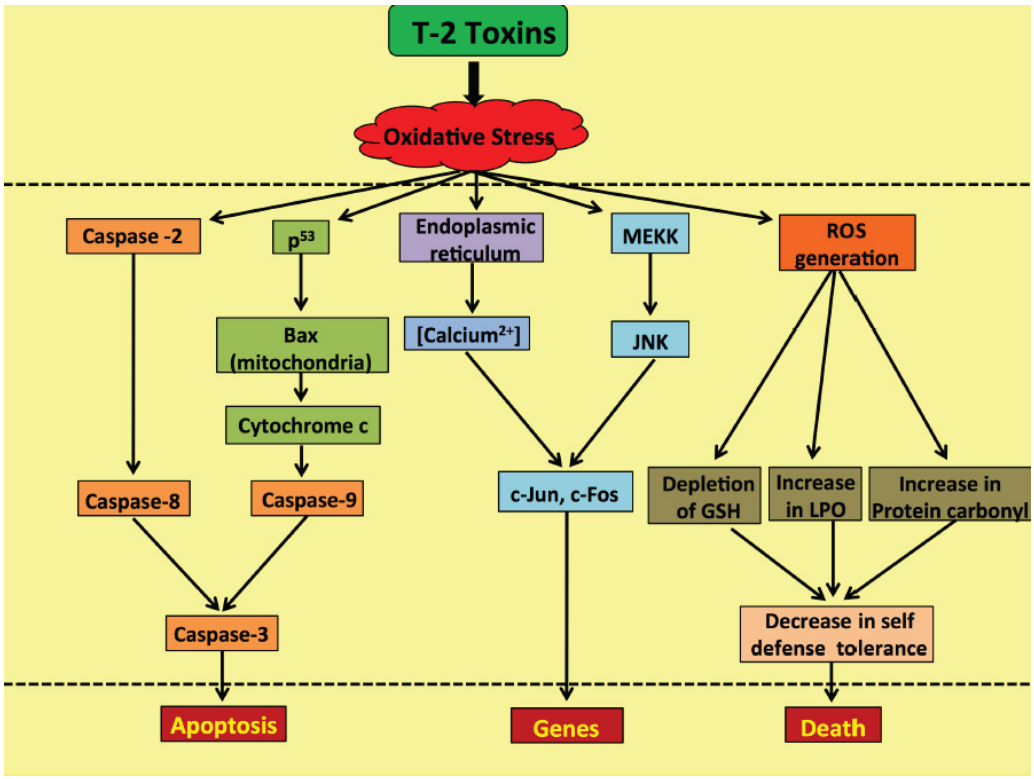

Figure 4: Effects of T-2 toxin through the induction of oxidative stress on cells *(Adhikari, et al., 2017)*

Exposure to T-2 toxin causes hematologic and immunotoxic disorders, by inducing leukopenia, cell depletion in the lymphoid organs and inhibition of erythropoiesis in the spleen and bone marrow. That also includes hematopoietic precursors as granulocyte and monocyte colony forming cells. The immunosuppressant potency is indicated by reduction of lymphocyte proliferation and disturbed maturation process of dendritic cells. Also, continuous levels of low dose T-2 toxin ingestion change the induction of the toll-like receptors by downgrading the ability of the immune system to notice pathogen associated molecular patterns derived from different microbes. (Adhikari, et al., 2017) (Seeboth, et al., 2012) (Sokolović, et al., 2008)

In addition, it appears that T-2 toxin influence the T-cell mediated functions by inhibiting their production of interleukin-2 (IL-2) and interleukin-5 (IL-5). Furthermore, induction of mRNA expression for specific proinflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6) was reported. Interleukins are important messengers of the immune system. They stimulate or inhibit the differentiation, proliferation and mitosis of specific cells of the immune system. Antagonistic effects on lymphocyte proliferation and immunoglobulin synthesis could be noticed after exposure to trichothecenes. Studies have shown that in pigs, continuous low-dosage exposure to T-2 toxin have negative effect on the secondary immune response and the humoral response mediated by B lymphocytes. (Adhikari, et al., 2017) (Seeboth, et al., 2012) (Shokri, et al., 2000)

Interleukin-6 belongs to the group of proinflammatory cytokines and is produced immediately as a response to tissue injuries and infections by fibroblasts and macrophages. It is a soluble mediator with a multifunctional effect on inflammation, immune response and hematopoiesis. The synthesis of IL-6 begins at the infectious lesion or in the area of tissue damage and acts as a warning signal to the rest of the body by inducing various responses (**Figure 5**). Following the synthesis of IL-6, it reaches the liver with the bloodstream and induces the production of acute phase proteins, such as C-reactive protein, serum amyloid A and fibrinogen, while the production of albumin and transferrin is decreased. In the bone marrow, IL-6 promotes megakaryocyte maturation, thus increasing the release of platelets. Measuring the change of acute phase proteins and platelet numbers is widely used in clinical laboratory examination for inflammations. Also, IL-6 elevates the specific differentiation of CD4+ T cells, therefore they hold an essential role in the connection of acquired and innate immune response. Furthermore, IL-6 induces the transformation of the CD8+ T cells into cytotoxic T cells and the transformation of activated B cells into plasma cells that produce antibodies. (Tanaka, et al., 2014)

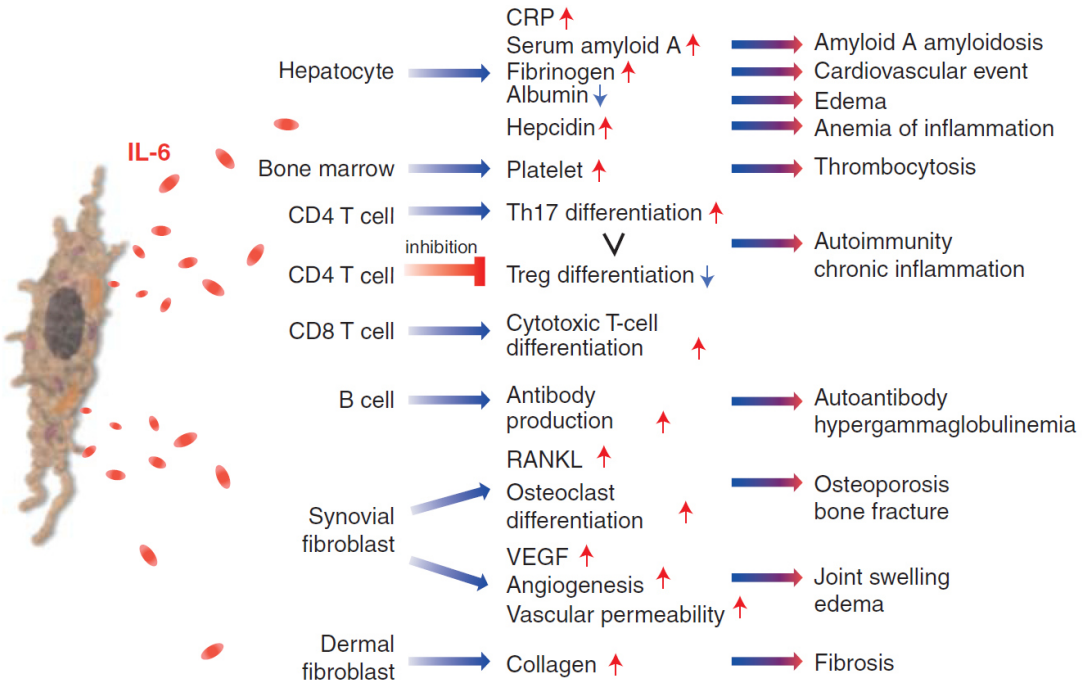

Figure 5: IL-6 in its pleiotropic function in inflammation, immunity and disease (Tanaka, et al., 2014)

5. Hypothesis and aims

T-2 toxin is one of the most toxic trichothecene mycotoxins. The exposure to T-2 toxin results in various pathological disorders in the liver, the primary organ of xenobiotic metabolism. T-2 toxin can elevate the level of ROS production, thus induces oxidative stress in the cells that can lead to lipid peroxidation, the inhibition of protein and nucleic acid synthesis and the initiation of apoptosis.

The aim of the present study was to determine the cellular effects of T-2 toxin in 3D hepatocyte-non-parenchymal cell co-cultures of chicken origin. Effects of T-2 toxin on the cultured chicken liver cells were examined by exposing the hepatocyte-NP cell co-cultures to different T-2 toxin concentrations (100, 500 and 1000 nM) for 24, 48 and 72 hours of incubation time. To determine the pathological effects on the liver cells, different parameters were measured, such as cellular metabolic activity, the amount of damaged or dead cells, and the concentration of interleukin-6.

6. Materials and Methods

All reagents were purchased from Merck KGaA (Darmstadt, Germany). Animal procedures were performed according to the international and national guidelines, as well as the institutional guidelines. This was 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.

6.1 Preparation of the solutions

First, the solutions needed for the isolation were prepared and heated up for 40°C for the multistep perfusion. The solutions for the perfusion were the following:

- 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

The HBSS stock solution was tenfold diluted with sterile distilled water, then 4,7 ml 7,5% $NaHCO_3$ was added to 1000 ml buffer. To prepare to EGTA containing HBSS, 1,052 ml of EGTA was added to 200 ml buffer to set the concentration to 0,5 mmol/l. To prepare a collagenase containing solution, 1 ml $MgCl_2$, 1 ml $CaCl_2$ (with a concentration of 7 mmol/l) and 250 mg of collagenase was added to 100 ml HBBS.

6.2 Chicken liver cell and non-parenchymal cell isolation

The isolation of the hepatic cells was performed from six weeks old male Ross-308 broiler chicken. After the decapitation of the animal in CO_2 narcosis, the chicken was fixated in a recumbent position, the feathers of the ventral area were removed, and laparotomy was performed. The hepatic perfusion was performed by the cannulation of the *gastropancreaticoduodenal* vein as the inflowing branch, and the *cardium* as the draining branch to collect the buffers. The liver was perfused in a three-step perfusion with a flow rate of 30 ml/minutes. The used buffers were warmed up to 40ºC and were freshly oxygenated with Carbogen. First, the liver was perfused by 150 ml of 0,5 mmol/L EGTA containing HBSS buffer to remove the calcium. EGTA is a chelat forming agent that binds the magnesium and calcium ions, thus helps loosening up the cell-cell conjuctions, the disintegration of the tissue and the isolation of the cells. Then, the liver was flushed by 150 ml EGTA free HBSS followed by 130 ml collagenase solution which is responsible for the degradation of the extracellular matrix. The effluent collagenase solution was collected in a sterile trypsinization flask which then was recirculated until the liver was dissolved. After the excision, the liver was placed in a sterile beaker. The next steps were performed on ice and in 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%). Then the suspension was filtered through three layers of sterile gauze into a sterile centrifugation tube. The primary cell suspension was incubated in BSA containing HBBS buffer on ice for 40-50 minutes to stop the collagenase enzymes and avoid the aggregation of the cells.

Next, the cell suspension was centrifuged three times for 3 minutes in low-velocity (100×g) in Williams Medium E supplemented with 0,22% NaHCO3, 50 mg/ml gentamycin, 2mM glutamine, 4 µg/l dexamethasone, 20 IU/l insulin and 5% FBS.

Afterwards, the viability of the cells and total cell number were determined in a Bürker Chamber by trypan blue staining. For this we added 200 µl concentrated cell suspension to 800 µl Williams Medium E, then we took 200 µl from the diluted cell suspension and added the same amount of trypan blue stain. With this staining, the dead and living cell ratio can be determined. The principle of this method is that the trypan blue can only enter the cells with damaged membrane, thus staining them blue. Then the cell suspension was diluted to get 10^6 cells/ml concentration.

To isolate the non-parenchymal cells, the previously achieved supernatant was centrifuged multiple times. First on 350×g for 10 min, then three times on 800×g for 10. The sediment was resuspended in 5 ml Williams Medium E and the viability, as well as the cell number were determined as described above. The suspension was then diluted to a final concentration of 10^6 cells/ml.

By mixing 10 ml non-parenchymal cell suspension with 60 ml hepatocyte suspension, 70 ml co-culture suspension was produced. In this co-culture, the hepatocyte and non-parenchymal cell ratio was 6:1.

6.3 3D cell cultures and treatments

The special equipment and chemicals which are necessary for the preparation of magnetic 3D cell cultures were obtained from Greiner Bio-One Hungary Kft. (Mosonmagyaróvár, Hungary). For the magnetization of the cells, 800 µL magnetic nanoparticles (NanoShuttle™-PL) were mixed into 8 ml cell suspension. NanoShuttle™-PL consists of gold, iron oxide and poly-L-lysine. These nanoparticles magnetize cells by electrostatically binding to the cell membranes. The magnetized cells will appear peppered with dark nanoparticles after incubation. NanoShuttle™-PL can stay attached to the cell membrane for up to 8 days. They are biocompatible and have no effect on the cellular metabolism, proliferation and inflammation responses.

After the incubation with the NanoShuttle™-PL, 100 µL of the cell suspension were transferred into each well onto a cell repellent 96-well plate. The plate was incubated for an hour at 37ºC. During this time, the NanoShuttle™-PL particles could bind to the cell surface. Then the plate was placed on top of a so called Spheroid Drive, that contains small magnets under each well of the plate, and was incubated on 37ºC with CO_2 concentration set to 5%. **Figure 6** shows hepatocytes, after incubation on the Spheroid Drive, aggregated to a 3D spheroid. 24 hours later the culture medium was changed to serum-free medium, while using a Holding Drive. This drive has bigger magnets between four wells of the 96-well plate. After another 24 hours of incubation, the cells were treated with three different (100, 500 and 1000 nM) T-2 toxin concentrations. Every 24 hours the medium was changed during the experiment.

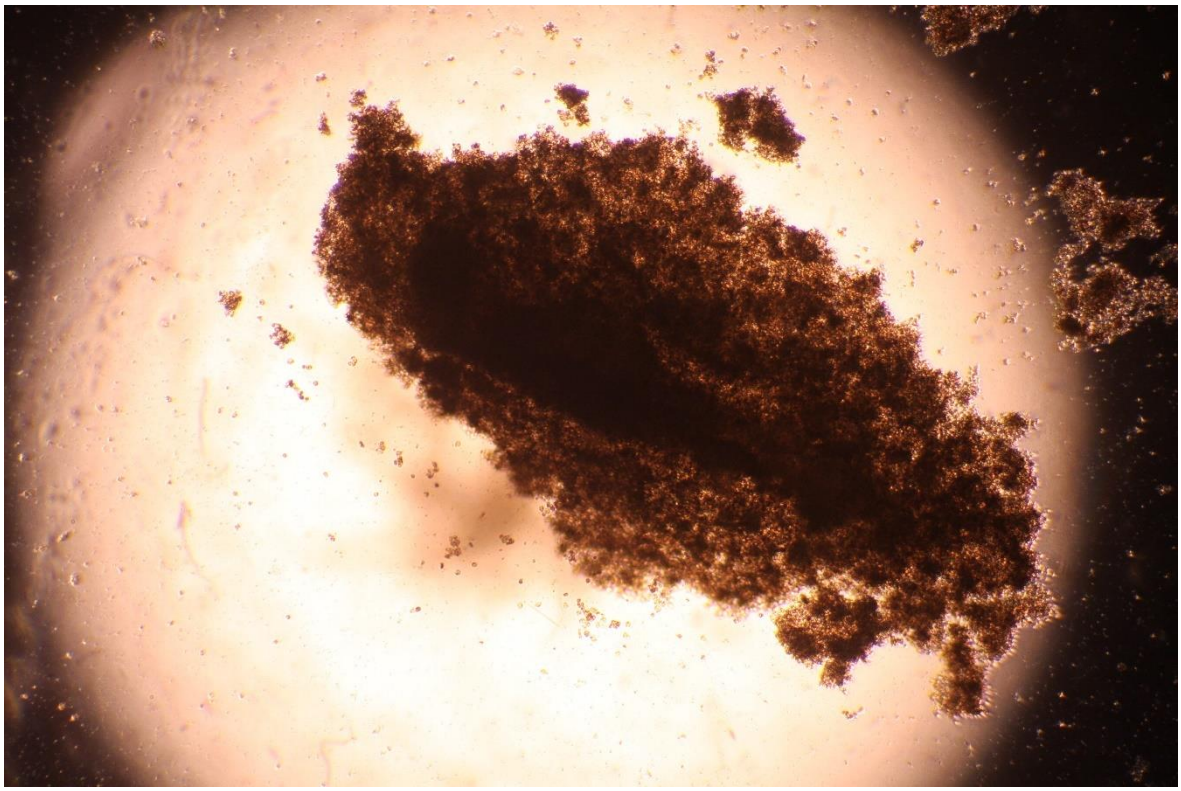

Figure 6: 3D Cell culture of hepatic cells of chicken origin under the light microscope with a magnification of 100x

6.4 Hematoxylin & eosin staining

Hematoxylin and eosin staining (HE) is a widely used staining technique for microscopic examination of tissues for over a century. Before the staining, the tissue must be fixed, processed, embedded and sectioned. It is a combination of two stains: eosin stains proteins nonspecifically in pink and hematoxylin stains nucleic acids in deep blue/purple by building a complex.

This results in the nuclei being stained blue, while the cytoplasm and ECM appears in different shades of pink, depending on other structures embedded in them. Therefore, this staining technique provides a clear differentiation between cytoplasmic and nuclear parts of the cells. H&E staining is often used in medical diagnosis in histopathology. (Fischer, et al., 2008) (Cardiff, et al., 2014)

6.5 Measurements

6.5.1 Metabolic activity

After each incubation times (24, 48, 72 hours) metabolic activity of the cells was evaluated with CCK-8 (Cell Counting Kit-8) assay. CCK-8 is a colorimetric method in which the change in colour intensity is detected. CCK-8 reagent contains WST-8 (Water Soluble Tetrasolium Salt) which is reduced into orange coloured formazan by the coenzymes produced by the viable cells as **Figure 7** is showing. The amount of produced formazan is directly proportional to the metabolic activity of the cells, thus their viability.

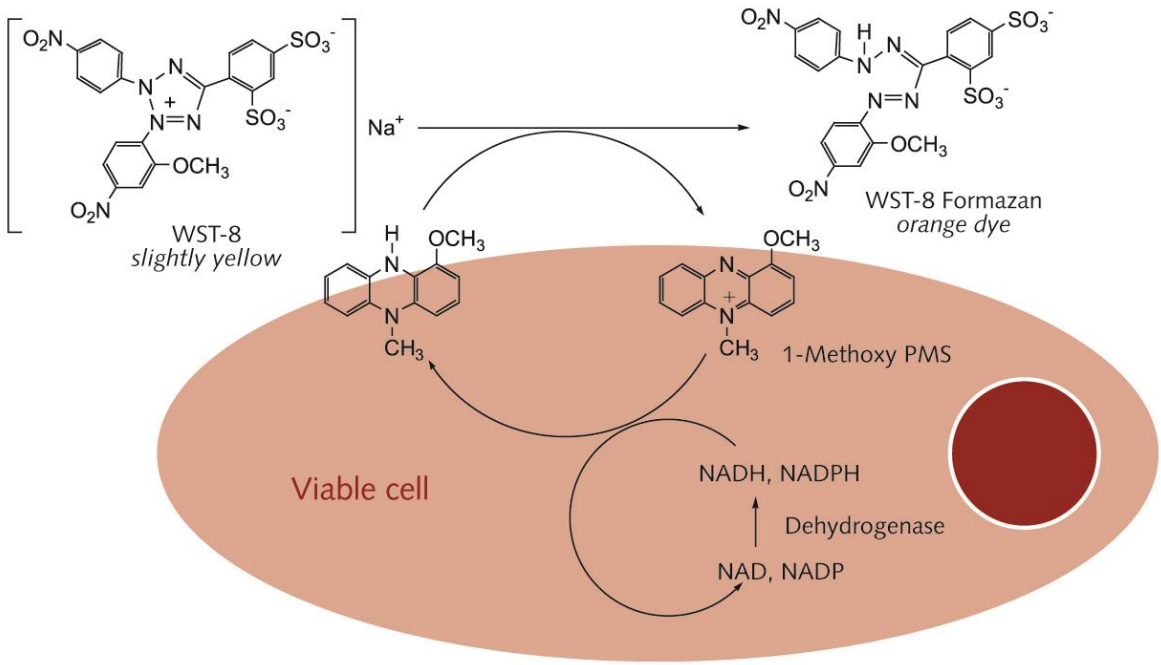

Figure 7: Assay mechanism of WST-8 conversion by dehydrogenases in viable cells, Instruction Manual by PromoCell (PromoCell GmbH, WST-8 Instruction Manual, Heidelberg, Germany)

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. This was followed by an incubation time of two hours at 37°C. The absorbance was measured at 450 nm with Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

6.5.2 Lactate dehydrogenase

The lactate dehydrogenase (LDH) assay (Diagnosticum Ltd., Budapest, Hungary) is a method to evaluate the change in the cell viability by measuring the activity of a cytoplasmatic enzyme, the lactate dehydrogenase by an enzymatic kinetic photometric assay. LDH is rapidly released into the medium in case of membrane damage because of apoptosis, necrosis or other cellular damage. Therefore, the amount of LDH released from the cells is a good indicator for the number of viable cells.

In this method, yellow-coloured tetrazolium salt (iodonitrotetrazolium, INT) is reduced by NADH into red formazan shown in **Figure 8**. The amount of produced formazan correlates linearly with the number of apoptotic cells.

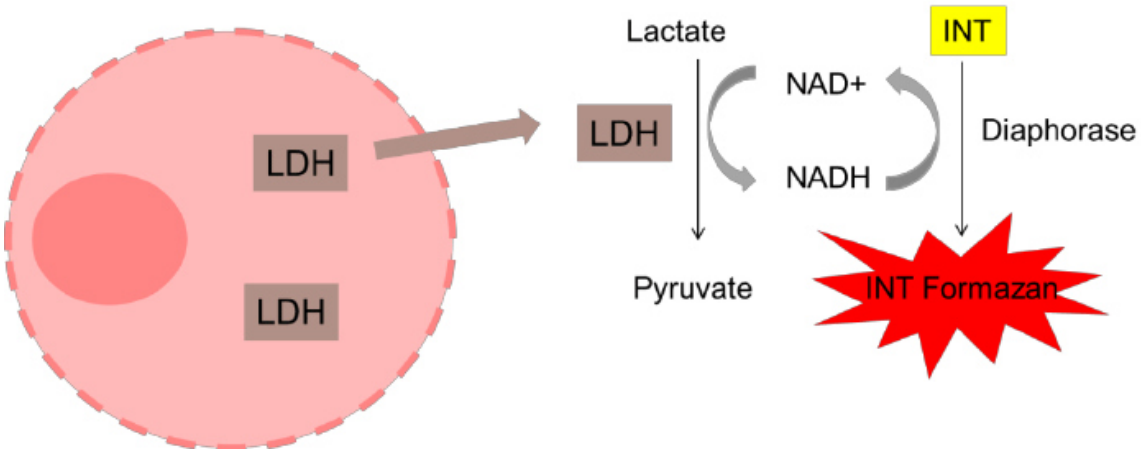

Figure 8 Schematic representation of the principle of the LDH release assay *(Forest, et al., 2015)*

200 µL of the working reagent (consisting of 56 mM phosphate buffer, 1.6 mM pyruvate and 240 µM NADH) was mixed with 10 µL of cell culture medium in a 96-well plate. The plate was incubated for 1 minute on room temperature, then the absorbance was read with Multiskan GO 3.2 plate reader on 340 nm in every minute for 6 minutes.

6.5.3 Enzyme-linked immunosorbent assay of IL-6

Enzyme-linked immunosorbent assay (ELISA) is used for measuring antigen or antibody concentrations. ELISA method works with an antigen-antibody reaction that is followed by an enzymatic reaction with visible colour change. With this method, the presence and quantity of an antigen or an antibody can be determined. Direct ELISA and sandwich ELISA methods detect antigens, while indirect ELISA and competitive ELISA methods detect antibodies.

The IL-6 concentration was measured by double antibody sandwich ELISA method following the manufacturer's protocol. This technique is more sensitive than direct ELISA methods, because two antibodies participate in the reaction, and both are specific for the target antigen. In this method, the plate is pre-coated with antibodies specific for one epitope of the target and can bind it. The second, biotin-labeled antibody can bind to another epitope of the target. Next, horseradish enzyme and avidin is added to the complex that can bind to the biotin-labeled antibodies. In the last step, the substrate of the enzyme is added, and the change in the coloration can be measured by spectrophotometry.

First, the IL-6 standard series and negative controls were prepared. 100 µL of the IL-6 standards and samples were added into each well and the plate was incubated for 90 minutes at 37 ºC. After the incubation time, the wells were washed with washing buffer and 100 µL IL-6 antibodies were measured in each well. The plate was incubated for 60 minutes at 37 ºC, followed by washing. Next, 100 µL of enzyme conjugate was measured into each well and incubated for another 30 minutes at 37 ºC. After washing,100 µL of colour reagent was added into each well and the plate was incubated for 20 minutes at 37 ºC. Finally, 100 µL of stop solution was added to each well. The absorbance was measured at 450 nm with Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

6.6 Statistics

The complete data analysis was performed using the R 3.5.3. software (GNU General Public License, Free Software Foundation, Boston, MA, USA). On the 96-well plates 15 wells were included to one treatment group regarding the dilution of the T-2 toxin.

Differences between the various groups were evaluated by using the one-way variance analysis (ANOVA) and then post-hoc tests were performed for pairwise comparisons. The results were evaluated as the mean ± standard error of the mean (SEM). Differences were assumed significant at $p < 0.05$, while all the T-2 concentration groups were compared to the control group.

7. Results

7.1 Hematoxylin & eosin staining

Figure 9 shows cell cultures consisting of hepatocyte-NP cells stained by H&E. The nuclei are stained blue, while the ECM and the cytoplasm are stained in different shades of pink.

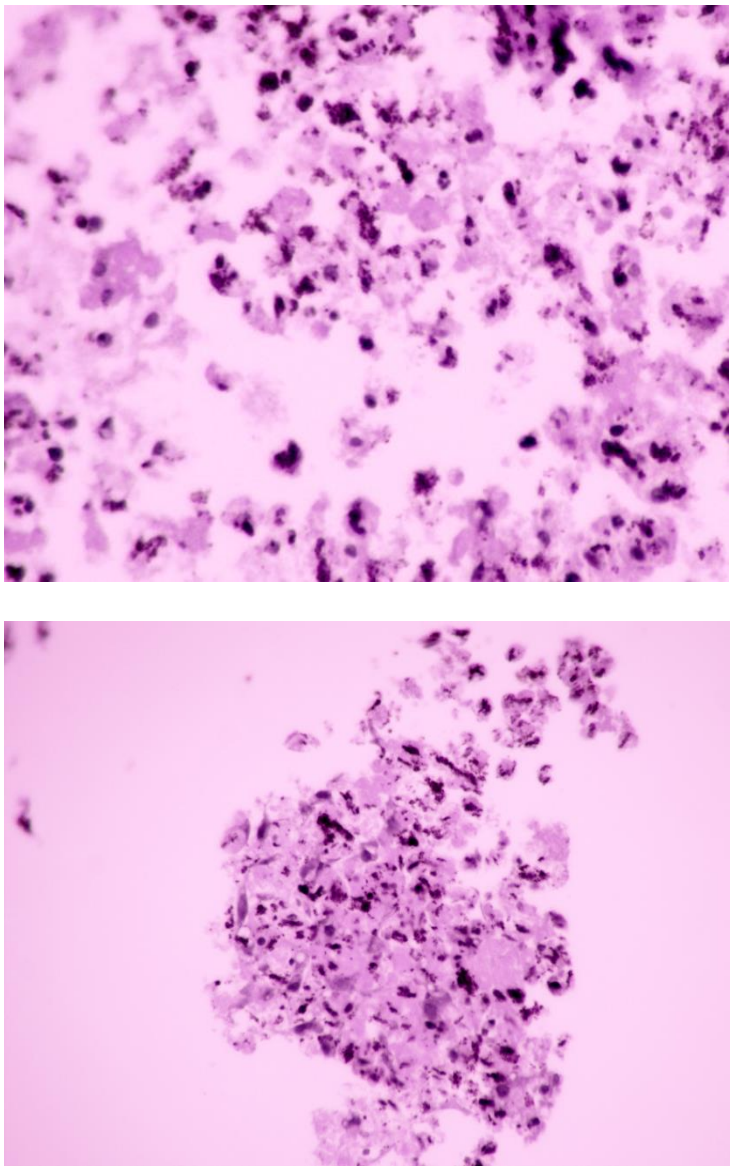

Figure 9: Hematoxylin & eosin staining of hepatocyte and non-parenchymal cells

7.2 Metabolic activity

The cellular metabolic activity of the cultured cells was determined by CCK-8 assay.

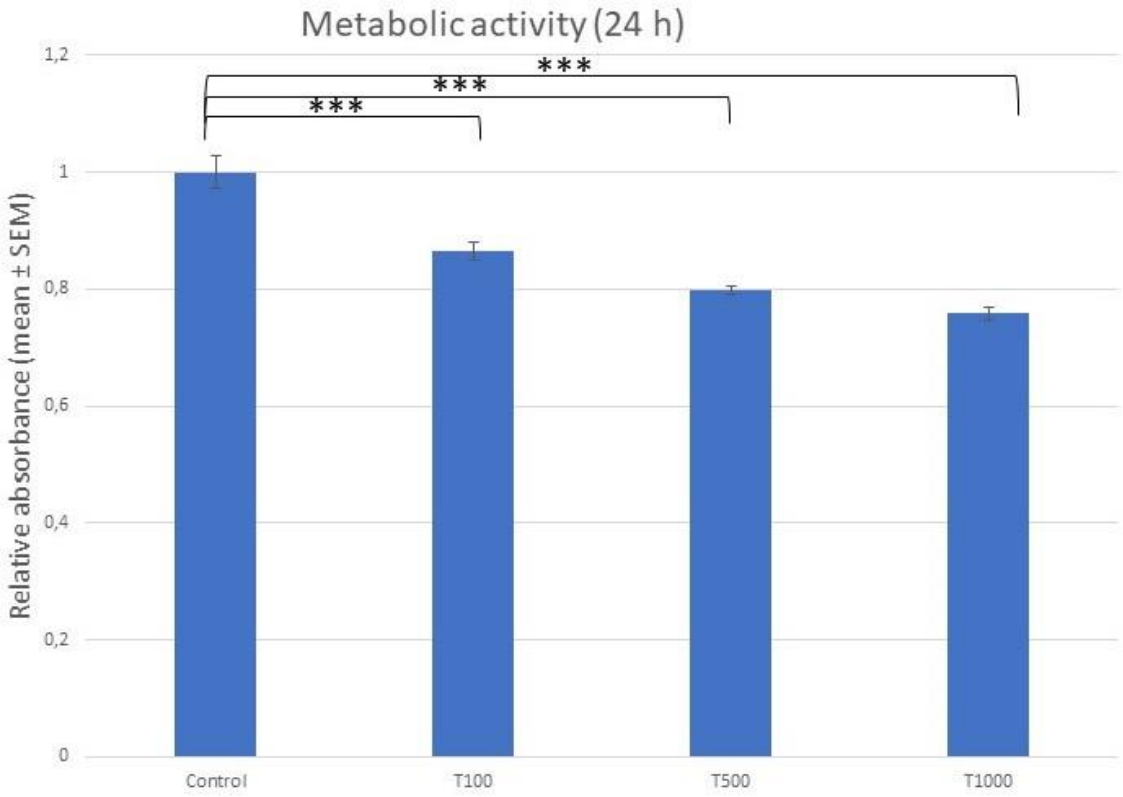

Figure 7: Effects of 24 hours T-2 toxin treatment on the metabolic activity of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. Cellular metabolic activity was measured by CCK-8 test. Relative absorbances were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM.; *** $p < 0.001$.

Effects of 24 hours T-2 toxin treatment on the metabolic activity of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 10**. T-2 exposure significantly decreased the metabolic activity of the cells in every treatment group after 24 h incubation time.

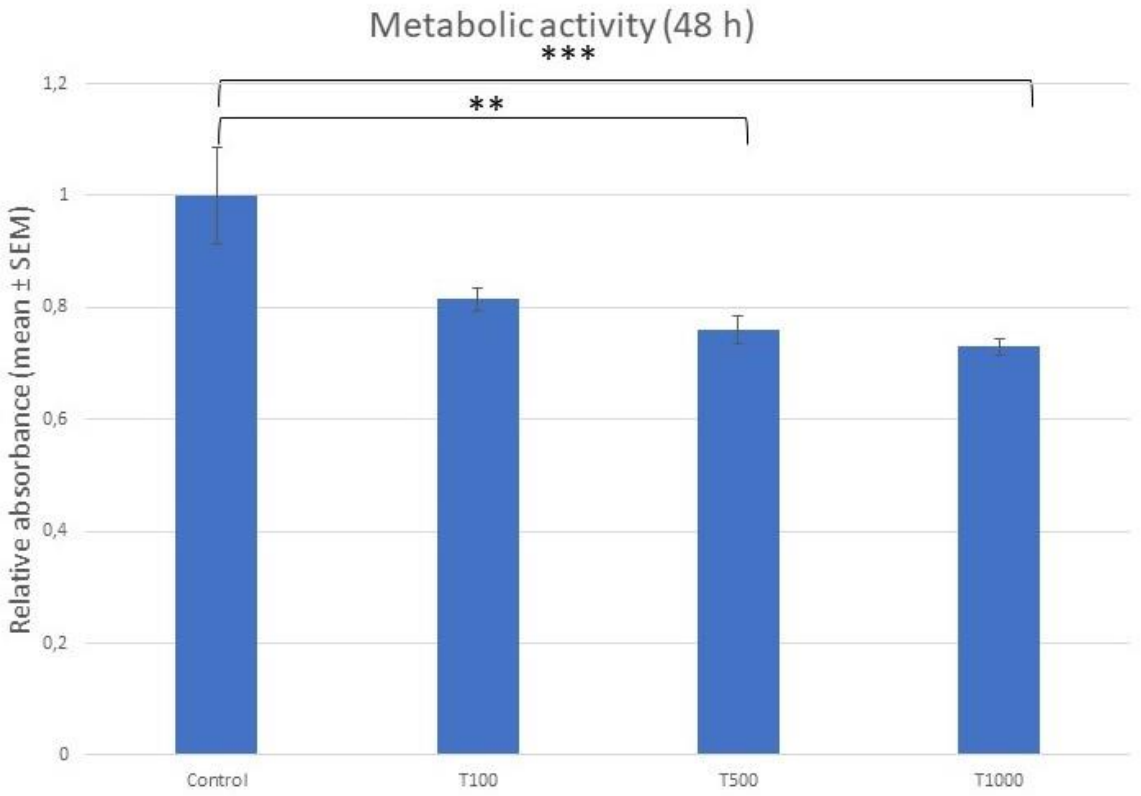

Figure 8: Effects of 48 hours T-2 toxin treatment on the metabolic activity of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. Cellular metabolic activity was measured by CCK-8 test. Relative absorbances were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM.; $** p < 0.01$; $*** p < 0.001$.

Effects of 48 hours T-2 toxin treatment on the metabolic activity of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 11**. After 48 h incubation, the higher concentrations of T-2 toxin (500 nM and 1000 nM) significantly decreased the cell's metabolic activity.

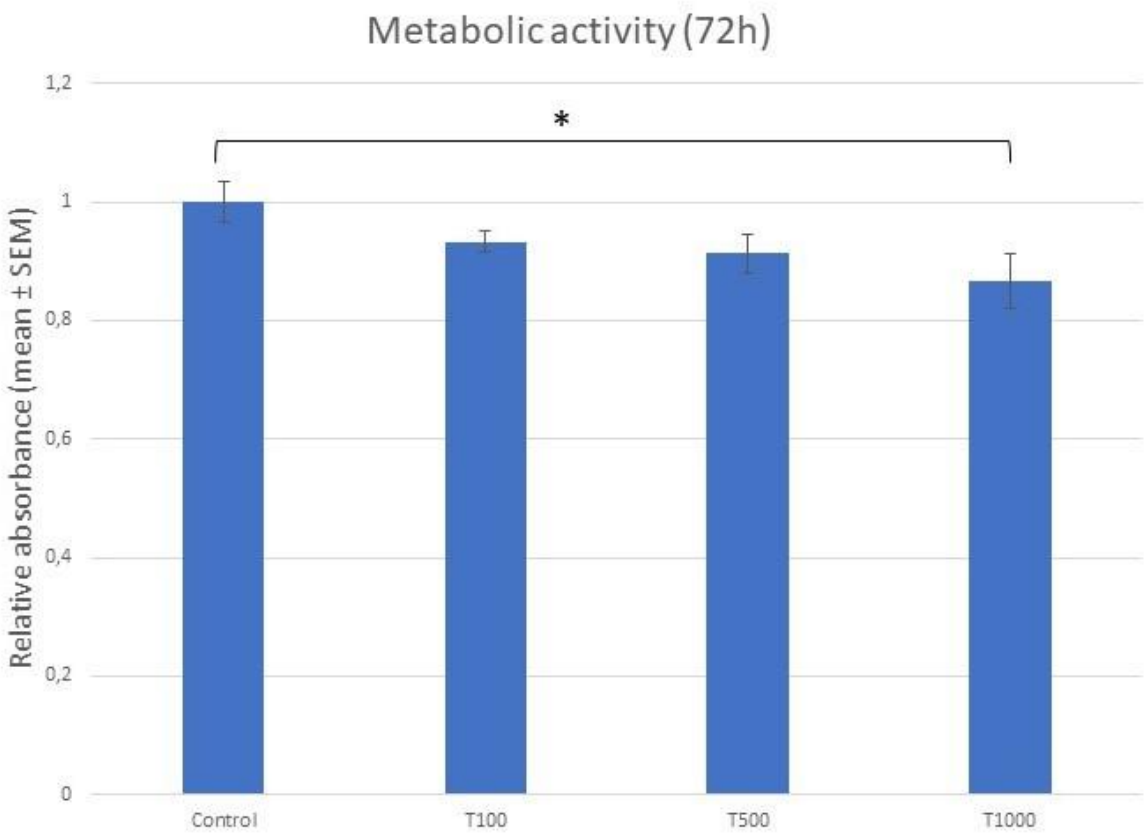

Figure 9: Effects of 72 hours T-2 toxin treatment on the metabolic activity of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. Cellular metabolic activity was measured by CCK-8 test. Relative absorbances were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. $* p < 0.05$.

Effects of 72 hours T-2 toxin treatment on the metabolic activity of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 12**. After 72 hours of incubation, the 1000 nM T-2 concentration decreased the metabolic activity of the cells.

7.3 Lactate dehydrogenase

The extent of cell necrosis was determined by measuring the LDH activity in the medium.

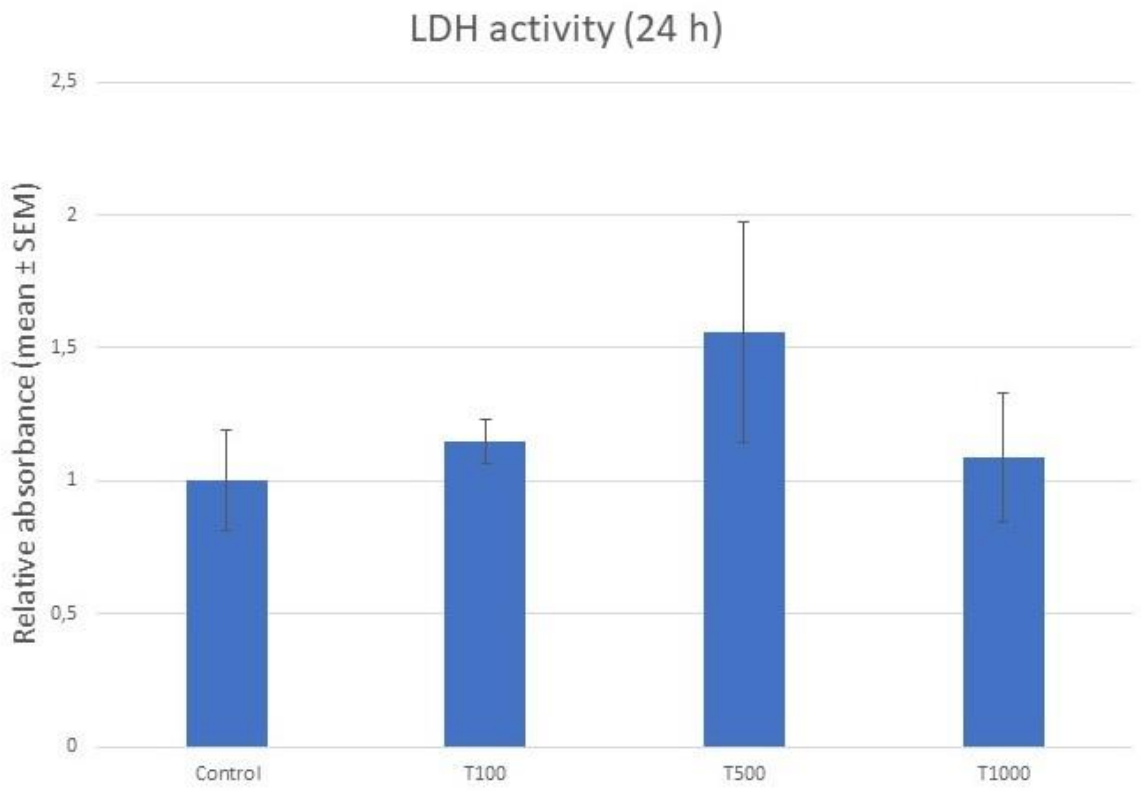

Figure 10: Effects of 24 hours T-2 toxin treatment on the extracellular lactate dehydrogenase (LDH) activity of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. Relative absorbances were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM.

Effects of 24 hours T-2 toxin treatment on the extracellular lactate dehydrogenase (LDH) activity of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 13**. Every T-2 toxin treatment elevated the level of LDH activity, but it was not statistically significant.

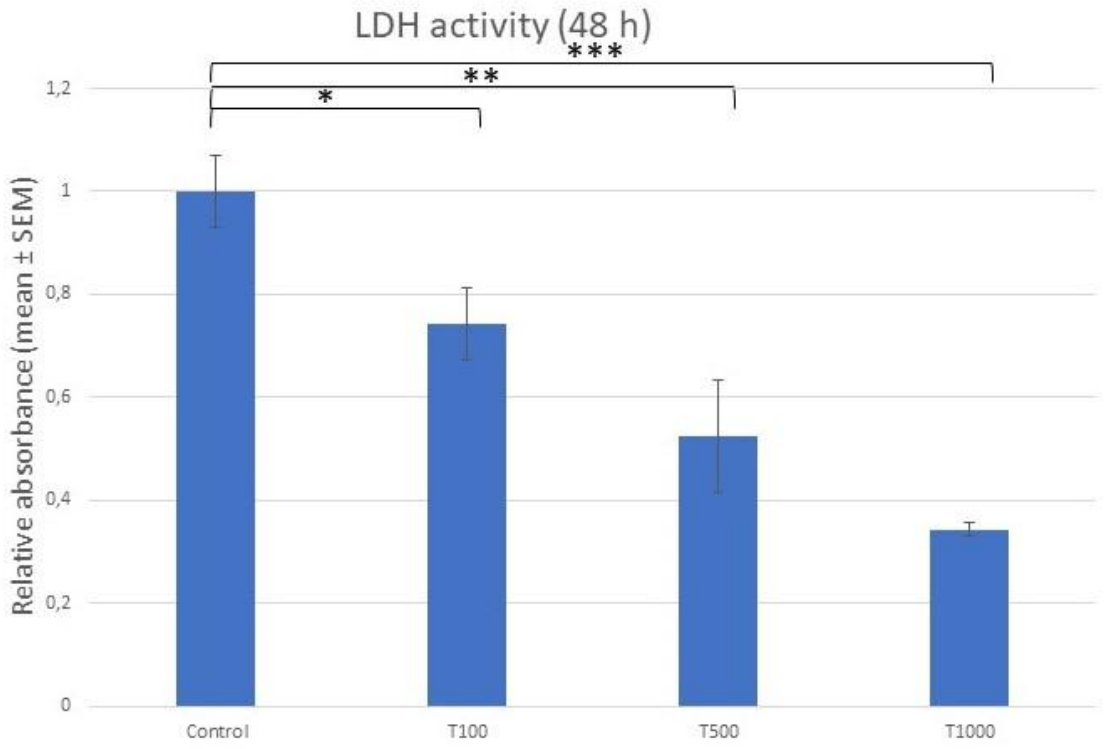

Figure 11: Effects of 48 hours T-2 toxin treatment on the extracellular lactate dehydrogenase (LDH) activity of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. Relative absorbances were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. *$* p < 0.05; ** p < 0.01; *** p < 0.001$*.

Effects of 48 hours T-2 toxin treatment on the extracellular lactate dehydrogenase (LDH) activity of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 14**. All three of T-2 concentrations significantly decreased the LDH activity in the cell's medium.

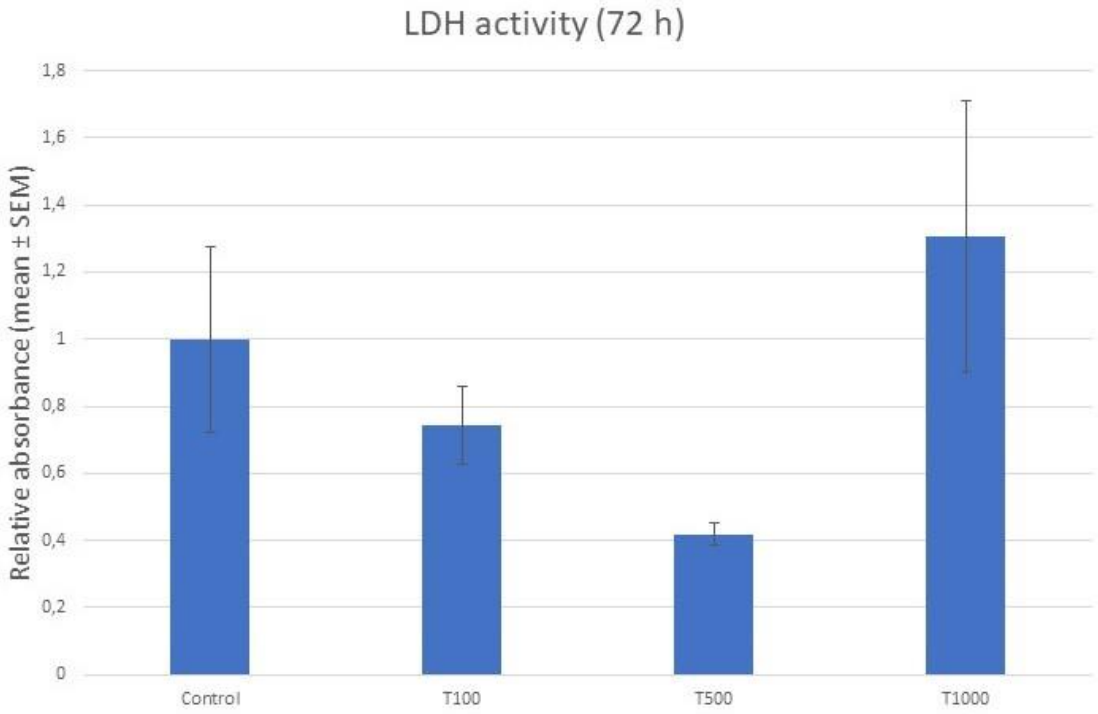

Figure 12: Effects of 72 hours T-2 toxin treatment on the extracellular lactate dehydrogenase (LDH) activity of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. Relative absorbances were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM.

Effects of 72 hours T-2 toxin treatment on the extracellular lactate dehydrogenase (LDH) activity of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 15**. The lower concentrations of T-2 toxin (100 nM and 500 nM) decreased, and the 1000 nM concentration increased the LDH activity of the cells.

7.4 Enzyme-linked immunosorbent assay of IL-6

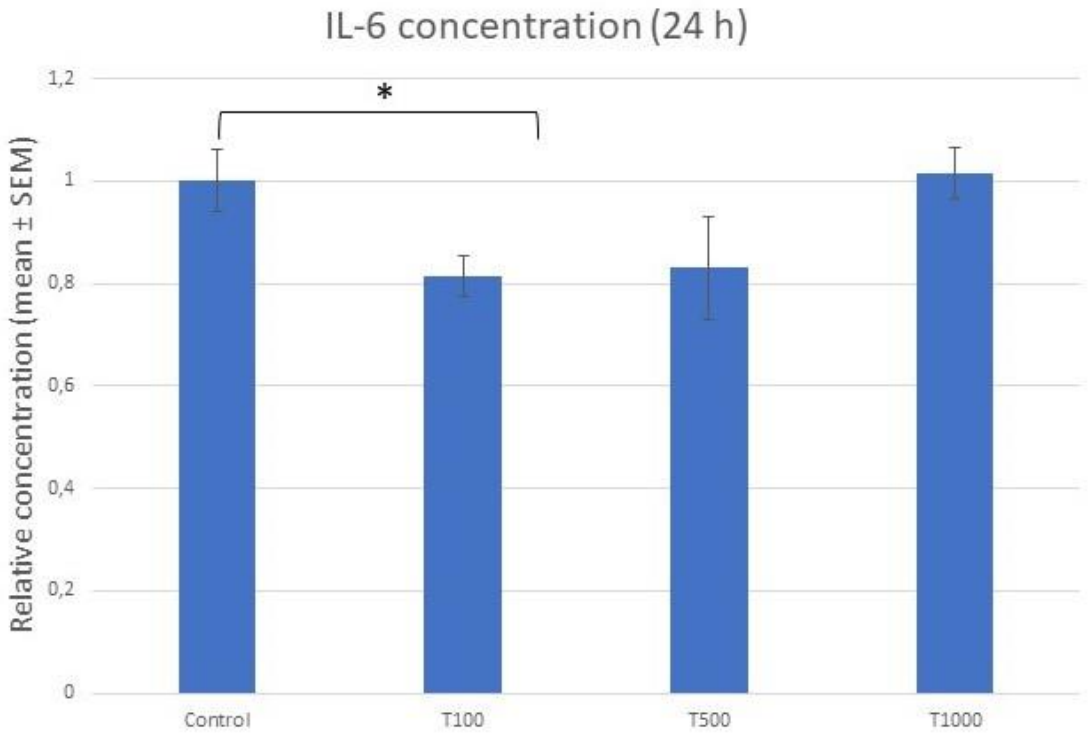

Figure 13 Effects of 24 hours T-2 toxin treatment on the interleukin-6 (IL-6) production of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. IL-6 concentration was measured by chicken specific ELISA test. Relative concentrations were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. * $p < 0.05$.

Effects of 24 hours T-2 toxin treatment on the interleukin-6 (IL-6) production of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 16**. The 100 nM concentration significantly decreased the IL-6 production of the cells.

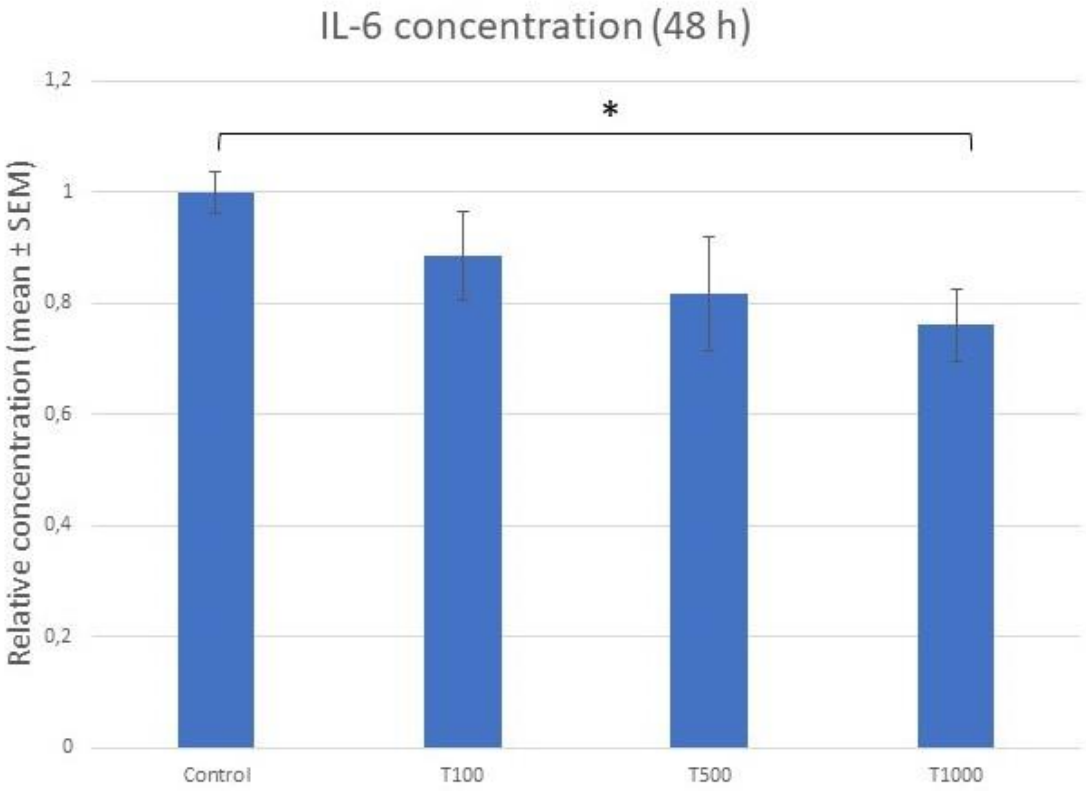

Figure 14 Effects of 48 hours T-2 toxin treatment on the interleukin-6 (IL-6) production of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. IL-6 concentration was measured by chicken specific ELISA test. Relative concentrations were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. * $p < 0.05$.

Effects of 48 hours T-2 toxin treatment on the interleukin-6 (IL-6) production of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 17**. The 1000 nM concentration of T-2 toxin significantly decreased the IL-6 concentration in the medium.

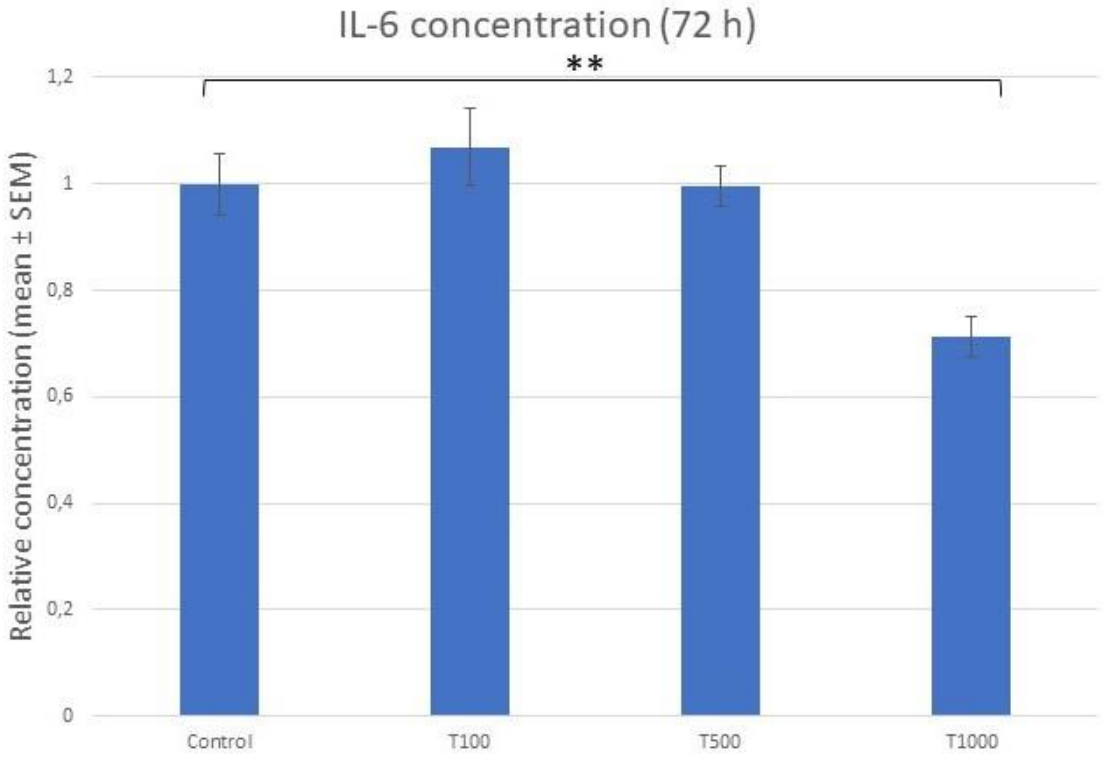

Figure 15 Effects of 72 hours T-2 toxin treatment on the interleukin-6 (IL-6) production of chicken derived primary hepatocyte-NP cell 3D co-cultures with the ratio of 6:1. Cells were treated with different concentrations of T-2 toxin. Control: without T-2 treatment; T100: 100 nM T-2 toxin treatment; T500: 500 nM T-2 toxin treatment; T1000: 1000 nM T-2 toxin treatment. IL-6 concentration was measured by chicken specific ELISA test. Relative concentrations were calculated by considering the mean value of the Control group as 1. Results are expressed as mean ± SEM. $** p < 0.01$.

Effects of 72 hours T-2 toxin treatment on the interleukin-6 (IL-6) production of chicken derived primary hepatocyte-NP cell 3D co-cultures are shown in **Figure 18**. The 1000 nM T-2 concentration signifanctly decreased the IL-6 production of the cells after 72 hours of incubation.

8. Discussion

T-2 toxin belongs to the group of the trichothecene mycotoxins. Trichothecenes are produced by different *Fusarium* species which can contaminate various crops. This makes it rather difficult to avoid mold contamination and therefore mycotoxin exposure. After entering the body, T-2 toxin reaches the liver, the main organ for the metabolism of the toxin. Hence, hepatocyte-NP cell co-cultures are excellent *in vitro* models to study the cellular effects of T-2 toxin. In the present study, the hepatocyte to non-parenchymal cell ratio was set to 6:1. This model could represent a state of mild hepatic inflammation with moderate intrahepatic macrophage migration. (Pomothy, et al., 2021) (Mackei, et al., 2020)

T-2 toxin have cytotoxic and immunological effects on different cell lines of animals and humans. (Adhikari, et al., 2017) (Shokri, et al., 2000) (Kankkunen, et al., 2009) The cytotoxic effect of T-2 is primarily connected to the inhibition of protein synthesis and the consequently impaired DNA and RNA synthesis. Furthermore, through the production of ROS and the following induction of oxidative stress, T-2 toxin exposure can lead to apoptosis. (Adhikari, et al., 2017) (Hossam El-Din, 2013) In addition, T-2 toxin can induce DNA damage in chicken peripheral lymphocytes and chromosomal aberrations in mice *in vivo*. (Sokolović, et al., 2008)

Effects of T-2 toxin on the immune system are immunomodulatory and can be either stimulating or inhibiting. The mode of action is time and dose dependent; therefore, a high dose is resulting in immunosuppression and a lower dose in immunostimulation. These effects can influence the gene expression of proinflammatory cytokines, such as IL-6. (Sokolović, et al., 2008)

The aim of this study was to examine the cellular effects of T-2 toxin in a 3D hepatocyte-NP cell co-culture system of chicken origin. Cellular metabolic activity, LDH leakage and the concentration of IL-6 was determined after three incubation times (24, 48 and 72 hours) with different T-2 toxin concentrations (100, 500 and 1000 nM).

Metabolic activity of the cells was measured by CCK-8 assay. The number of viable cells that produce $NADH+H^+$ can be determined with this method. In all cell cultures treated with T-2 toxin after 24, 48 and 72 (**Figure 10, 11 and 12**) hours of incubation a significant decrease of metabolic activity was detected, which is inversely correlated with the increase of the T-2 toxin concentration. Metabolic adaption was shown by the liver cells as metabolic

depressions was alleviated after a longer incubation period. After 72 (**Figure 12**) hours of incubation (Figure the cell viability was higher than the cell viability after 24 and 48 hours (**Figure 10** and **Figure 11**). This indicates a cytotoxic effect of T-2 toxin on the hepatic cells. These results are in line with findings of other publications indicating a cytotoxic effect of T-2 toxin on different cell lines of various animal species (Adhikari, et al., 2017)(Hossam El-Din, 2013). Furthermore, a significant decrease in cellular viability after T-2 toxin exposure was measured by Weidner et al. (Weidner, et al., 2013).

To measure the cellular and plasma membrane damage thus the amount of apoptotic cells, LDH leakage of the cell is measured with the LDH assay. After 24 hours (**Figure 13**), the cell cultures treated with T-2 toxin had an increased LDH leakage, although only in the groups treated with 100 nM and 500 nM T-2 toxin were significantly increased. The LDH activity after 48 hours of T-2 exposure (**Figure 14**) was significantly decreased in every treatment group compared to the control group. Similarly, after 72 hours (**Figure 15**) of T-2 toxin exposure, in case of the 100 nM and 500 nM treatment the LDH leakage was lower, but the 1000 nM treatment elevated the LDH activity compared to the control group. This can be explained by the process of autophagy, which allows the degradation of cellular components and maintains the homeostasis in the liver. This cytoprotective process results in a decreased LDH leakage as damaged organelles of hepatocytes are non-selectively removed through a lysosome-dependent mechanism. (Sebök, et al., 2021) In case of the 1000 nM treatment group, the result suggest that the number of damaged cells caused by high T-2 toxin concentration was much higher than the number of cells that were eliminated by autophagy.

The immunomodulation of T-2 toxin was assessed by measuring the amount of IL-6, a proinflammatory cytokine, by double antibody sandwich ELISA method. IL-6 synthesis is induced through inflammation and virus infection. High concentrations of IL-6 can affect the B- and T cell differentiation and the production of acute phase proteins.

The relative concentration of IL-6 was decreased after 24 hours of incubation time (**Figure 16**) in the 100 nM and 500 nM treatment groups, but only in the case of the 100 nM treatment group it was significant. The 1000 nM concentration elevated the IL-6 production. The decreased levels of IL-6 production are consistent with the results of Wu et al., which showed that the gene expression of IL-6 after T-2 toxin treatment was increased significantly

after 8-12 hours of exposure and then decreased to almost zero after 24 hours of incubation (Wu, et al., 2014).

After 48 and 72 hours of incubation (**Figure 17, Figure 18**), the measured IL-6 concentration was significantly decreased in case of the 1000 nM concentration of T-2 toxin. This fits the time and dose dependent effect of T2-toxin on the immune system, which is the inhibition of the immune system by higher dosages over a longer period.

9. Conclusion

Based on the results of this study and the collected and reviewed data, T-2 toxin has a cytotoxic and immunomodulatory effect *in vitro* on hepatocyte-NP cell co-cultures of chicken origin.

In this present study the results showed a significant decrease in the metabolic activity of the cells after the treatment with different T-2 toxin concentrations, which shows that T-2 toxin has a clear effect on the viability of the chicken hepatocytes. After longer incubation times the hepatocytes showed the potential to adapt to the metabolic depression. Furthermore, the results regarding the LDH activity suggest an increasing effect of T-2 toxin on autophagy processes in liver cells. In addition, T-2 toxin have an immunomodulating effect on the immune system: it can stimulate or inhibit the immune system in a dose- and time-dependent manner.

These results build a sufficient foundation for further studies regarding the cellular effects of T-2 toxin on hepatocytes of chicken origin, however additional *in vivo* studies are crucial for scientific research and empiric observations in the future.

10. Abstract

The T-2 toxin is a type A trichothecene, produced by various *Fusarium* species and it is one of the most toxic trichothecene mycotoxins for humans and animals. Therefore, the investigation of the effects of T-2 toxin on the animals is highly important for the veterinary medicine and the agriculture. According to literature, the toxic effects of T-2 can be classified as immunomodulatory, genotoxic and cytotoxic. The affected cells belong to the liver, the digestive tract, the nervous system and the skin.

Several studies showed that T-2 toxin has a cytotoxic and immunomodulatory effect on different cell lines in 2D cell cultures. However, data concerning the effect of T-2 toxin on cells in a 3D cell culture are limited. The aim of present study was to examine the cellular effects of T-2 toxin in 3D spheroids, consisting of hepatocyte-NP cells of chicken origin in a ratio of 6:1.

For the examination of the effect of the T-2 toxin on the hepatocytes, the viability and the inflammatory response was tested after exposing the hepatocyte-NP cell co-cultures to several concentrations (100, 500 and 1000 nM) of T-2 toxin for different incubation times (24, 48 and 72 hours). The change in the viability was measured by the CCK-8 assay and showed a significant decrease in the viability of the cultured cells in every treatment group and incubation time. After longer incubation times the liver cells showed an adaptive response to the metabolic depression of the T-2 toxin. The number of cells with membrane damage was determined by measuring the LDH leakage in the medium. The results of the LDH assay indicated an increasing amount of LDH leakage after the first 24 hours through cell damage caused by T-2 toxin and then a significant decrease of LDH activity after 48 and 72 hours, which can be explained by the induction of cytoprotective autophagy processes after longer exposure times of liver cells to T-2 toxin. The inflammatory reaction was determined by measuring the concentration of IL-6 with chicken specific ELISA test. This confirmed that the T-2 toxin has an immunomodulatory effect, due to the significant decrease in the IL-6 production that correlated with higher concentrations of T-2 toxin and longer incubation times. Based on the results of the present study study, T-2 toxin has a cytotoxic and immunomodulatory effect *in vitro* on chicken derived hepatocyte-NP cell co-cultures. These findings provide a sufficient foundation for further studies regarding the cellular effects of T-2 toxin on hepatocytes of chicken origin, however further experiments are needed for scientific research and empiric observations in the future.

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I hereby confirm that I am familiar with the content of the thesis entitled

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