Immunomodulatory interactions of heat stress, certain mycotoxins and bacterial lipopolysaccharides in chicken

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Final report

I. Introduction

Several environmental and nutritional factors can contribute to immunomodulation in poultry farming, which commonly diminish animal health and production by affecting the cellular inflammatory and stress response. For instance, heat stress triggered by elevated ambient temperatures, mycotoxin contamination of the feed and bacterial cell wall components coupled to enteric infections are among the major concerns in broiler chickens, severely deteriorating animal welfare, resulting in significant production losses and making the animals more sensitive to complex, multifactorial diseases. Notwithstanding that liver is highly susceptible to the aforementioned stressors and also plays a central role in the maintenance of metabolic, inflammatory and oxidative homeostasis, the effects of heat stress, mycotoxins and bacterial endotoxins on the chicken liver are not fully elucidated.

Among the numerous issues, heat stress is of primary importance in intensive poultry production, with increasing significance due to global climate change (Surai, 2015). Factors that increase broiler susceptibility to high ambient temperatures include continuous selection for effective and rapid weight gain, the absence of sweat glands, and feathering (Song et al., 2015). Even moderate elevation of optimal room temperature, especially in combination with high relative air humidity, can result in severe alterations of the function and structure of cellular proteins, lipids, and nucleic acids (Belhadj Slimen et al., 2015). Therefore, it is of great importance to understand the cellular consequences of heat stress to successfully alleviate its harmful effects in broiler farming (Altan et al., 2003).

Excessively high temperatures initiate a specific defense mechanism, called the heat shock response (HSR) that aims to restore the cellular homeostasis by complex alterations of several signaling and metabolic pathways (Richter et al., 2010). Oxidative distress is commonly linked to the HSR, occurring mainly due to intense reactive oxygen species (ROS) release, being one of the most significant consequences of increased heat exposure (Lin et al., 2006). Elevated ROS production may interrupt the antioxidant defense system, inducing lipid peroxidation and oxidative damage of proteins, resulting in increased malondialdehyde (MDA) production and the generation of protein carbonyl derivates, respectively (Schieber and Chandel, 2014; Fernando et al., 2016).

As high temperature is considered to impair protein stability, maintaining the physiological conformation of proteins and preventing the aggregation of non-native proteins are of special importance (Horowitz and Robinson, 2007). Heat-shock proteins (HSP-s) as major protective molecules play a crucial role in the maintenance of physiological processes under stress conditions and are required for the effective cellular alterations involved in the HSR (Yadav et al., 2020). Small heat shock proteins (sHSP-s) belong to a highly relevant group of molecular chaperones, involved in the efficient cellular adaptation to different stress conditions (Haslbeck et al., 2019), but their exact role in restoring cell function in heat stressed chickens remained unclear. As an immunomodulatory factor, heat stress was also reported to cause functional changes in the immune response by altering the gene expression of pro-inflammatory cytokines, such as increasing splenic interleukin (IL-)4 and IL-12 concentrations in chicken (Ohtsu et al., 2007). Further, the cellular immune system may also

get diminished by heat stress, reflected by decreased total white blood cell count and macrophage activity (Mashaly et al., 2004).

Beside heat stress, the contamination of the feed with mycotoxins has also an exceptional importance in intensive poultry farming. The T-2 toxin, one of the most noxious members of the trichothecene group, can provide a serious hazard in broiler nutrition as well, threating both animal and human health by contaminating the food chain (Milicevic et al., 2010). Avian species are relatively tolerant to trichothecenes in comparison with mammals; however, the presence of T-2 toxin in the feed serves as a relevant problem in poultry industry worldwide. Although several studies exist about the effects of T-2 toxin in various poultry species, there are numerous questions regarding the mode of action on the molecular level and considering the species-specific differences in the effects of the toxin.

On cellular level, T-2 toxin can inhibit the protein synthesis by binding the peptidyl transferase enzyme in the 60S ribosomal subunit (Pestka, 2007), and it can induce DNA fragmentation, contributing to genetic disorders (Chaudhari et al., 2009). Another highly important non-ribosomal effect of T-2 toxin is the intensive ROS production and the oxidative stress associated harmful effects, such as nuclear and mitochondrial DNA damage, elevated lipid peroxidation and disturbances in the cell signalling and inflammatory pathways (Wu et al., 2014). In most cases, T-2 toxin significantly increases the level of reactive oxygen species (ROS) and induces changes in the antioxidant status of the cells (Chaudhari et al., 2009), while in other studies beside the intensive cellular damage oxidative stress was not detected (Rezar et al., 2007).

Furthermore, some HSP-s (such as HSP70) can show a correlation with the cytoprotective mechanisms against different toxic effects (El Golli-Bennour et al., 2011), but data addressing the effects of trichothecenes on influencing HSP expression are limited. Elevated HSP70 expression caused by T-2 toxin was observed in placenta of pregnant rat *in vivo* (Sehata et al., 2005), and similarly, T-2 toxin induced HSP70 protein production *in vitro* in Vero cells (El Golli et al., 2006), but no data is available concerning avian species. Regarding the effects of T-2 toxin on cytokines and on other inflammatory mediators, conflicting results can be found in the literature. *In vitro* studies described reduced interleukin (IL)-1β and tumor necrosis factor (TNF)-α concentrations in primary porcine macrophages (Seeboth et al., 2012), while other experiments suggested, that the toxin synergistically activated IL-1β and IL-18 mediated inflammatory response in human macrophages *in vitro* (Kankkunen et al., 2009).

Broiler chickens with an immature immune system are prone to enteric bacterial infections, and disruption of the intestinal barrier integrity would lead to leakage of microbial toxins and byproducts through the epithelial barrier leading to inflammation. The liver serves as the major organ barrier for the gut-derived antigenic load and protects the systemic circulation against both residual oxidative and pathogen burden originated from the gastrointestinal tract. Modeling these pathologies *in vitro* is of high relevance to study the molecular mechanisms of the hepatic inflammatory response, and to find potential molecules for the prophylaxis of gut-originated pathogen burden induced inflammatory and oxidative damage of the liver.

The most important and most common pathogen-associated molecular patterns causing inflammatory response are lipopolysaccharide (LPS) type endotoxins derived from the Gram-negative bacterial cell wall (Dickson et al., 2019). Along with its Gram-positive bacterial counterpart, lipoteichoic acid (LTA), it can either modulate signal transduction via toll-like receptor (TLR) activation or can be bound nonspecifically by type-I scavenger receptor in the liver (Gao et al., 2008). According to recent studies, these endotoxins might play an equal role in septic shock, tissue damage, and multiorgan failure synergistically with other cell wall components like peptidoglycans in Gram-positive septicemia (Dickson et al.,

2019). In chicken, both LTA- and LTA-triggered TLR signaling can strongly stimulate interleukin synthesis (Kannaki et al., 2010). The LPS from O55:B5 chicken pathogen *Escherichia coli* or from *Salmonella enterica* serovar. Typhimurium are most frequently used *in vitro* on chicken cell cultures (Kannaki et al., 2010), while *Staphylococcus aureus* originated LTA proved to induce oxidative burst in chicken heterophil granulocytes (Farnell et al., 2003).

Concerning further bacterial products, flagellin can be derived from the disintegration or the leaky assembly of the bacterial flagellum. The highly conserved hidden core regions of this motor protein can induce the activation of the non-specific immune system considerably via the TLR pathway (Fliegmann and Felix, 2016). Beyond its chloride ion channel activator nature, the heat-labile enterotoxin of *E. coli* has also proved to contribute in bacterial inflammations by modulating T cell function and increasing the major histocompatibility class II (MHCII) and CD25 expression (De Haan and Hirst, 2004). As a further potent pro-inflammatory agent, polyinosinic-polycytidylic acid (Poly I:C) shows a similar structure to viral double-stranded RNA characteristic for *Reoviridae* and *Birnaviridae* virus families with abundant poultry specific strains. Hence, Poly I:C can be applied to mimic the cellular inflammatory response related to viral infections.

Based on the aforementioned data, heat-, mycotoxin- or bacterial infection-associated distress of the liver, due to its central role in the metabolism of nutrients and xenobiotics, may be critical for the whole organism by destructing the maintenance of metabolic health. On cellular level, monitoring the functions of different cell types, particularly those of hepatocytes and non-parenchymal (NP) cells, primarily macrophages in the complex regulation of stress and inflammatory response could provide novel data on the pathomechanism of stress-associated multifactorial diseases, also highlighting new ways of improving animal health and productivity.

II. Aims, materials and methods of the studies

Study 1. Developing and characterizing a novel primary hepatocyte – NP cell co-culture of chicken origin

Aims

To study the effects of environmental and nutritional immunomodulatory factors, such as heat stress, mycotoxins and bacterial by-products on the function of different liver cells in chickens, novel hepatic cell culture models were aimed to be developed. Our research group has already established and characterized a primary co-culture comprised of hepatocytes and NP cells (mostly Kupffer cells) of pig origin, which can serve as a proper tool for investigations on the cellular inflammatory and stress response (Mátis et al., 2016). Since no similar avian liver cell culture models have been prepared yet, the first main goal of the present study was to develop a hepatic parenchymal – NP cell co-culture from chickens. Due to the difference in size and structure of hepatic cells in birds and mammals, cell isolation procedures had to be adapted to chickens, and separated cell fractions needed to be characterized.

Cell isolation and culturing

To fulfill the abovementioned goals, liver cells were freshly isolated from three-week-old male broiler chickens of the Ross-308 strain reared and fed according to the Ross technology [14], and obtained from Gallus Ltd. (Devecser, Hungary). All experimental procedures with the animals were carried out in accordance with the national and EU laws as well as with the institutional guidelines, and were confirmed by the Local Animal Welfare Committee of the University of Veterinary Medicine, Budapest (permission number: PEI/001/1430-4/2015). All chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany) except when otherwise specified.

The animals were slaughtered in carbon dioxide narcosis by decapitation, and the liver was perfused via the gastropancreaticoduodenal vein of the hepatic portal system. All perfusion buffers were previously warmed up to 40^{o}C and were freshly oxygenated with Carbogen (95% O_2, 5% CO_2); the velocity of the perfusion was set to 30 ml/min. In the first stage of the multi-step perfusion, 150 ml ethylene glycol bis (2-aminoethyl ether) tetraacetic acid (EGTA, 0.5 mM) containing Hanks' Balanced Salt Solution (HBSS) buffer (previously supplemented with 0.035% $NaHCO_3$) was applied, followed by 150 ml EGTA-free HBSS. In the final step, 100 ml HBSS buffer, freshly supplemented with 100 mg collagenase type IV (Nordmark, Uetersen, Germany), 7 mM $CaCl_2$ and 7 mM $MgCl_2$ was perfused into the liver to disintegrate hepatic parenchymal cells.

After excision of the liver, the capsule was disrupted, and the gained liver cell suspension was filtered through three layers of sterile gauze and was incubated in bovine serum albumin (BSA, 2.5%)-containing HBSS buffer on ice for 45 min to avoid cell aggregate formation. Thereafter, the cell suspension was centrifuged three times at 100 x g for 3 min, and the hepatocyte-enriched sediment was resuspended in Williams' Medium E, previously supplemented with 0.22% $NaHCO_3$, 50 mg/ml gentamycin, 2 mM glutamine, 4 µg/l dexamethasone, 20 IU/l insulin and 5% foetal bovine serum (FBS).

The NP cell fraction (containing mostly macrophages, primarily Kupffer cells) was separated from the supernatants gained in the low-speed (100 x g) centrifugation steps. The supernatants were centrifuged at 350 x g for 10 min to sediment the remaining hepatocytes, cell detritus and red blood cells, and the newly gained supernatant was centrifuged again at 800g for 10 min. The final sediment, containing NP cells, was also resuspended in Williams' Medium E. The viability of hepatocytes and NP cells was confirmed by the trypan blue exclusion test, and cell yield was examined by cell counting in Bürker's chamber to adjust the

appropriate cell concentrations (hepatocyte mono-cultures: 10^6 cells/ml; co-cultures: 8.5 x 10^5 cells/ml hepatocytes, 1.5 x 10^5 cells/ml NP cells).

All cell cultures were prepared on 6-well, 96-well (Greiner Bio-One, Frickenhausen, Germany) and lumox x-well (Sarstedt, Nümbrecht, Germany) cell culture dishes, depending on the aim of the later experiments and measurements, previously coated with collagen type I (10µg/cm^2) according to the manufacturer's instructions. The NP cells were seeded at first, and after their rapid attachment to the plate surface in 20 min, to prepare hepatocyte – NP cell co-cultures, the culture medium was removed and hepatocytes were seeded in the cell ratio of 6:1 (hepatocyte to NP cells). Hepatocyte mono-cultures were also prepared by seeding hepatocyte-enriched fraction onto cell culture dishes. The seeding volume was 1.5 ml/well on 6-well plates, 100 µl/well on 96-well plates and 300 µl/well on lumox x-well dishes. All cell cultures were incubated at 38.5°C in humid atmosphere with 5% CO_2. Culture media were changed 4 h after seeding, and confluent monolayers were gained following 24 h culturing.

Characterization of cell cultures

To confirm cell morphology, 48-h-cultured confluent monolayers on 6-well plates were stained with Giemsa, and to assess the presence and the ratio of various liver cell types in different cell culture models, immunocytochemical analyses were applied with chicken specific antibodies. Albumin was detected with a chicken specific, fluorescein isothiocyanate coupled anti-albumin antibody (Cedarlane, Burlington, Canada). The macrophages (mainly Kupffer cells as the resident liver macrophages) in NP cell fractions were labelled by using a chicken macrophage-specific phycoerythrin coupled antibody (Southern Biotech, Uden, The Netherlands).

Isolated cells and cell cultures (following 48 h culturing) on lumox x-well plates were fixed in phosphate buffered saline (PBS) containing 4% formaldehyde for 30 min at room temperature (21°C). After rinsing the fixed cells in PBS (3 times 5 min), they were permeabilized with Triton-X (0.25%) containing PBS for 20 min, and were subsequently blocked in PBS supplemented with 5% goat serum, 3% BSA and 0.1% Triton-X for 60 min at room temperature. Antibodies were dissolved (in the ratio of 1:100 for anti-albumin and 1:50 for macrophage specific antibody) in PBS containing 1% BSA and were applied overnight at 4°C. After counterstaining with 4',6-diamidino-2-phenylindole (DAPI), cell cultures were analyzed with an Olympus CKX-41-type fluorescent microscope and a Canon EOS 1100D camera.

Hepatocyte enriched and NP cell containing fractions were examined with flow cytometry in the Institute of Physiology and Nutrition, Georgikon Campus, Hungarian University of Agriculture and Life Sciences, Keszthely, Hungary. Cell suspensions with approximately 1 x 10^6 /ml cell concentration were filtered prior to data acquisiton with Sysmex CellTrics filters (30 µm, Ref No. 04-0042-2316), then subsequently analyzed with a Beckman Coulter FC 500 flow cytometer equipped with an air-cooled 20 mW, 488 nm Argon ion laser. Forward and side scatter values were recorded in the corresponding photodetectors. The flow rate was set to „low" (10 µl/min). A total of 10 000 events were collected per sample.

Data analysis was done with Flowing free software (version 2.5.1, www.flowingsoftware.com) and FCS Express 7 Plus (version 7.00.0037, www.denovosoftware.com) by drawing two-dimensional plots, showing forward (FS) versus side scatter (SS). Both parameters were displayed on log axes.

Study 2. Effects of acute heat stress on chicken hepatic cell culture models

Aims

The molecular effects of a shorter (1 h) and a longer (2 h) heat exposure on the metabolic activity, oxidative status, HSP70 and pro-inflammatory cytokine production were aimed to be assessed on the newly established primary liver cell cultures from chickens. Applying mono-cultures of hepatocytes and co-cultures of parenchymal and NP cells could highlight the role of different cell types in stress response, and the established co-culture as an inflammatory model could contribute to understand the link between hepatic inflammation and distress.

Treatments of cell cultures

Hepatic cell cultures were prepared as described in Study 1. After 24 h culturing, culture media were changed to fresh FBS-free Williams' Medium E, and confluent mono- and co-cultures on 6-well and 96-well plates were incubated at 43°C for 1 or 2 h to mimic acute heat stress, while control cells were incubated further at 38.5°C. The incubation conditions were set based on literature data and our pilot studies, considering that birds have higher physiological body temperature than mammals; however, cells isolated from avian species are often cultured at temperatures similar to mammalian cells. Normal incubation temperatures of avian cell cultures are ranging from 37°C to 41.5°C, and temperatures mimicking heat stress are varied between 40°C and 45°C hence, as a compromise, 38.5°C was chosen for maintaining control cells, and 43°C for studying acute heat stress.

Laboratory analyses

Following heat exposure, the metabolic activity of cells on 96-well plates was monitored by the CCK-8 assay according to the manufacturer's instructions, detecting the amount of $NADH+H^+$ gained in the catabolic pathways. Briefly, 10 µl CCK-8 reagent and 100 µl fresh Williams' Medium E were given to the cultured cells, and the absorbance was measured at 450 nm with a Multiskan GO 3.2 reader after 2 h incubation at 38.5°C. In order to monitor cytotoxicity, extracellular lactate dehydrogenase (LDH) activity was measured by a specific photometric assay (Diagnosticum Ltd., Budapest, Hungary). First, 200 µl working reagent (containing 56 mM phosphate buffer, pH = 7.5; 1.6 mM pyruvate and 240 µM $NADH+H^+$) was mixed with 10 µl cell culture medium. The enzyme activity was assessed by a kinetic method, measuring the absorbance of samples at 340 nm with a Multiskan GO 3.2 reader.

Culture media of 6-well plates were collected directly after the applied treatments for measuring extracellular ROS (H_2O_2), HSP70, IL-6 and IL-8 concentrations. Following the removal of media, cultured cells from both 6-well and 96-well plates were gently washed in PBS and were subsequently lysed in M-PER solution supplemented with 1% Halt Protease Inhibitor Cocktail and 1% ethylene diamine tetraacetic acid (EDTA) (Thermo Fisher Scientific, Waltham, USA) for assaying total protein concentrations. All culture media and cell lysate samples were stored at -80°C until further processing.

The H_2O_2 concentration of cell supernatants was assessed with the Amplex Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, USA). The applied substrate (Amplex Red) reacts with H_2O_2 in a horseradish peroxidase (HRP) catalyzed reaction, producing highly fluorescent resorufin. After 30 min incubation of 50 µl culture media with 50 µl freshly prepared, Amplex Red (100 µM) and HRP (0.2 U/ml) containing working solution at room temperature, fluorescence was detected with a Victor X2 2030 fluorometer (λex = 560nm; λem = 590nm). The concentrations of HSP70, IL-6 and IL-8 were measured in the culture media of 6-well dishes by chicken specific ELISA kits (Cat. No. MBS734158, MBS268769 and MBS013823, respectively; MyBioSource, San Diego, CA,

USA) following the manufacturer's instructions. The absorbance values were quantified at 450 nm with a Multiskan GO 3.2 reader. Total protein concentration of cell lysates was assessed with the PierceTM Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, USA) as indicated by the manufacturer, applying BSA as a standard, adding 25 µl sample to 200 µl reagent mixture and measuring the absorbance after 30 min incubation at 37°C at 562 nm with a Multiskan GO 3.2 reader.

Study 3. Effects of T-2 toxin on chicken hepatic cell culture models

Aims

The main aim of this study was to investigate the cellular effects of T-2 toxin on the previously developed hepatic cell culture models of chicken origin. Further, as other studies provided different and often conflicting results depending on the investigated organs or cell types, another goal of the trial was to compare the effects of T-2 toxin on primary liver-derived hepatocyte mono-cultures and hepatocyte – NP cell (predominantly resident macrophages, Kupffer cells) co-cultures of chicken origin *in vitro*. According to the hypothesis of our study, T-2 toxin may alter metabolic activity, redox status and pro-inflammatory cytokine (IL-6 and IL-8) production of the examined cell culture models.

Treatments of cell cultures

Hepatocyte mono-cultures and hepatocyte – NP cell co-cultures were prepared as described previously in Study 1. After 24 h culturing, confluent monolayers were challenged to T-2 toxin in different concentrations. Williams' Medium E was supplemented with 0 nmol/l (control), 10 nmol/l, 100 nmol/l or 1000 nmol/l T-2 toxin. Treatment with toxin containing media lasted either for 8 h or for 24 h, respectively, in all of the applied cell culture models. Later, samples were taken from culture media of the 6-well plates after both incubation times and cells were lysed using M-PER solution supplemented with 1% Halt Protease Inhibitor Cocktail and 1% EDTA. Samples were stored until further analysis at -80°C.

Laboratory analyses

Following T-2 toxin challenge, metabolic activity of cells seeded onto 96-well plates was monitored by CCK-8 assay. Extracellular H_2O_2 concentration was determined in the culture medium using Amplex Red method. Production of HSP70, IL-6 and IL-8 was measured by chicken-specific ELISA tests in the collected medium. In order to standardize values gained from the previously mentioned methods, total protein concentration of cell lysates was determined by Pierce BCA Protein Assay. Laboratory analyses were performed as previously described in Study 2.

Study 4. Effects of pathogen-associated molecular patterns on chicken hepatic cell culture models

Aims

The primary aim of this study was to induce inflammation on hepatic cell cultures of chicken origin by testing different potentially pro-inflammatory compounds, while illuminating the mechanism of action of the investigated pathogen-associated molecular patterns. Within this study, LPS and LTA type endotoxins, the beta subunit of the heat-labile enterotoxin of *E. coli*, flagellin, Poly I:C, serving as a model of viral infections, and PMA as a general pro-inflammatory substance were aimed to be applied. Further, a novel three-dimensional (3D) cell culture model comprised of hepatocytes and NP cells was also developed to provide an even more reliable model of the *in vivo* inflammatory conditions. Hence, the effects of the applied substances on cellular metabolic activity and pro-

inflammatory cytokine release were compared on 2D monolayers and the newly established 3D cultures. These results would serve as indispensable data to set and characterize an inflammatory chicken hepatic cell model suitable for challenging substances with antioxidant and immunomodulatory effects.

Cell culturing

The 2D hepatocyte – NP cell co-cultures were prepared on collagen-coated 96-well plates as indicated in Study 1. For the 3D cell culturing, cells were magnetized with 500 µL magnetic nanoparticles (NanoShuttleTM-PL, Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) added to 5 ml co-culture suspension (previously mixing cell suspensions in the ratio of 6:1, hepatocyte to NP cells). The cells were seeded onto 96-well cell repellent plates (Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) without collagen coating, the seeding volume was 100 µL/well. The plates were incubated at 37°C for 1 h to get the nanoparticles attached to the cell membrane. Thereafter, the plates were placed on a magnetic drive with magnets under each well (Spheroid Drive, Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) and were incubated at 37°C in humid atmosphere with 5% CO_2.

Culture media were changed after 24 h incubation for both 2D and 3D cultures. For the latter ones, the plate was placed on a magnetic Holding Drive (Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary). Then, the 3D cell cultures were incubated for an additional 24 hours on the Spheroid Drive. The plate was left on the Spheroid Drive altogether for 48 h to produce adequate spheroids. Culture medium contained 5% FBS only in the first 24 hours of culturing, all of the other supplements added to the medium during the entire experiment were the same as in the medium used at the seeding.

Treatments of cell cultures

The 2D cell cultures were treated when reaching confluency after 24 h incubation, while 3D cultures were exposed to the investigated compounds after 48 h when spheroid formation finished. The medium of the 2D and 3D cell cultures was supplemented with the followings:

- None (controls)
- 10 or 50 µg/mL LTA from *Staphylococcus aureus*
- 10 or 50 µg/mL LPS from *Escherichia coli*
- 20 or 50 µg/mL B subunit of the heat-labile enterotoxin derived from *Escherichia coli*
- 100 or 250 ng/mL *Salmonella* Typhimurium derived flagellin
- 100 or 1000 ng/mL PMA
- 50 or 100 µg/mL Poly I:C. To achieve re-annealing, Poly I:C was previously heated at 50°C for 3 minutes then cooled down to 37°C.

Laboratory analyses

The metabolic activity of the cells was measured on 96-well plates by CCK-8 assay. In order to investigate the treatment solutions' cytotoxicity, LDH activity in the culture media was measured by an enzyme kinetic photometric assay. The concentrations of IL-6 and IL-8 were measured in the culture media by chicken specific ELISA kits following the manufacturer's protocol, as described in Study 2.

Study 5. Effects of fermented wheat germ extract as a redox modulator on cultured rat hepatocytes

Aims

Primary hepatic cell culture models also enable the investigation of suggested protective substances against the deteriorative action of the already studied immunomodulatory stressors. Serving as a basis of further studies related to anti-inflammatory and antioxidant candidates, a preliminary trial was carried out to assess the potential of effects wheat germ extract (FWGE) as a redox modulator on cultured rat hepatocytes.

Cell isolation and culturing

The preparation of primary rat hepatocytes was performed using 8-week-old Wistar rats (approx. 200-250 g). Animals were kept and fed according to the actual Hungarian and European animal welfare laws. After carbon dioxide narcosis, median laparotomy was performed followed by the cannulation of the *vena portae* and the thoracic section of the *vena cava caudalis*. Similarly to chickens, the liver was flushed and exsanguinated through the portal system, using different buffers and multi-step perfusion. To perfuse the liver, 300 mL EGTA (0.5 mM) containing HBSS buffer, 200 mL EGTA-free HBSS, and finally, 130 mL EGTA-free HBSS supplemented with 50 mg type IV. collagenase (Serva, Duisburg, Germany), 2.5 mM $CaCl_2$ and $MgCl_2$ were used.

After excision of the liver and disruption of the capsule, cell suspension was filtered using sterile gauze sheets. Cell suspension was placed for 50 min into 25 mg/mL BSA containing ice-cold HBSS in order to avoid undesired cluster formation. Hepatocytes were isolated using low speed multistep differential centrifugation (3 times, 100 x g, 2 min) and the gained pellets were resuspended in Williams' Medium E supplemented as described in Study 1 for chicken hepatic cells. After the final resuspendation, viability of hepatocytes was tested by trypan blue exclusion test, cell counting was carried out in Bürker's chamber, and the cell suspension was diluted to 10^6 cells/mL. Hepatocytes were seeded onto 96- and 6-well plates previously coated with collagen type I (10µg/cm^2). Cultures were incubated at 37ºC and 100% relative air humidity. Cell culture media were changed after 4 h, and confluent monolayer cell cultures were gained after 24 h incubation.

Treatments of cell cultures

After 24 h culturing cells were treated using cell culture media supplemented with 0 (control) or 10 µg/mL *Salmonella* Typhimurium derived LPS for 2 and 8 h incubation time. Further, in both of the control and LPS-challenged cultures, subgroups were prepared using 0.1% and 1% FWGE prepared from Immunovet®, silymarin (50 µg/mL) or ursodeoxycholic acid (UDCA, 200 µg/mL) containing cell culture medium. In the latter two cases, cultures were treated with proved hepatoprotective and antioxidant substances. In case of the 6-well plates, following either 2 or 8 h incubation, samples were taken from the cell culture media. Thereafter, wells were washed with PBS and cells were lysed using M-PER solution supplemented with 1% Halt Protease Inhibitor Cocktail and 1% EDTA. All of the collected samples were stored until further analysis at −80ºC.

Laboratory analyses

Following the treatments, metabolic activity of cells cultured on 96-well plates was checked using CCK-8 assay; extracellular H_2O_2 concentration was detected in the culture medium using the fluorimetric Amplex Red method as indicated in Study 2. The MDA concentration as a marker of lipid peroxidation was monitored in cell culture media with a specific colorimetric test. According to the protocol, 300 µL freshly prepared thiobarbituric acid (TBA) stock solution was mixed with 100 µL diluted sample. Solutions were incubated

at 95°C for 1 h followed by 10 min cooling in ice. Absorbance was measured at 532 nm with a Multiskan GO 3.2 reader. As one of the most prominent member of the antioxidant defense system, activity of glutathione peroxidase (GPx) enzyme of the cell lysates was also determined using a colorimetric kinetic assay. At first, GPx Assay Buffer was prepared according to the manufacturer's protocol and 455 µL buffer was mixed with 25 µL of NADPH Assay Reagent supplemented with 5 µL Substrate solution (tert-Butyl Hyperoxide). Decrement of absorbance was continuously monitored (initial delay: 15 sec; reading interval: 10 sec; number of readings: 6; wavelength: 340 nm). Enzyme activity was calculated using the formula provided by the manufacturer.

Study 6. The role of small heat shock proteins in the modulation of redox homeostasis under acute heat stress in chickens

Aims

Based on the results obtained in Study 2, an *in vivo* study was planned to investigate certain effects of acute heat stress in the parenchymal organs of broiler chickens under *in vivo* conditions. Since there are numerous questions regarding the exact effects of heat stress on the molecular level in broiler chickens, especially surrounding the specific function of sHSP molecules in heat-triggered alterations of the redox homeostasis, the main aim of this study was to monitor the impact of acute heat stress on cellular oxidative status and sHSP expression in the liver, kidney, and spleen. To gain a comprehensive understanding of the redox state of the animals, several redox parameters, including MDA, reduced glutathione (GSH), and protein carbonyl concentrations, as well as GPx enzyme activities, were examined. To assess the function and role of sHSP-s in the heat shock response of chickens, HSP27, αA-crystallin, and αB-crystallin protein expressions were determined in all three of the mentioned organs. This study reveals novel data concerning the complex interplay of sHSP-s and heat-provoked oxidative damage in different tissues of chickens, while also reflecting key points for alleviating the adverse effects of heat stress.

Animal trial

Raising and housing of the animals was carried out in the Institute of Physiology and Nutrition, Georgikon Campus, Hungarian University of Agriculture and Life Sciences, Keszthely, Hungary. Hundred male, one-day-old Ross 308 broiler chicks were obtained from a commercial hatchery (Gallus Ltd., Devecser, Hungary), and group-housed on wheat straw litter in floor pens. Climatic conditions and housing were established according to the requirements of the Ross technology (Aviagen, 2018) over the entire study, except the day of the treatment. Feed and drinking water were provided ad libitum and the growth performance of the animals matched the parameters described in the Broiler Management Handbook for Ross 308 broilers (Aviagen, 2018).

Housing, feeding, and treatment of the chickens were carried out in strict accordance with the national and international laws as well as with the institutional guidelines. Experimental procedures were approved by the Government Office of Zala County, Food Chain Safety, Plant Protection, and Soil Conservation Directorate, Zalaegerszeg, Hungary (number of permission: GK-419/2020; approval date: 11 May 2020).

At the age of day 27, randomly selected animals were allocated to individual cages and subdivided into three different treatment groups (n = 8 animals per group). On day 32 of life, animals were challenged to 37°C environmental heat exposure with 50% relative humidity (temperature humidity index, THI = 88) either for 4 h or 8 h period of time, respectively. In the control group, climatic conditions remained unchanged and corresponded completely with the breeder's recommendations (22 ± 1°C).

After the treatment periods, cloacal temperatures were registered and chickens were slaughtered in carbon dioxide narcosis, followed by sampling from the left lobe of the liver, the spleen, and the left caudal division of the kidney. The samples were immediately shock-frozen in dry ice and stored at -80°C until further analysis.

Laboratory analyses

For sample processing and laboratory analyses, all reagents were purchased from Merck KGaA (Darmstadt, Germany), except when otherwise specified. Samples were freshly thawed on ice, then homogenized in T-PER lysis buffer (adding 1 ml buffer to 100 mg tissue) freshly supplemented with 1% Halt Protease Inhibitor Cocktail by Potter-Elvehjem tissue homogenizer. Homogenates were centrifuged at 5,000 x g for 10 min, and supernatants were used for further measurements.

Total protein concentration of homogenates was assayed by PierceTM Bicinchoninic Acid (BCA) Protein Assay using BSA as standard, similarly to the method described in Study 2. As a marker of lipid peroxidation, MDA concentration was measured by a specific colorimetric test as indicated in Study 5. As a prominent member of the antioxidant defense system, reduced glutathione (GSH) concentration was determined by specific colorimetric tests. Following the manufacturer's instructions, 40 µL standard solution or sample was pipetted to each well, supplemented with 120 µL Buffer solution. The plate was incubated for 1 h at 37ºC and 20 µL Substrate solution was pipetted to all the wells afterward. Lastly, 20 µL Coenzyme working solution as well as 20 µL Enzyme working solution was added followed by a 10 min long incubation at 37ºC. Absorbance was measured at 412 nm. The GPx enzyme activity was also determined using a colorimetric kinetic assay on the same way as in Study 5.

To achieve information about protein damage caused by oxidative stress, Protein Carbonyl Content Assay Kit was applied. First, 100 µL DNPH (2,4-dinitrophenylhydrazine) solution was added to 100 µL sample, and Eppendorf tubes were incubated for 10 min at room temperature. Thereafter, 30 µL 100% trichloroacetic acid solution was pipetted to each sample followed by a 5 min long incubation in ice. After a 2 min long centrifugation at 13,000 x g, supernatant was removed, and freshly formed pellet was dissolved and incubated at -20ºC in 500 µL ice-cold acetone for 5 min. After the second centrifugation (13,000 x g; 2 min) acetone was removed and pellet was dissolved in 200 µL of 6 M guanidine solution. Absorbance values of 100 µL samples were measured at 375 nm using a Multiskan GO 3.2 microplate reader.

In order to detect αA-crystallin, αB-crystallin and HSP27 concentrations, chicken specific ELISA kits were used (MyBioSource, San Diego, CA, USA; MBS7231952, MBS7241579, MBS700383, respectively). For the purpose of detecting the concentration of αA-crystallin protein by competitive ELISA method, firstly 100 µL sample, blank (PBS with pH 7.0-7.2) or standard solutions were measured into the wells. As it was described by the manufacturer's protocol, 10 µL Balance Solution was only added to the samples. Thereafter, 50 µL Conjugate was pipetted to each well except blanks, and plates were incubated for 1 h at 37ºC. After multiple washing steps 50 µL Substrate A and 50 µL Substrate B Solutions were added followed by 20 min incubation at 37ºC. Reaction was stopped by adding 50 µL Stop Solution, and absorbance values were measured at 450 nm.

In case of chicken specific αB-crystallin competitive ELISA, 100 µL standard, sample or blank solutions were measured into the wells followed by a 2 h long incubation at 37ºC and the addition of 100 µL Detection Reagent A. After several washing steps 100 µL Detection Reagent B was incubated for 1 h followed up by washing procedure. After the incubation of 90 µL Substrate solution for 20 min at 37ºC, reaction was terminated by Stop Solution and absorbance was measured at 450 nm.

Regarding HSP27 sandwich ELISA, firstly 100 µL standard or sample solutions were added to the wells, whilst blanks remained empty. After 90 min incubation at 37ºC and two

washing steps, 100 µL Biotinylated chicken HSP27 antibody was added to each well and plate was incubated for 60 min at 37ºC. Next, following three times washing, 100 µL Enzyme-conjugate Solution was added to each well except blanks and plate was put back to incubator for 30 min at 37ºC. Lastly, wells were washed five times and 100 µL Colour Reagent was pipetted to each of them. Within 30 min incubation at 37ºC, the reaction was stopped by adding 100 µL Colour Reagent C and absorbance values were immediately detected at 450 nm.

*Statistics (for **Study 1-6**)*

In case of the *in vitro* trials (Study 1-5), all treatments were applied in triplicates on 6-well plates, and 6 wells were included in each group on 96-well plates. The measured data on 6-well-plates were all standardized to the total protein concentrations of cell lysates. Data were analyzed with one-way ANOVA and with post-hoc tests, correlations were assayed with Pearson's test of correlation, applying the R 3.5.3 software. All results are expressed as mean ± SEM or in boxplots, level of significance was set at $P<0.05$.

The overview of the performed studies is summarized in **Table 1**.

Table 1. Overview of the performed studies.

Study No	Study type	Applied cell cultures/animals	Major topic	Laboratory analyses (measured parameters)	Place of the study
Study 1	*in vitro*	Chicken primary hepatocyte mono-culture and hepatocyte – NP cell co-culture	Development and characterization of novel chicken hepatic cell culture models	Characterization with immunofluorescence and flow cytometry	Budapest, Keszthely
Study 2	*in vitro*	Chicken primary hepatocyte mono-culture and hepatocyte – NP cell co-culture	The effects of acute heat stress on chicken hepatic cell cultures	Metabolic activity, extracellular LDH activity, H_2O_2, HSP70, IL-6 and IL-8 concentration	Budapest
Study 3	*in vitro*	Chicken primary hepatocyte mono-culture and hepatocyte – NP cell co-culture	The effects of T-2 toxin on chicken hepatic cell cultures	Metabolic activity, extracellular H_2O_2, HSP70, IL-6 and IL-8 concentration	Budapest
Study 4	*in vitro*	Chicken primary hepatocyte – NP cell co-culture and 3D spheroids prepared from co-culture	The hepatic pro-inflammatory action of pathogen-associated molecular patterns	Metabolic activity, extracellular LDH activity, IL-6 and IL-8 concentration	Budapest
Study 5	*in vitro*	Rat primary hepatocyte mono-culture	Fermented wheat germ extract as a redox modulator in the hepatic inflammatory response	Metabolic activity, extracellular H_2O_2 and MDA concentration, intracellular GPx activity	Budapest
Study 6	*in vivo*	Broiler chickens	The role of small heat shock proteins in chickens under acute heat stress	MDA, protein carbonyl, glutathione, HSP27, αA-crystallin, αB-crystallin in liver, spleen and kidney homogenates	Budapest, Keszthely

III. Main results

Study 1. Developing and characterizing a novel primary hepatocyte – NP cell co-culture of chicken origin

After staining confluent hepatocyte mono-cultures and hepatocyte – NP cell co-cultures with Giemsa, typical morphological features of parenchymal and NP liver cells could be observed (**Figure 1**).

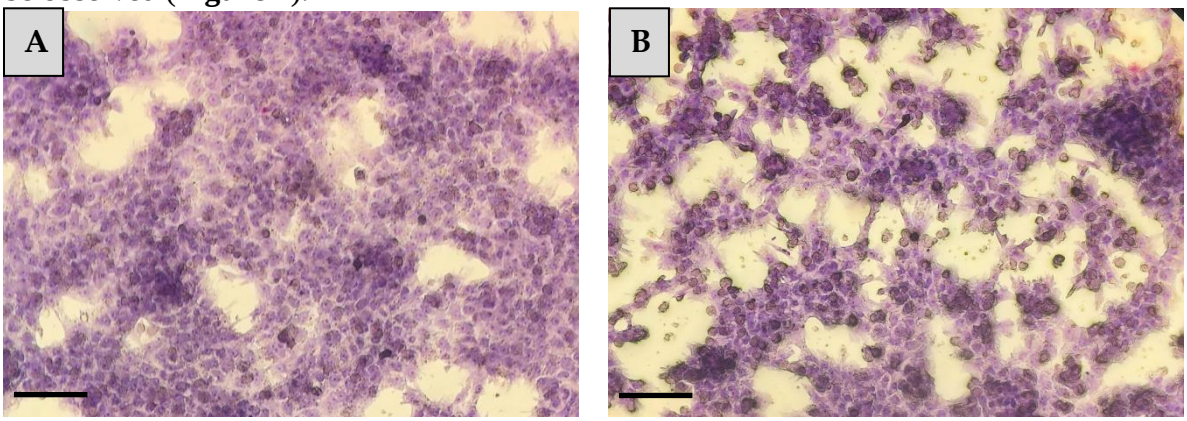


Figure 1. Giemsa staining of **A.** hepatocyte mono-cultures and **B.** hepatocyte – non-parenchymal cell co-cultures after 48 h culturing (20x magnification, bar = 100 μm).

Immunocytochemical detection of albumin revealed that cultured parenchymal cells showed strong positivity indicated by the green fluorescence of the applied FITC coupled anti-albumin antibody, confirming the presence of hepatocytes in both freshly isolated cell suspensions and cells after 48 h culturing (**Figure 2**). Besides, the majority of isolated and cultured NP cells were positively stained by the macrophage specific PE conjugated antibody, reflected by the red fluorescence (**Figure 3**).

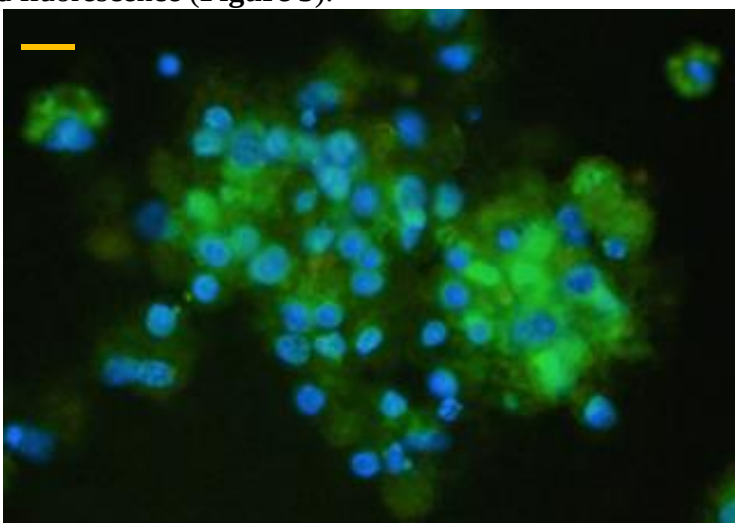


Figure 2. Immunofluorescent detection of hepatocytes in a hepatocyte – non-parenchymal cell co-culture after 48 h culturing with a chicken specific, fluorescein isothiocyanate (FITC) coupled anti-albumin antibody (40x magnification, bar = 40 μm). Blue colour indicates cell nuclei with DAPI staining, while green colour refers to hepatocytes detected with the FITC conjugated antibody.

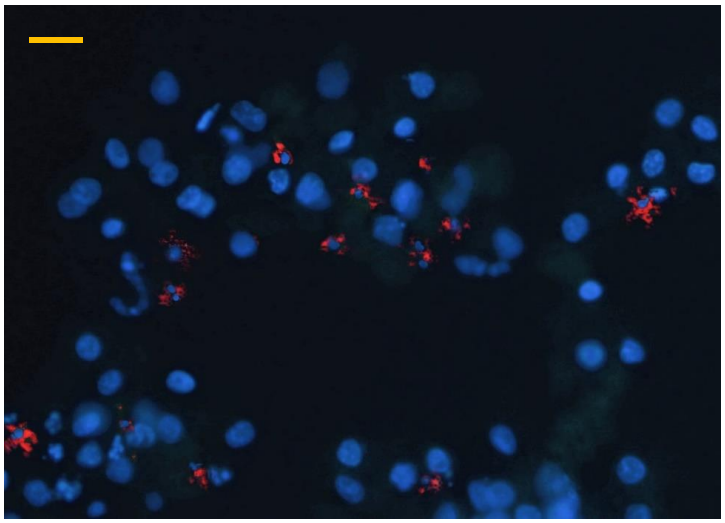


Figure 3. Immunofluorescent detection of macrophages in a hepatocyte – non-parenchymal cell co-culture after 48 h culturing with a phycoerythrin (PE) coupled chicken macrophage specific antibody (40x magnification, bar = 40 μm). Blue colour indicates cell nuclei with DAPI staining, while red colour refers to macrophages detected with the PE conjugated antibody.

When investigating the isolated cells with flow cytometry, and applying scatter profiles to monitor the characteristics and homogeneity of the separated cell suspensions, two well-defined, reproducible fractions were isolated (**Figure 4**). The type of cells comprising these fractions was confirmed with immunofluorescent staining of isolated and cultured cells as described above, using FITC coupled anti-albumin and PE conjugated macrophage specific antibodies.

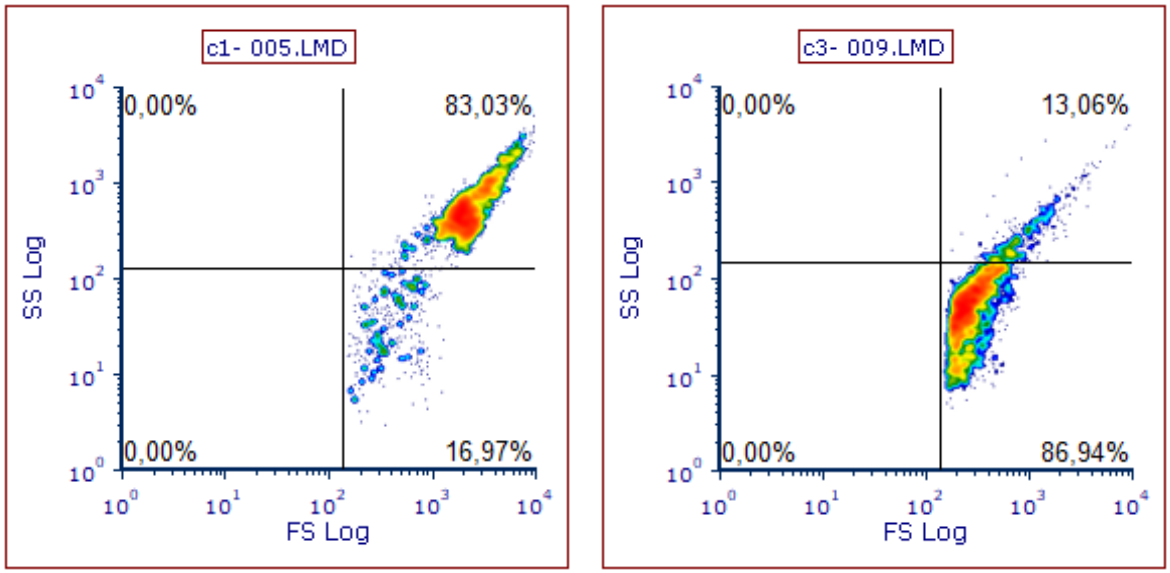


Figure 4. A representative density plot figure showing the forward (FS) and side (SS) scatter profiles of hepatocytes (left panel) and non-parenchymal cells (right panel).

Study 2. Effects of acute heat stress on chicken hepatic cell culture models

The metabolic activity of cultured cells, monitored with the CCK-8 assay (**Figure 5**), was higher in co-cultures compared to hepatocyte mono-cultures, independently from the heat exposure ($P<0.001$). The shorter, 1 h lasting heat stress increased the catabolic activity of hepatocyte mono-cultures and hepatocyte – NP cell co-cultures compared to controls ($P<0.001$). The longer, 2 h heat exposure elevated the metabolic activity of hepatocyte mono-cultures ($P=0.006$), but with a lower extent than the 1 h heat stress, whereas it reduced metabolic activity of co-cultured cells ($P=0.004$).

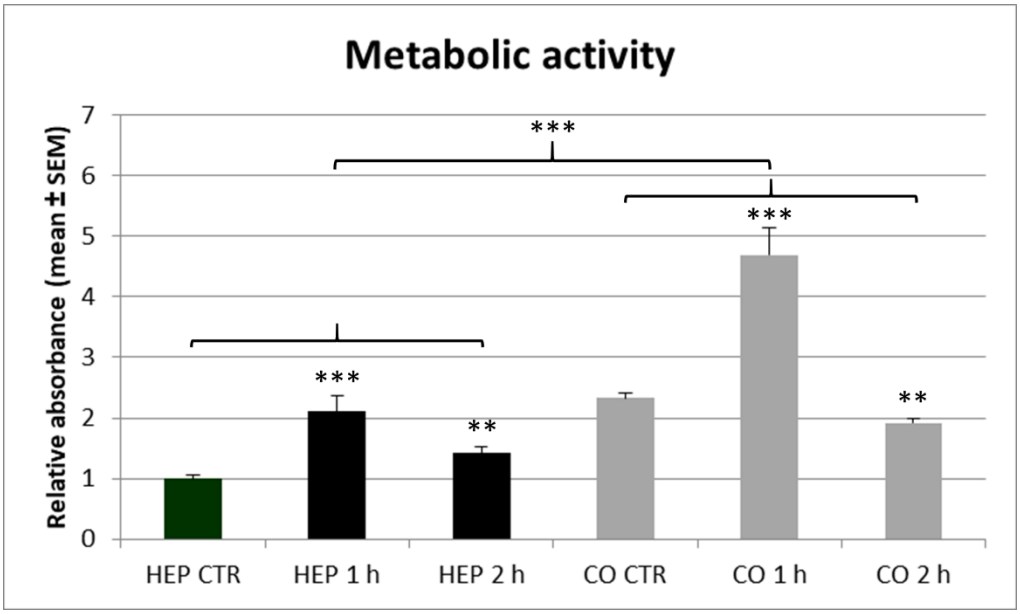


Figure 5. The metabolic activity of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") as indicated by the CCK-8 assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43°C for 1 h or 2 h, respectively. Results are expressed as mean ± standard error of the mean (SEM), n=6/group. Asterisks over bars of "1 h" and "2 h" refer to significant differences compared to "CTR" cells within the same cell culture model. Significant differences between cell culture models are indicated with asterisks on the clamps. **P<0.01, ***P<0.001.

The extracellular LDH activity indicating the amount of necrotic cells is shown in **Figure 6**. According to our results, LDH activity was not affected by heat exposure on both cell culture models. In comparison with the mono-cultured hepatocytes, significantly lower ($P<0.001$) LDH activity was detected in case of co-cultures independently from the heat stress.

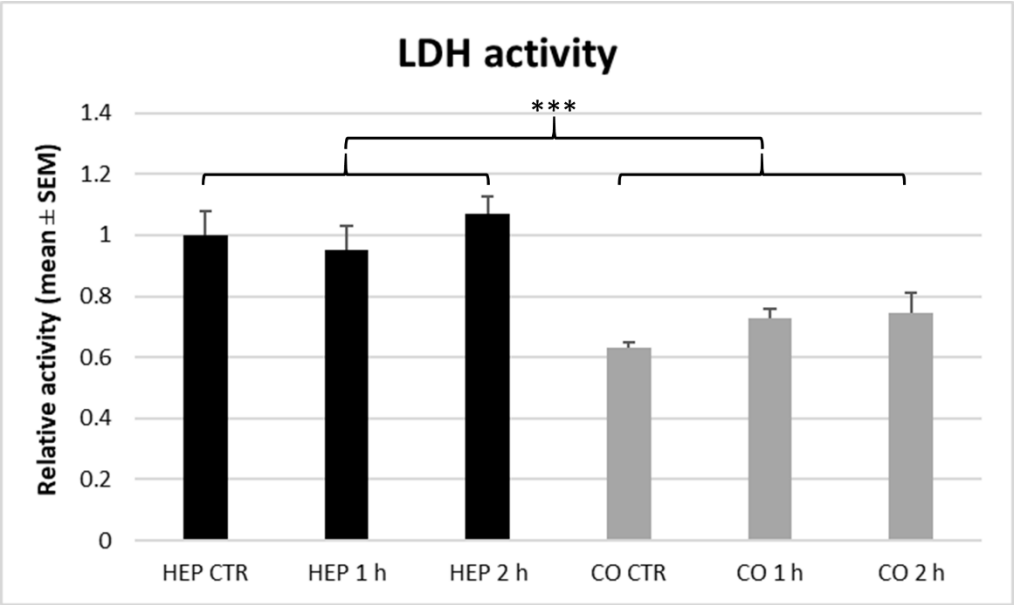


Figure 6. The LDH activity of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") as indicated by specific photometric assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43°C for 1 h or 2 h, respectively. Relative absorbances were calculated by considering the mean value of control hepatocyte mono-cultures as 1. Results are expressed as mean ± standard error of the mean (SEM), n=3/group. Asterisks over bars of "1 h" and "2 h" refer to significant differences compared to "CTR" cells within the same cell culture model. Significant differences between cell culture models are indicated with asterisks on the clamps. ***P<0.001.

The extracellular ROS production of cell cultures was investigated by measuring the H_2O_2 concentration of culture media with the Amplex Red assay (**Figure 7**). When comparing hepatocyte mono-cultures and hepatocyte – NP co-cultures, slightly, but significantly lower extracellular ROS concentration was detected in the latter case (P=0.048). The shorter term (1 h) heat exposure increased the ROS release of hepatocyte mono-cultures (P=0.004) and hepatocyte – NP cell co-cultures (P=0.003) compared to controls, while the longer (2 h) heat stress did not influence the H_2O_2 concentration of cell supernatants on both cell culture models.

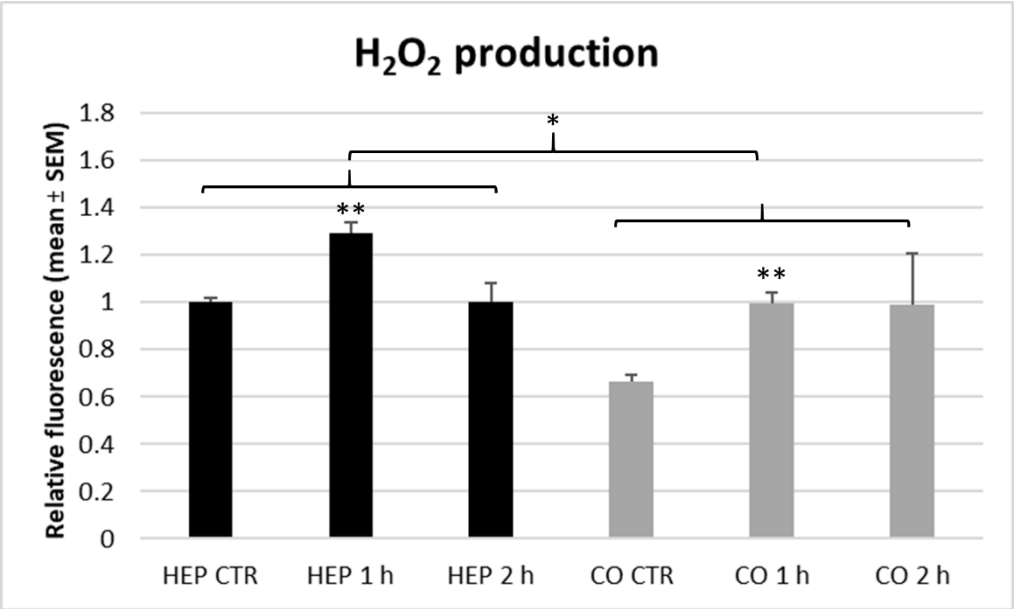


Figure 7. The hydrogen peroxide (H_2O_2) production of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") as indicated by the Amplex Red assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43°C for 1 h or 2 h, respectively. Relative fluorescences were calculated by considering the mean value of control hepatocyte mono-cultures as 1. Results are expressed as mean ± standard error of the mean (SEM), n=3/group. Asterisks over bars of "1 h" and "2 h" refer to significant differences compared to "CTR" cells within the same cell culture model. Significant differences between cell culture models are indicated with asterisks on the clamps. *P<0.05, **P<0.01.

The HSP70 concentration of cell culture supernatants (**Figure 8**), measured by a specific ELISA assay, did not differ significantly on different cell culture models. The shorter (1 h) heat exposure strongly decreased the HSP70 release of both hepatocyte mono-cultures and hepatocyte – NP cell co-cultures ($P<0.001$ in both cases), while the HSP70 level was normalized after 2 h heat stress as no significant difference could be found in the HSP70 concentrations of control and 2 h long heat exposed cells in any cell cultures.

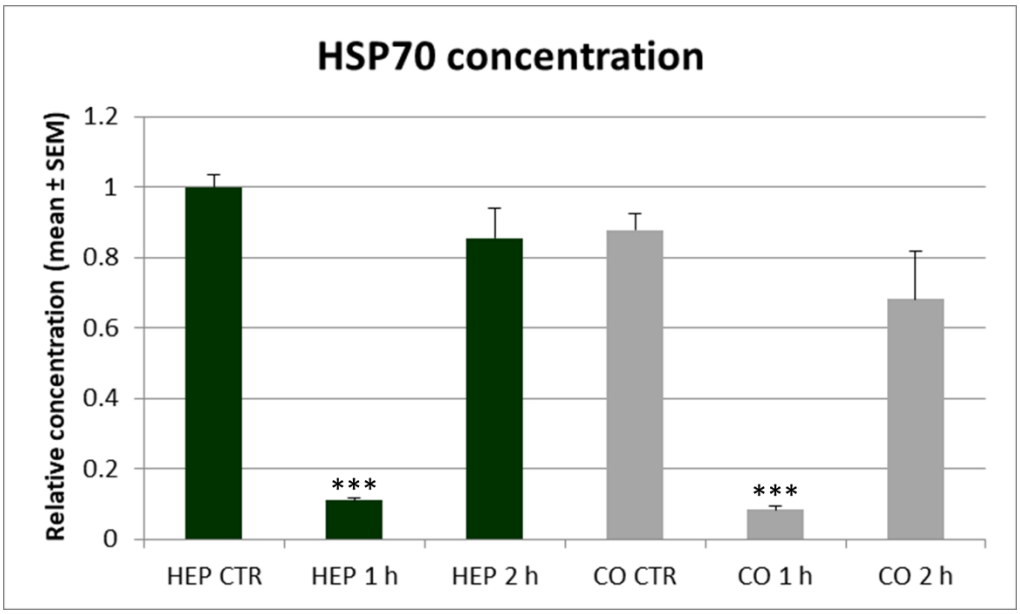


Figure 8. The heat shock protein 70 (HSP70) concentration in culture media of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") as measured by a chicken specific ELISA assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43°C for 1 h or 2 h, respectively. Relative concentrations were calculated by considering the mean value of control hepatocyte mono-cultures as 1. Results are expressed as mean ± standard error of the mean (SEM), n=3/group. Asterisks over bars of "1 h" and "2 h" refer to significant differences compared to "CTR" cells within the same cell culture model. ***P<0.001.

Similarly to HSP70, the concentration of pro-inflammatory cytokines IL-6 and IL-8 showed no significant differences between hepatocyte mono-cultures and co-cultures in culture media as measured by specific ELISA kits (**Figure 9.A-B**). A strong decrease in both IL-6 and IL-8 levels was detected after 1 h heat exposure on both hepatocyte mono-cultures and hepatocyte – NP cell co-cultures compared to controls (IL-6: P=0.007 and P=0.001, IL-8: P=0.005 and P=0.001, respectively). Following the longer, 2 h heat stress, interleukin concentrations tended to return to the baseline values as no significant differences were observed when comparing control and 2 h heat exposed cells.

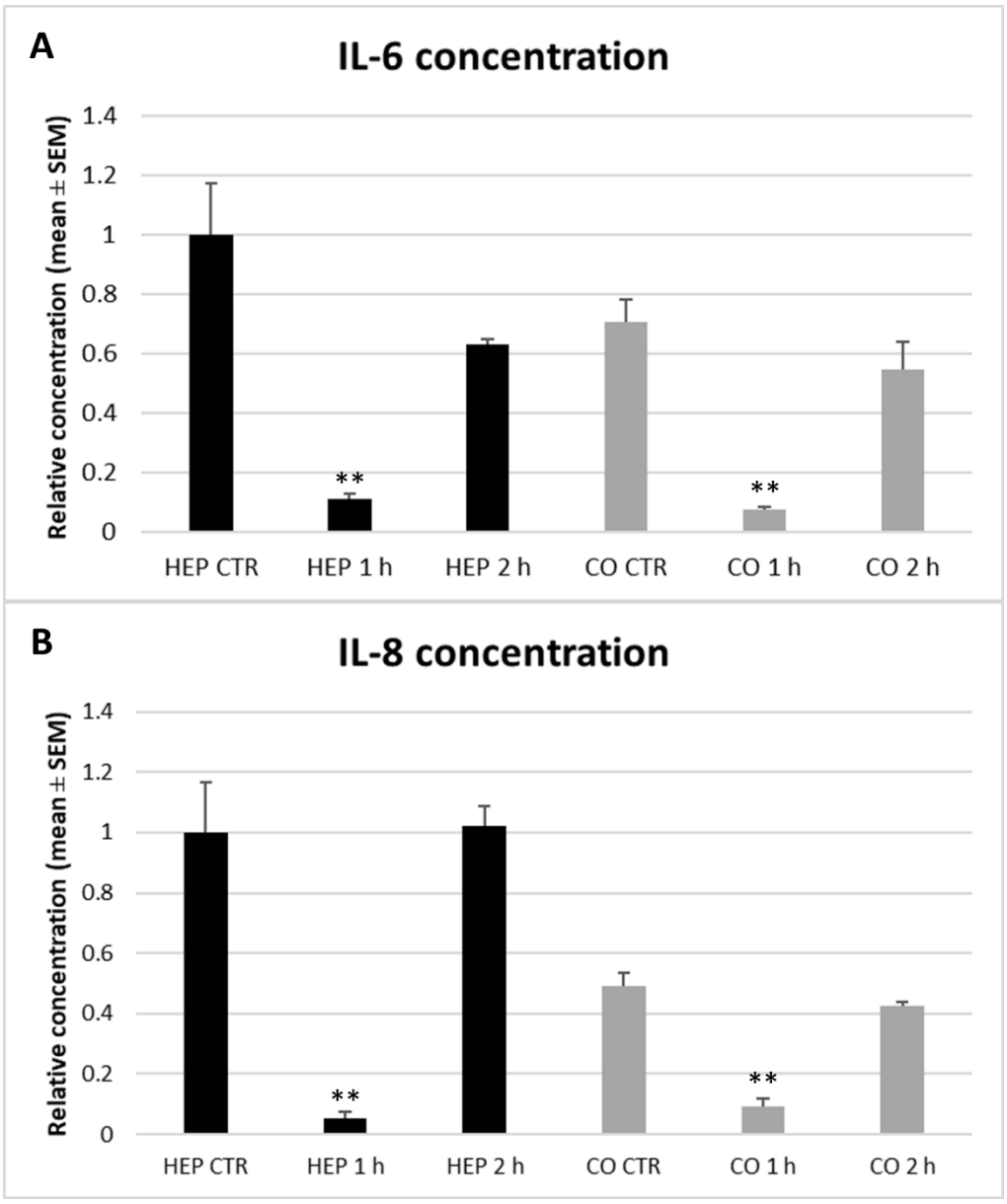
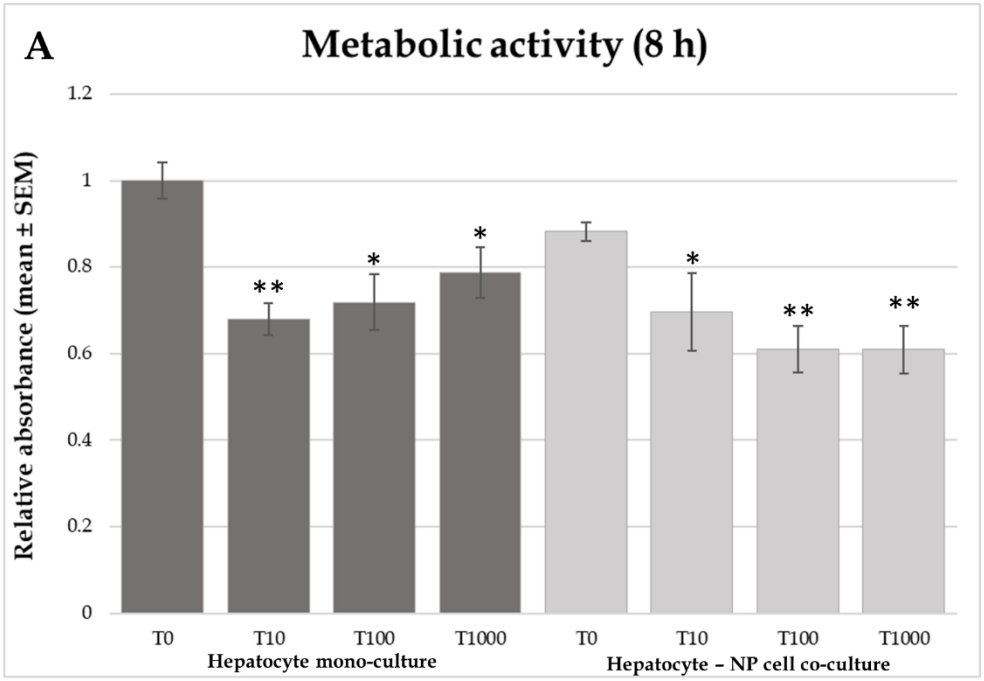


Figure 9. Interleukin-6 (IL-6, **A**) and interleukin-8 (IL-8, **B**) concentration in culture media of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") detected by a chicken specific ELISA assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43°C for 1 h or 2 h, respectively. Relative concentrations were calculated by considering the mean value of control hepatocyte mono-cultures as 1. Results are expressed as mean ± standard error of the mean (SEM), n=3/group. Asterisks over bars of "1 h" and "2 h" refer to significant differences compared to "CTR" cells within the same cell culture model. **P<0.01.

Study 3. Effects of T-2 toxin on chicken hepatic cell culture models

The T-2 toxin treatment decreased the metabolic activity of cultured cells at all applied T-2 toxin concentrations both in the hepatocyte mono-culture and in the hepatocyte – NP cell co-culture models, following both 8 h (**Figure 10.A**) and 24 h (**Figure 10.B**) incubation times (8 h incubation of hepatocyte mono-culture: P=0.00299; P=0.011; P=0.0272 for 10, 100 and 1000 nmol/L T-2 toxin, respectively; 8 h incubation of co-culture: P=0.0454; P=0.00201; P=0.00214 for 10, 100 and 1000 nmol/L T-2 toxin, respectively, 24 h incubation of hepatocyte mono-culture: P=0.00204; P<0.001; P=0.00154 for 10, 100 and 1000 nmol/L T-2 toxin, respectively, 24 h incubation of co-culture: P<0.001; P=0.0434; P<0.001 for 10, 100 and 1000 nmol/L T-2 toxin, respectively).



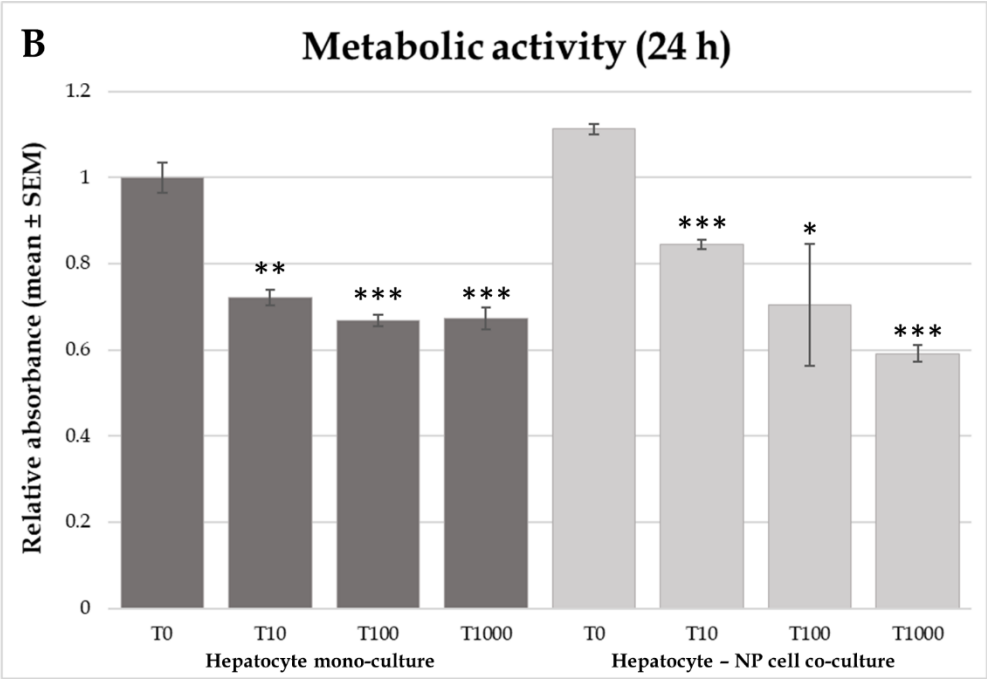


Figure 10. Effects of T-2 toxin treatment on cellular metabolic activity of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures with the cell ration of 6:1 (hepatocytes to non-parenchymal cells). Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h (**A**) or 24 h (**B**). "T0" refers to control group (without T-2 toxin treatment); "T10" refers to 10 nmol/L T-2 toxin treatment; "T100" refers to 100 nmol/L T-2 toxin treatment; "T1000" refers to 1000 nmol/L T-2 toxin treatment. Cellular metabolic activity was measured by CCK-8 test. Relative absorbances were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1. Results are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

According the present results, H_2O_2 production was not affected by T-2 toxin treatment using 8h or 24 h incubation in any of the applied cell culture models (**Figure 11**). Comparing the culture specific differences, a higher H_2O_2 production rate was observed in the hepatocyte mono-culture model than in the co-culture after 8 h of incubation (**Figure 11.A**; P<0.001).

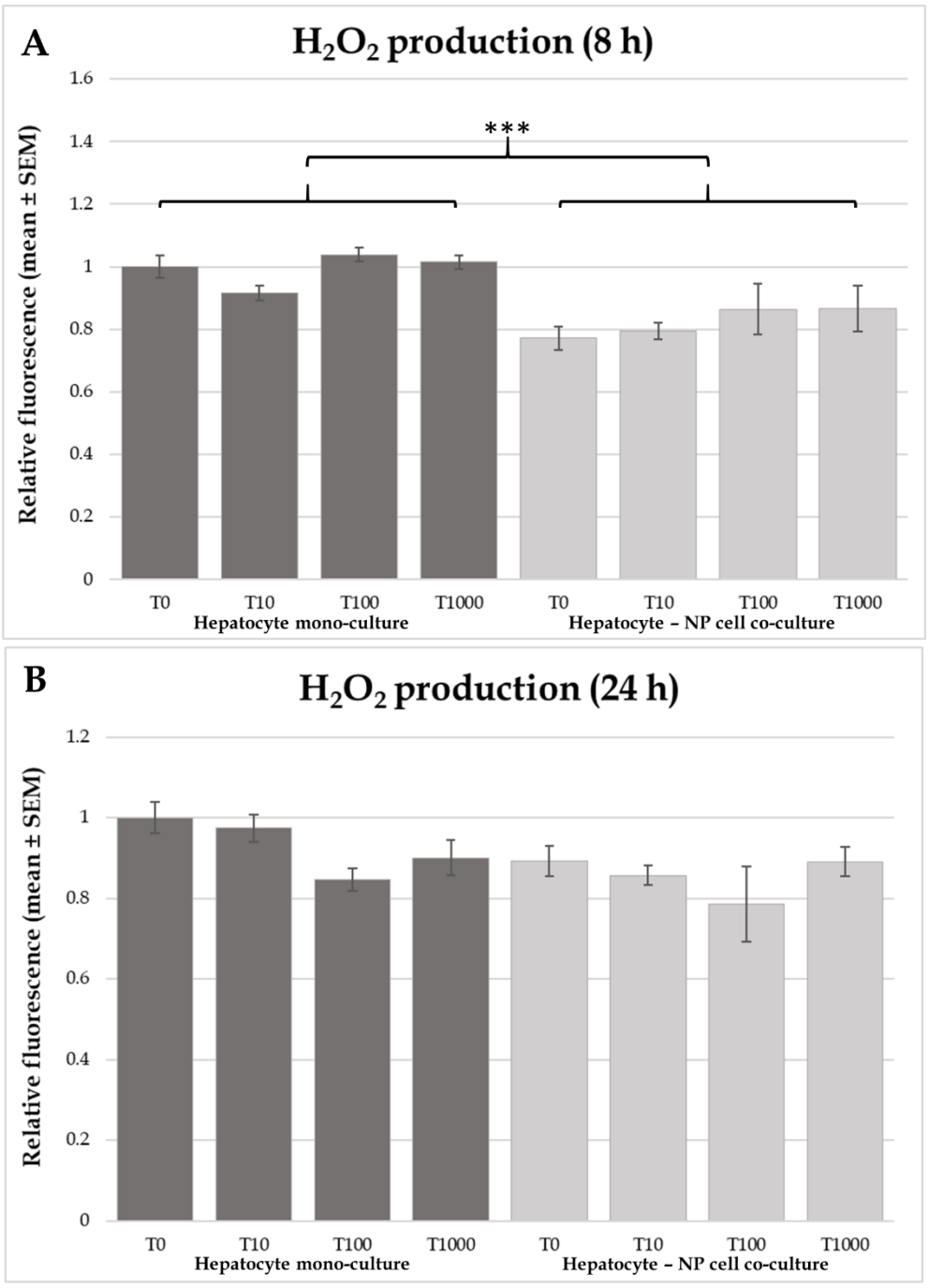


Figure 11. Effects of T-2 toxin treatment on H_2O_2 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures with the cell ration of 6:1 (hepatocytes to non-parenchymal cells). Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h (**A**) or 24 h (**B**). "T0" refers to control group (without T-2 toxin treatment); "T10" refers to 10 nmol/L T-2 toxin treatment; "T100" refers to 100 nmol/L T-2 toxin treatment; "T1000" refers to 1000 nmol/L T-2 toxin treatment. H_2O_2 production was measured by Amplex Red method. Relative fluorescences were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1. Results are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

Higher (P=0.025) HSP70 concentrations were found in the culture media of co-cultures compared to those of the hepatocyte mono-culture models (**Figure 12**). Due to methodological difficulties, no analysable data is available for HSP70 after 8 h incubation. Addition of T-2 toxin at 100 or 1000 nmol/L elevated HSP70 concentrations (P=0.0389 and

=0.0444) in the hepatocyte mono-cultures after 24 h incubation. However, no differences were detected in the co-cultures (**Figure 12**).

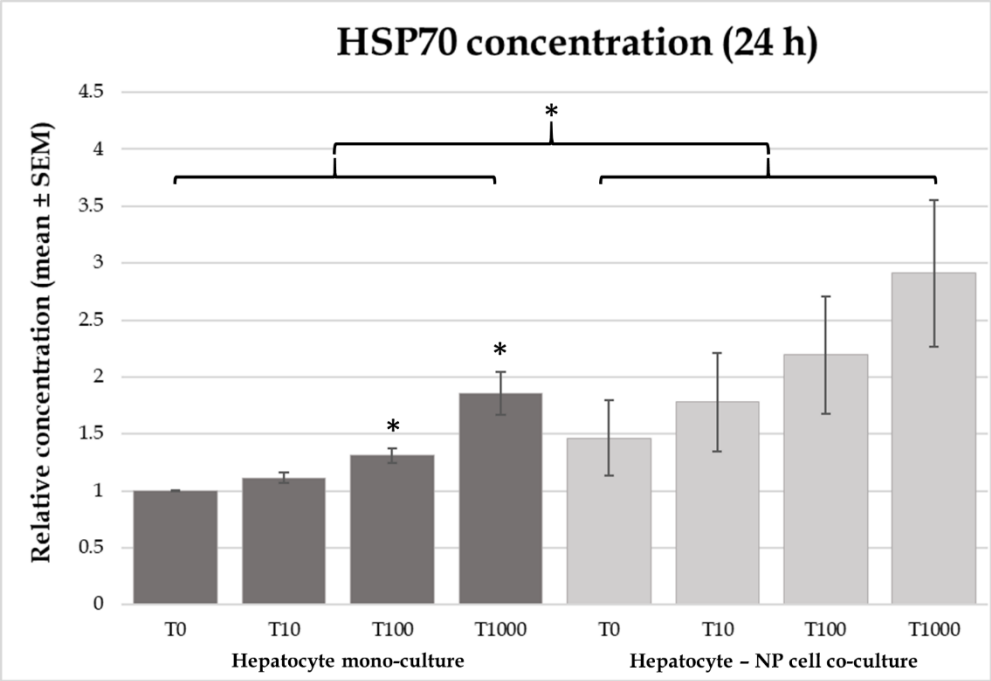


Figure 12. Effects of T-2 toxin treatment on HSP70 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures with the cell ration of 6:1 (hepatocytes to non-parenchymal cells). Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h (**A**) or 24 h (**B**). "T0" refers to control group (without T-2 toxin treatment); "T10" refers to 10 nmol/L T-2 toxin treatment; "T100" refers to 100 nmol/L T-2 toxin treatment; "T1000" refers to 1000 nmol/L T-2 toxin treatment. HSP70 concentrations were measured by chicken specific ELISA tests. Relative concentrations were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1. Results are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

The IL-6 concentration differed in both culture types when comparing 100 nmol/L or 1000 nmol/L T-2 toxin supplemented groups to the control after 8 h of incubation treatment in both hepatocyte mono-cultures and hepatocyte – NP cell co-cultures (hepatocyte mono-culture: P=0.0465; P=0.0153 for 100 and 1000 nmol/L T-2 toxin, respectively; co-culture: P=0.00752; P=0.00605 for 100 and 1000 nmol/L T-2 toxin, respectively). In the meanwhile, IL-6 concentrations were unchanged after 24 h of incubation time (**Figure 13**).

In the co-culture model higher IL-8 concentration was shown, in the culture media of cells treated with 1000 nmol/l T-2 toxin than in controls incubated for 8 h (P=0.0479), No such significant effect was found in the hepatocyte mono-culture model (**Figure 14.A**). In contrast, 24 h incubation with 1000 nmol/L toxin concentration resulted in higher IL-8 levels (P=0.018) in the hepatocyte mono-culture model. However, 24 h toxin incubation did not affect IL-8 levels in the co-cultures (**Figure 14.B**). Concerning the different models, higher IL-8 production (P=0.00677) was shown in the hepatocyte mono-cultures after 8 h incubation but not after 24 h (**Figure 14.A**).

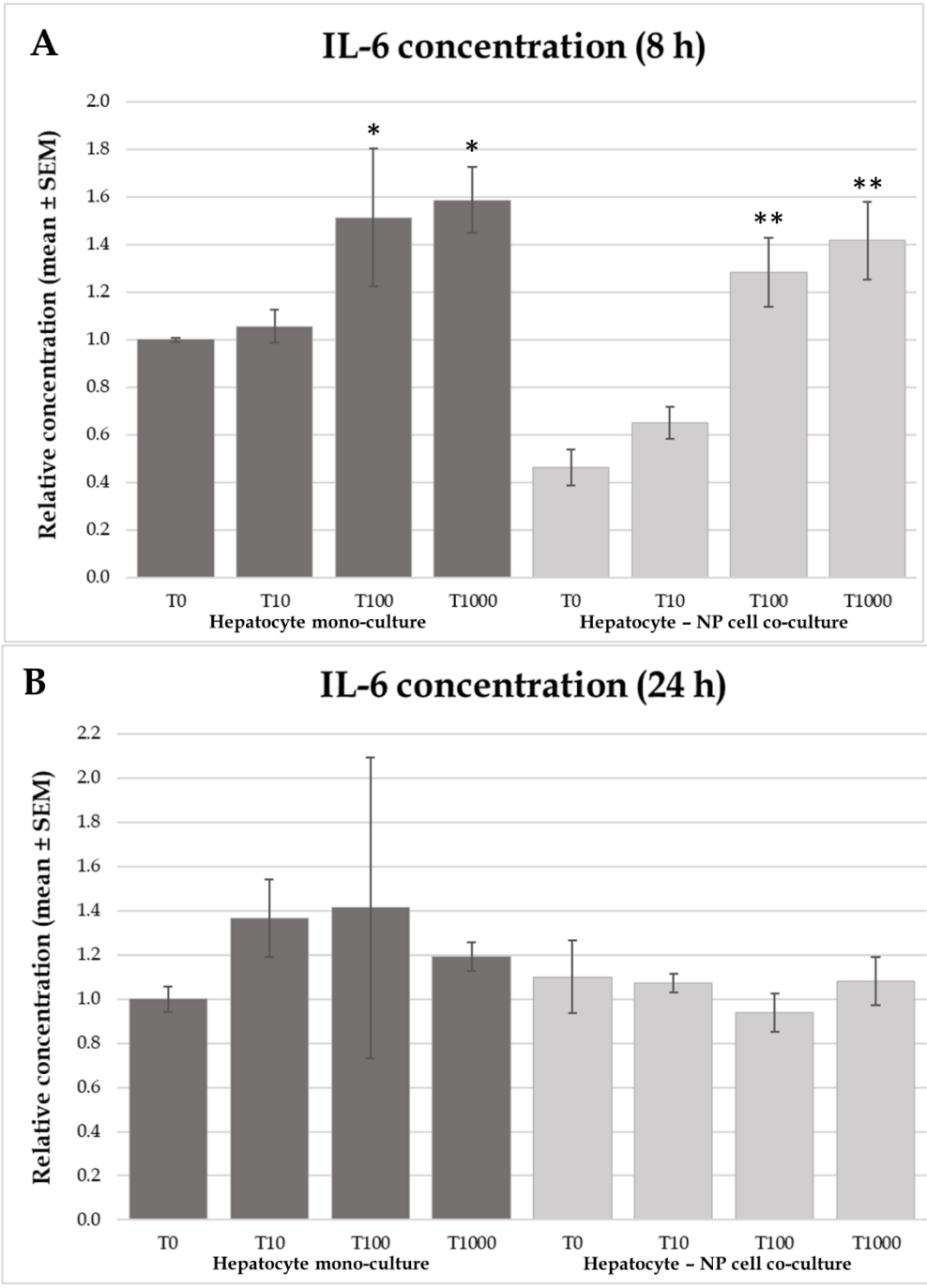


Figure 13. Effects of T-2 toxin treatment on IL-6 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures with the cell ration of 6:1 (hepatocytes to non-parenchymal cells). Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h (A) or 24 h (B). "T0" refers to control group (without T-2 toxin treatment); "T10" refers to 10 nmol/L T-2 toxin treatment; "T100" refers to 100 nmol/L T-2 toxin treatment; "T1000" refers to 1000 nmol/L T-2 toxin treatment. IL-6 concentrations were measured by chicken specific ELISA tests. Relative concentrations were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1. Results are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

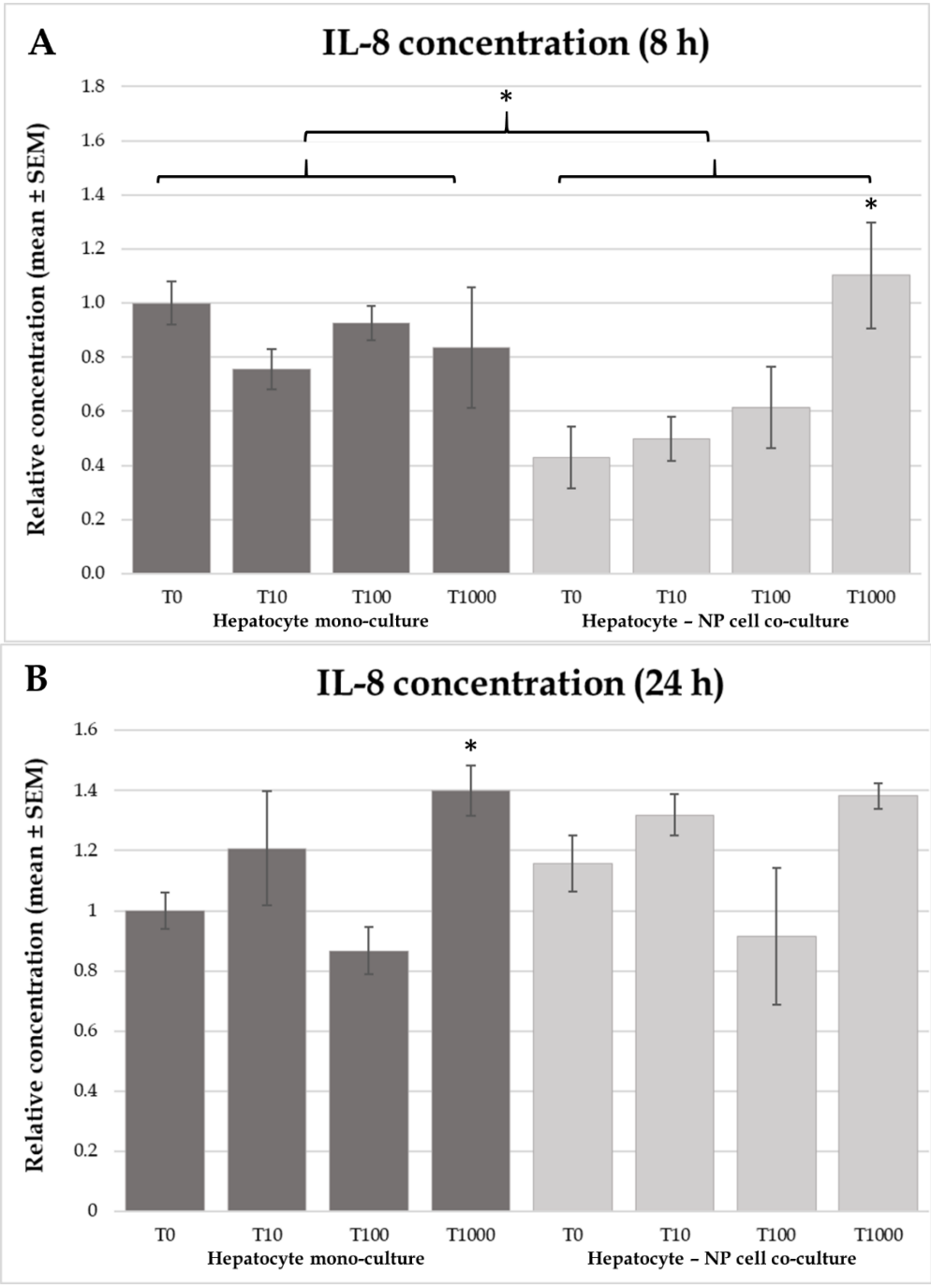


Figure 14. Effects of T-2 toxin treatment on IL-8 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures with the cell ration of 6:1 (hepatocytes to non-parenchymal cells). Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h (A) or 24 h (B). "T0" refers to control group (without T-2 toxin treatment); "T10" refers to 10 nmol/L T-2 toxin treatment; "T100" refers to 100 nmol/L T-2 toxin treatment; "T1000" refers to 1000 nmol/L T-2 toxin treatment. IL-8 concentrations were measured by chicken specific ELISA tests. Relative concentrations were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1. Results are expressed as mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

Study 4. Effects of pathogen-associated molecular patterns on chicken hepatic cell culture models

Metabolic activity measured with CCK-8 test after treatment with 10 and 50 µg/mL LPS and 50 µg/mL LTA concentration was significantly higher (P=0.005, P=0.030, P=0.020, respectively) in our 3D-cultured cells compared to control (**Figure 15**). No alteration was found in our 2D-cultured cells after LPS or LTA treatment (**Figure 15.A**). There was a significant increase after 20 and 50 µg/mL enterotoxin treatment (P<0.001, P=0.023), and a significant decrease after 100 and 1000 ng/mL PMA treatment (P=0.004, P=0.004) and 50 and 100 µg/mL Poly I:C treatment (P<0.001 in both cases) in our 2D-cultured cells (**Figure 15.B**). No significant effect was detected in our 3D-cells after these treatments (**Figure 15.D**).

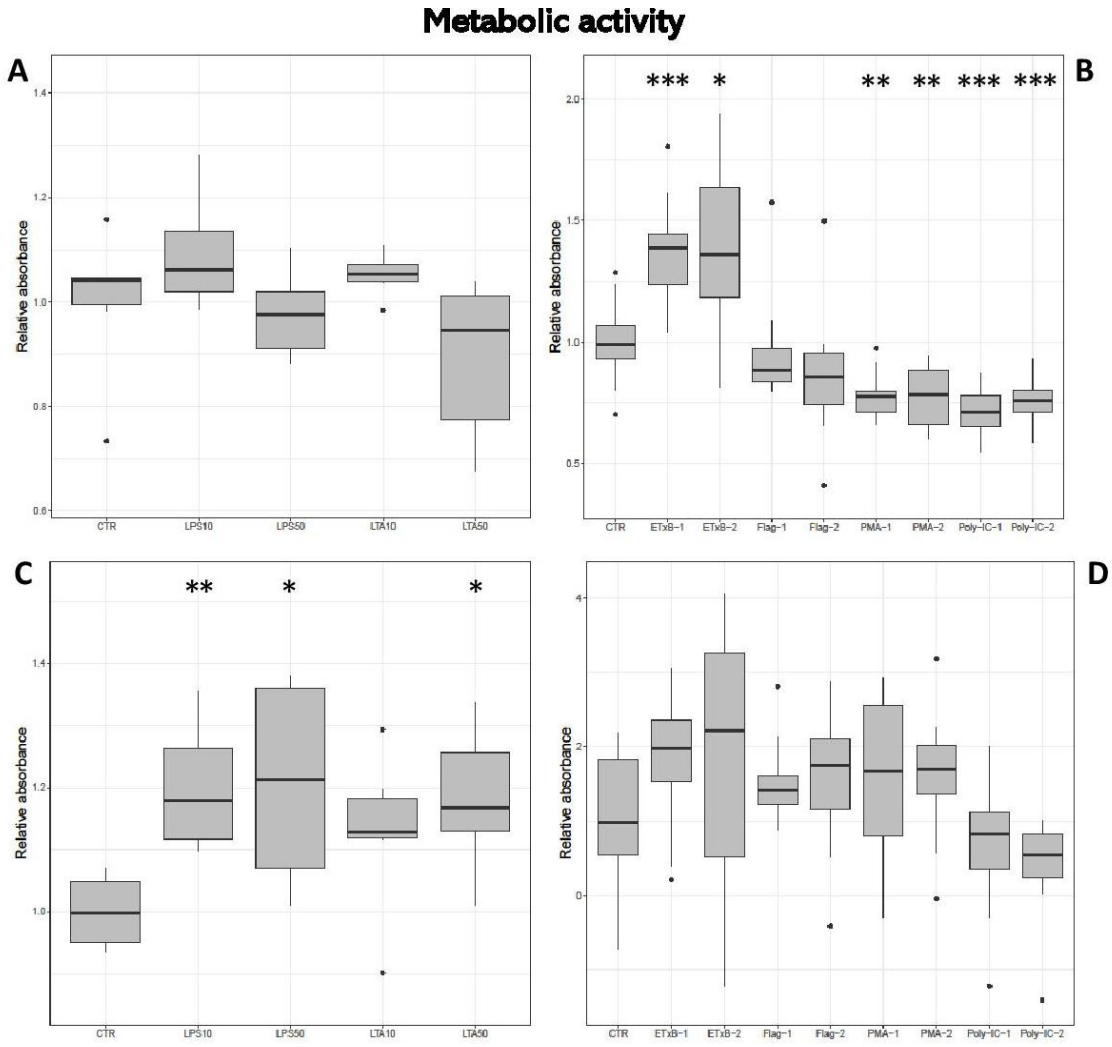


Figure 15. The metabolic activity of hepatocyte – non-parenchymal cells in 2D (**A** and **B**) and 3D (**C** and **D**) co-cultures as indicated by the CCK-8 assay. Relative absorbances were calculated by considering the mean value of control cultures as 1. The „CTR" refers to control cells that received none of the treatments. The treatments were: LPS10 and LPS50 = 10 and 50 µg/mL lipopolysaccharide (LPS) from *Escherichia coli*, LTA10 and LTA50= 10 and 50 µg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*, EtxB-1 and 2 = 20 and 50 µg/mL subunit B of heat-labile enterotoxin of *Escherichia coli*, Flag-1 and -2 = 100 and 250 ng/mL flagellin from *Salmonella* Typhimurium, PMA-1 and -2 = 100 and 1000 ng/mL phorbol myristate acetate (PMA), Poly-IC-1 and -2= 50 and 100 µg/mL polyinosinic-polycytidylic acid (Poly I:C). Asterisks over the boxes refer to significant differences compared to "CTR" cells within the same cell culture model and the same study. *P<0.05, **P<0.01, ***P<0.001.

Concerning the extracellular LDH activities, significant increase was found after both (10 and 50 µg/mL) LTA concentrations (P=0.026, P=0.004) in our 2D cultures (**Figure 16.A)**. On the contrary, LDH activities were decreased after 10 and 50 µg/mL LPS (P=0.002, P=0.005) and 10 and 50 µg/mL LTA treatment (P=0.016, P=0.005) in our 3D-cultured cells (**Figure 16.C)**. Significant elevation was detected after 50 ng/mL Poly I:C treatment (P=0.032) in our 2D cultures (**Figure 16.B)**, and after 50 and 100 µg/mL concentrations of Poly I:C (P=0.012, P=0.016) in our 3D cultures (**Figure 16.D)**.

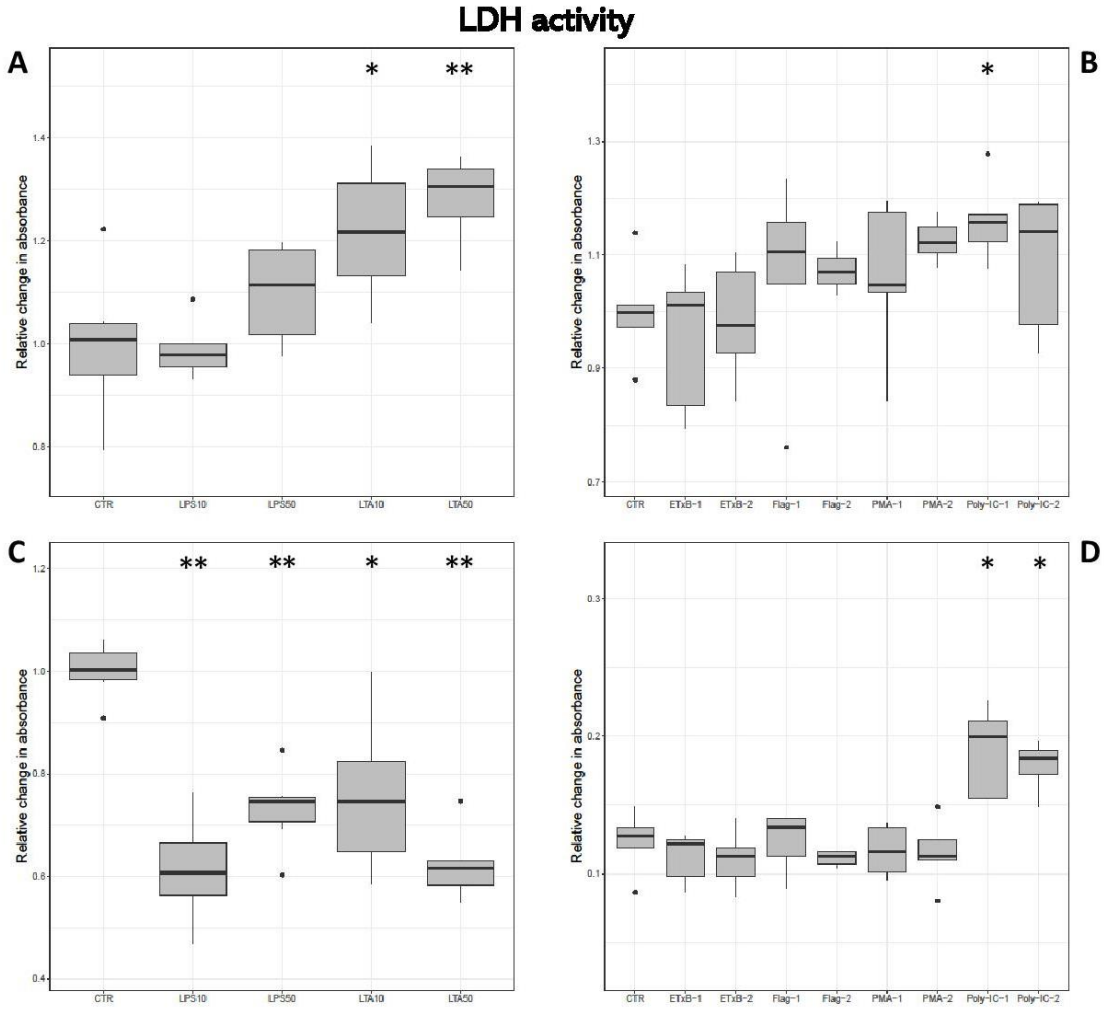


Figure 16. The lactate dehydrogenase (LDH) activity of hepatocyte – non-parenchymal cells in 2D (**A** and **B**) and 3D (**C** and **D**) co-cultures as indicated by a specific enzyme kinetic assay. Relative changes in absorbances were calculated by considering the mean value of control cultures as 1. The „CTR" refers to control cells that received none of the treatments. The treatments were: LPS10 and LPS50 = 10 and 50 µg/mL lipopolysaccharide (LPS) from *Escherichia coli*, LTA10 and LTA50= 10 and 50 µg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*, EtxB-1 and -2 = 20 and 50 µg/mL subunit B of heat-labile enterotoxin of *Escherichia coli*, Flag-1 and -2 = 100 and 250 ng/mL flagellin from *Salmonella* Typhimurium, PMA-1 and -2 = 100 and 1000 ng/mL phorbol myristate acetate (PMA), Poly-IC-1 and -2= 50 and 100 µg/mL polyinosinic-polycytidylic acid (Poly I:C). Asterisks over the boxes refer to significant differences compared to "CTR" cells within the same cell culture model and the same study. *P<0.05, **P<0.01.

The IL-6 concentration was significantly decreased after 50 µg/mL LTA treatment (P=0.016) in our 3D cultures (**Figure 17.C**), but no significant effect was detected in our 2D cultures (**Figure 17.A**). 50 ng/mL Poly I:C treatment significantly increased the IL-6

concentration (P=0.036) in our 3D cultures (**Figure 17.D**), and no significant change was observed in our 2D cultures (**Figure 17.B**).

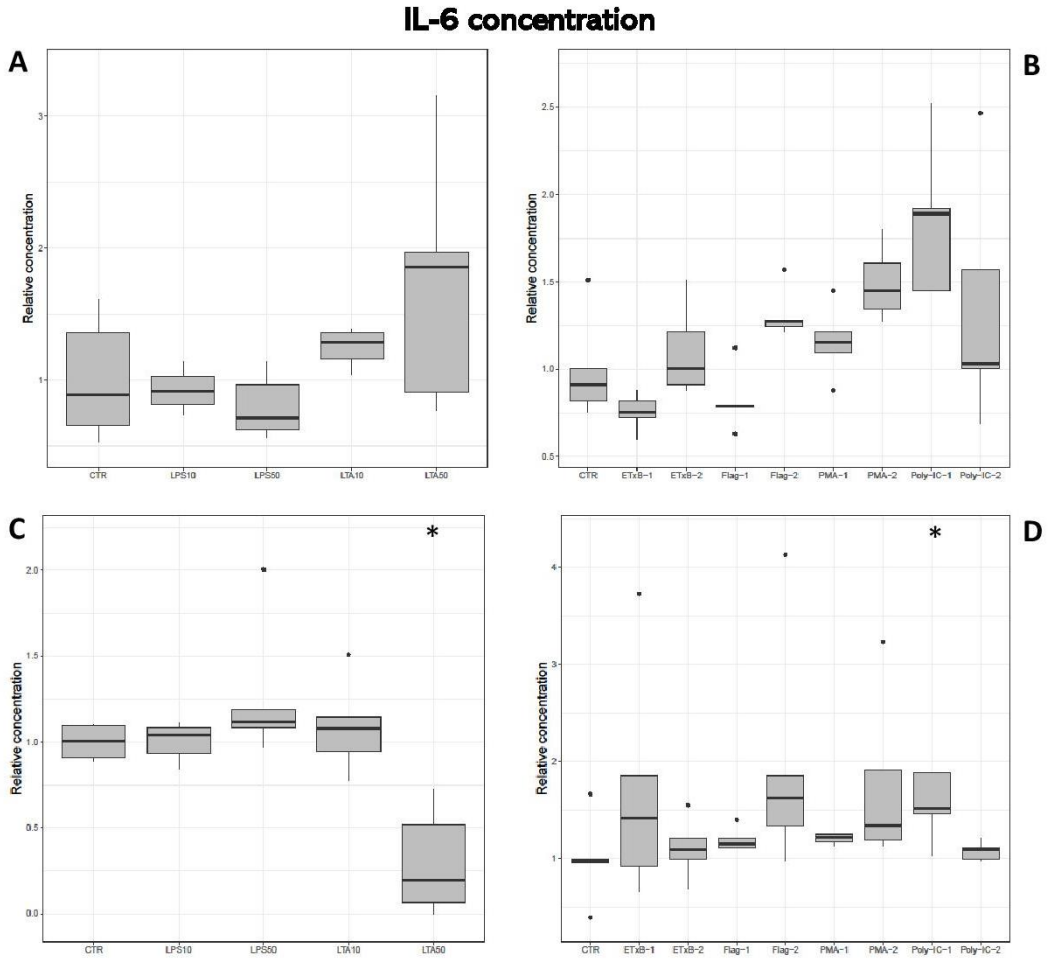


Figure 17. Interleukin-6 (IL-6) concentration in cell culture media of hepatocyte – non-parenchymal cells in 2D (**A** and **B**) and 3D (**C** and **D**) co-cultures detected by a chicken specific ELISA assay. Relative concentrations were calculated by considering the mean value of control cultures as 1. The „CTR" refers to control cells that received none of the treatments. The treatments were: LPS10 and LPS50 = 10 and 50 μg/mL lipopolysaccharide (LPS) from *Escherichia coli*, LTA10 and LTA50= 10 and 50 μg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*, EtxB-1 and -2 = 20 and 50 μg/mL subunit B of heat-labile enterotoxin of *Escherichia coli*, Flag-1 and -2 = 100 and 250 ng/mL flagellin from *Salmonella* Typhimurium, PMA-1 and -2 = 100 and 1000 ng/mL phorbol myristate acetate (PMA), Poly-IC-1 and -2= 50 and 100 μg/mL polyinosinic-polycytidylic acid (Poly I:C). Asterisks over the boxes refer to significant differences compared to "CTR" cells within the same cell culture model and the same study. * P<0.05.

The concentrations of IL-8 were significantly decreased after 10 and 50 μg/mL LPS (P=0.008, P=0.008) and 10 and 50 μg/mL LTA treatment (P=0.008, P=0.008) in our 3D cultures (**Figure 18.C)**, but there was no significant change in our 2D cultures (**Figure 18.A**). 50 μg/mL Poly I:C treatment significantly increased the concentration of IL-8 (P=0.036) in our 2D cultures (**Figure 18.B**), but had no significant effect in our 3D cultures (**Figure 18.D**).

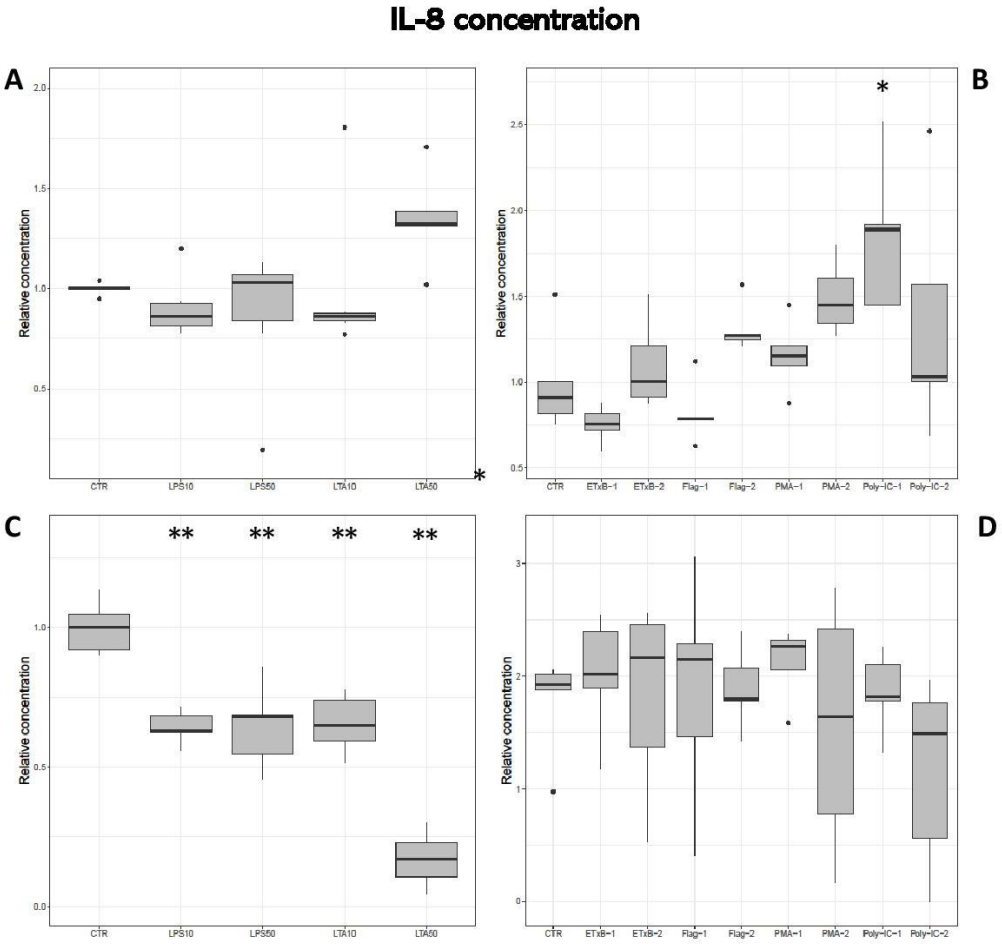


Figure 18. Interleukin-8 (IL-8) concentration in cell culture media of hepatocyte – non-parenchymal cells in 2D (**A** and **B**) and 3D (**C** and **D**) co-cultures detected by a chicken specific ELISA assay. Relative concentrations were calculated by considering the mean value of control cultures as 1. The „CTR" refers to control cells that received none of the treatments. The treatments were: LPS10 and LPS50 = 10 and 50 µg/mL lipopolysaccharide (LPS) from *Escherichia coli*, LTA10 and LTA50= 10 and 50 µg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*, EtxB-1 and -2 = 20 and 50 µg/mL subunit B of heat-labile enterotoxin of *Escherichia coli*, Flag-1 and -2 = 100 and 250 ng/mL flagellin from *Salmonella* Typhimurium, PMA-1 and -2 = 100 and 1000 ng/mL phorbol myristate acetate (PMA), Poly-IC-1 and -2= 50 and 100 µg/mL polyinosinic-polycytidylic acid (Poly I:C). Asterisks over the boxes refer to significant differences compared to "CTR" cells within the same cell culture model and the same study. * P<0.05, ** P<0.01.

Study 5. Effects of fermented wheat germ extract as a redox modulator on cultured rat hepatocytes

According to our results, the majority of the applied treatments were not able to affect the metabolic activity of the cultures, except the observed significant decrease in case of the 2 h long 0.1% FWGE (P=0.016; **Figure 19**) exposure in the LPS challenged groups, and the 8 h long silymarin and UDCA (P=0.029 and P<0.001; **Figure 19.B**) treatments of LPS free control cells.

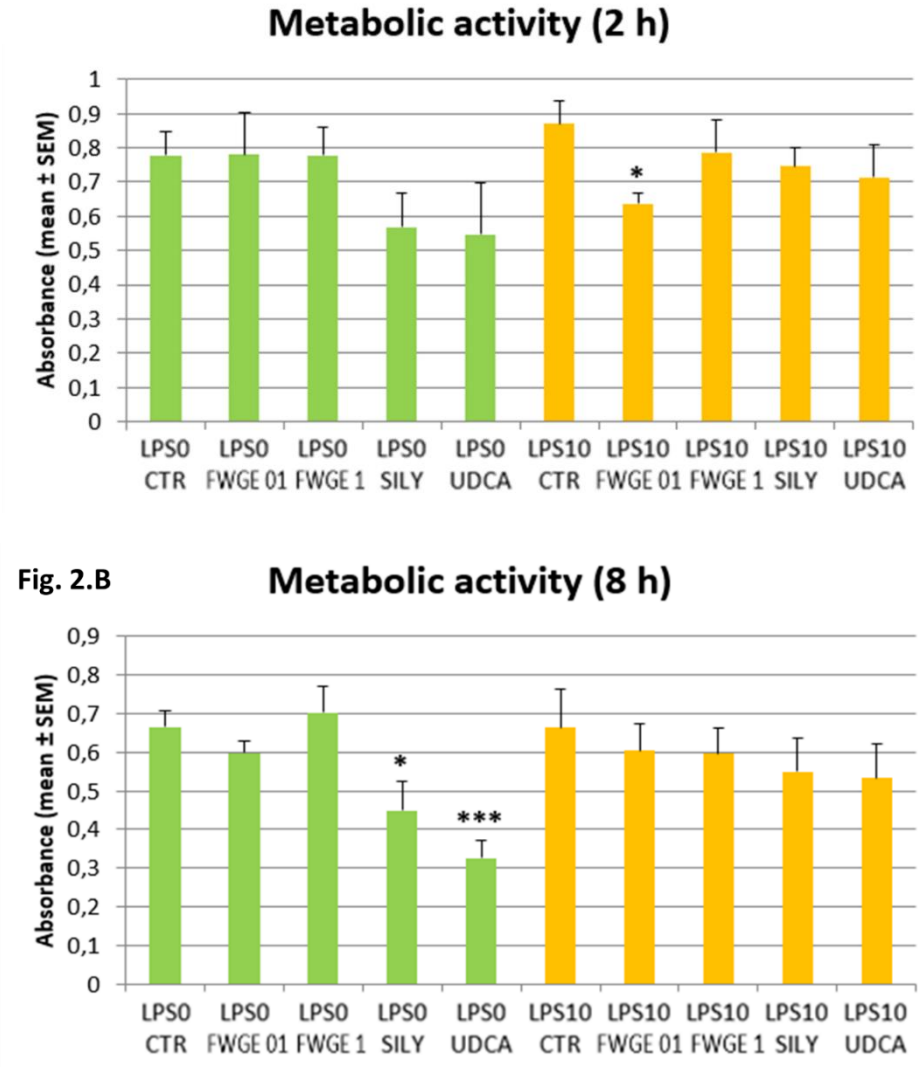


Figure 19. Metabolic activity of cultured cells after 2 h (**A**) and 8 h (**B**) incubation measured with the CCK-8 assay. LPS 0 = cultures with no LPS exposure, LPS 10 = LPS treated cultures (10 μg/mL); FWGE 01 = 0.1% FWGE, FWGE 1 = 1% FWGE, SILY = silymarin (50 μg/mL), UDCA = ursodeoxycholic acid (200 μg/mL). Mean ± SEM, *P<0.05, ***P<0.001.

The extracellular H_2O_2 (ROS) concentrations were elevated after both of the incubation times in the LPS treated groups (2 h incubation: P=0.0012; 8 h incubation: P=0.036; **Figure 20.A-B**). In cell cultures without LPS treatment, FWGE applied in 1% significantly increased ROS concentration of the cell culture media after both 2 h and 8 h incubation (P<0.001 and P=0.007; **Figure 20.A-B**). Similarly to these findings, ROS production was significantly and tendentiously increased as a result of 2 h silymarin and UDCA treatments without LPS application (silymarin: P=0.014; UDCA: P=0.058; **Figure**

20.A). On the other hand, LPS triggered elevation of the ROS levels were significantly decreased applying both FWGE and silymarin supplementation after 8 h incubation (0.1% FWGE: P=0.020; 1% FWGE: P=0.027; silymarin: P=0.006; **Figure 20.B**).

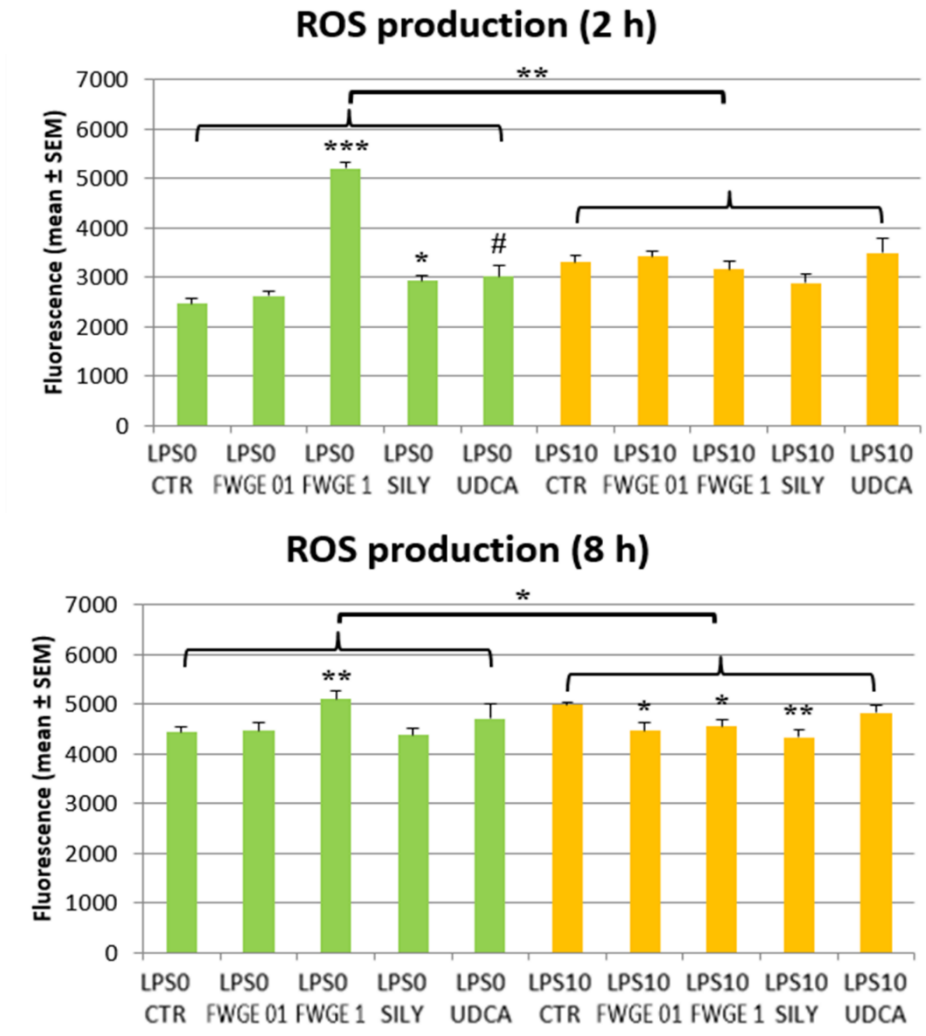


Figure 20. Extracellular ROS production of cell cultures after 2 h (**A**) and 8 h (**B**) incubation measured with the Amplex red method. LPS 0 = cultures with no LPS exposure , LPS 10 = LPS treated cultures (10 μg/mL); FWGE 01 = 0.1% FWGE, FWGE 1 = 1% FWGE, SILY = silymarin (50 μg/mL), UDCA = ursodeoxycholic acid (200 μg/mL). Mean ± SEM, #P<0.10, *P<0.05, **P<0.01, ***P<0.001.

In order to monitor the lipid peroxidation processes in the cell cultures, MDA concentration was measured in the media after both incubation times. In the cells with no LPS treatment, FWGE applied in 1% concentration caused significantly higher MDA level after 2 h as well as 8 h incubation time (2 h: P<0.001; 8 h: P=0.003; **Figure 21.A-B**). However, together with LPS treatment, 1% FWGE significantly decreased the production of MDA after both incubation times (2 h: P<0.001; 8 h: P=0.044; **Figure 21.A-B**). Similarly to these findings, following 2 h of LPS exposure, FWGE applied in lower concentration as well as silymarin and UDCA treatment significantly decreased the MDA concentration of the cell culture supernatants (0.1% FWGE: P=0.018, silymarin: P=0.004 UDCA: P<0.001; **Figure 21.A**).

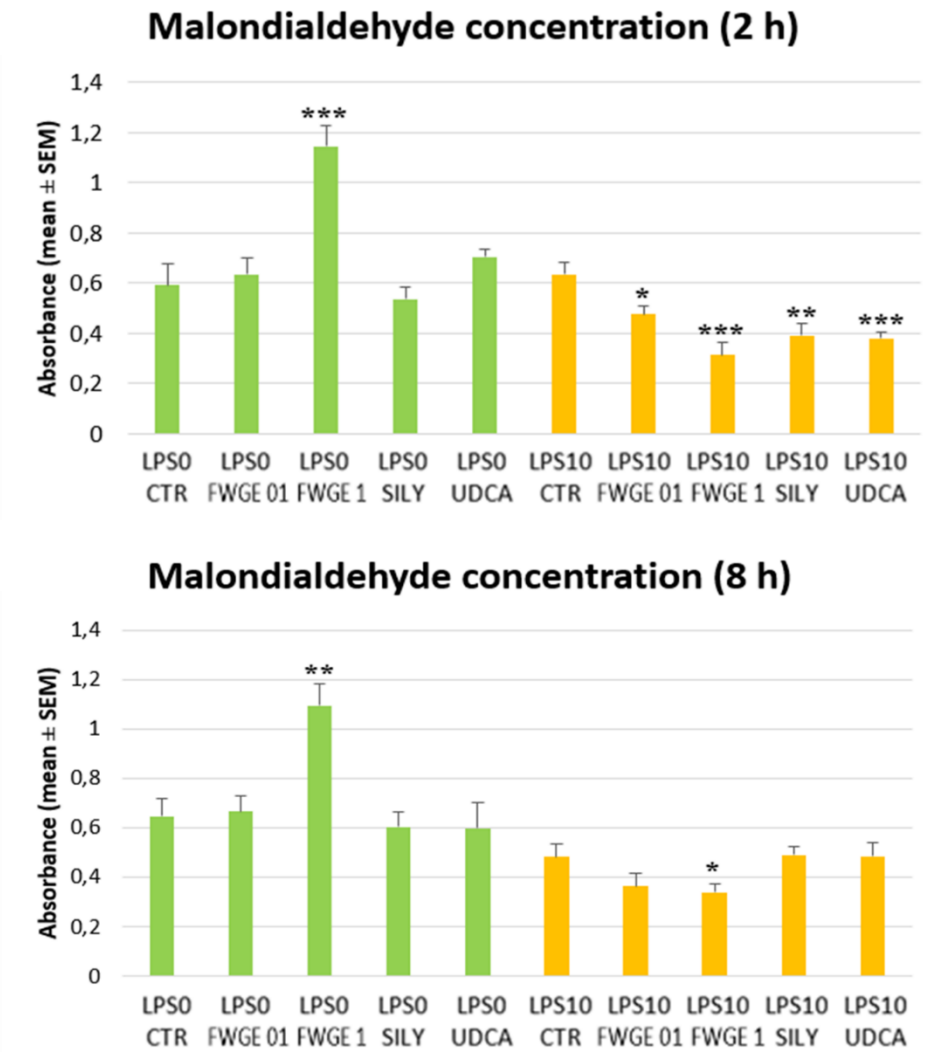


Figure 21. Malondialdehyde concentration measured in the cell culture media after 2 h (**A**) and 8 h (**B**) incubation. LPS 0 = cultures with no LPS exposure, LPS 10 = LPS treated cultures (10 μg/mL); FWGE 01 = 0.1% FWGE, FWGE 1 = 1% FWGE, SILY = silymarin (50 μg/mL), UDCA = ursodeoxycholic acid (200 μg/mL). Mean ± SEM, *P<0.05, **P<0.01, ***P<0.001.

The activity of glutathione peroxidase enzyme of the lysed cells was monitored after 8 h incubation. As the effect of LPS challenge, enzyme activity was significantly elevated (P<0.001; **Figure 22**). Both of the applied FWGE concentrations along with the silymarin and UDCA incubation decreased the activity of glutathione peroxidase in the cell cultures without LPS treatment (0.1% FWGE, silymarin and UDCA: P<0.001; 1% FWGE: P=0.004; **Figure 22**). In LPS exposed cells, only the UDCA treatment was able to decrease the glutathione peroxidase activity in a significant manner (P=0.002; **Figure 22**).

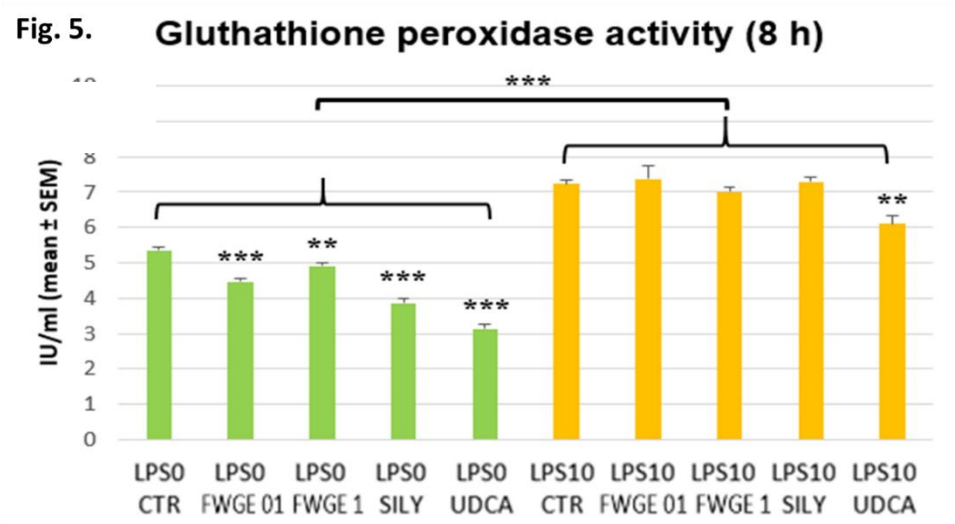


Figure 22. Glutathione peroxidase activity of the cultured cells after 8 h incubation. LPS 0 = cultures with no LPS exposure, LPS 10 = LPS treated cultures (10 μg/mL); FWGE 01 = 0.1% FWGE, FWGE 1 = 1% FWGE, SILY = silymarin (50 μg/mL), UDCA = ursodeoxycholic acid (200 μg/mL). Mean ± SEM, **P<0.01, ***P<0.001.

Study 6. The role of small heat shock proteins in the modulation of redox homeostasis under acute heat stress in chickens

Cloacal temperatures were elevated after both 4 h and 8 h heat exposure times (P<0.001 in both groups, respectively; **Figure 23**) compared to controls.

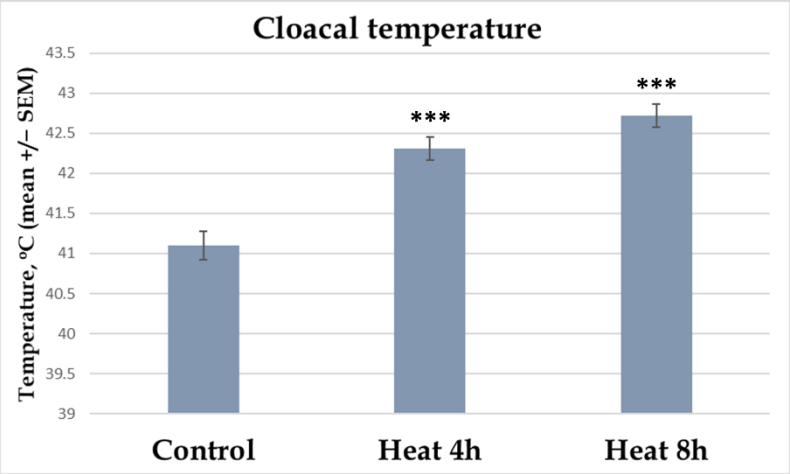


Figure 23. Cloacal temperatures registered following treatments; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. ***P < 0.001.

Hepatic MDA concentration was higher (P=0.020) after 8 h heat exposure compared to that of the control group; however, no difference was found between 4 h stressed and control chickens (**Figure 24.A**). No significant changes were either detected in correlation with any of the heat stressed and control groups in the spleen and kidney (**Figure 24.B-C**).

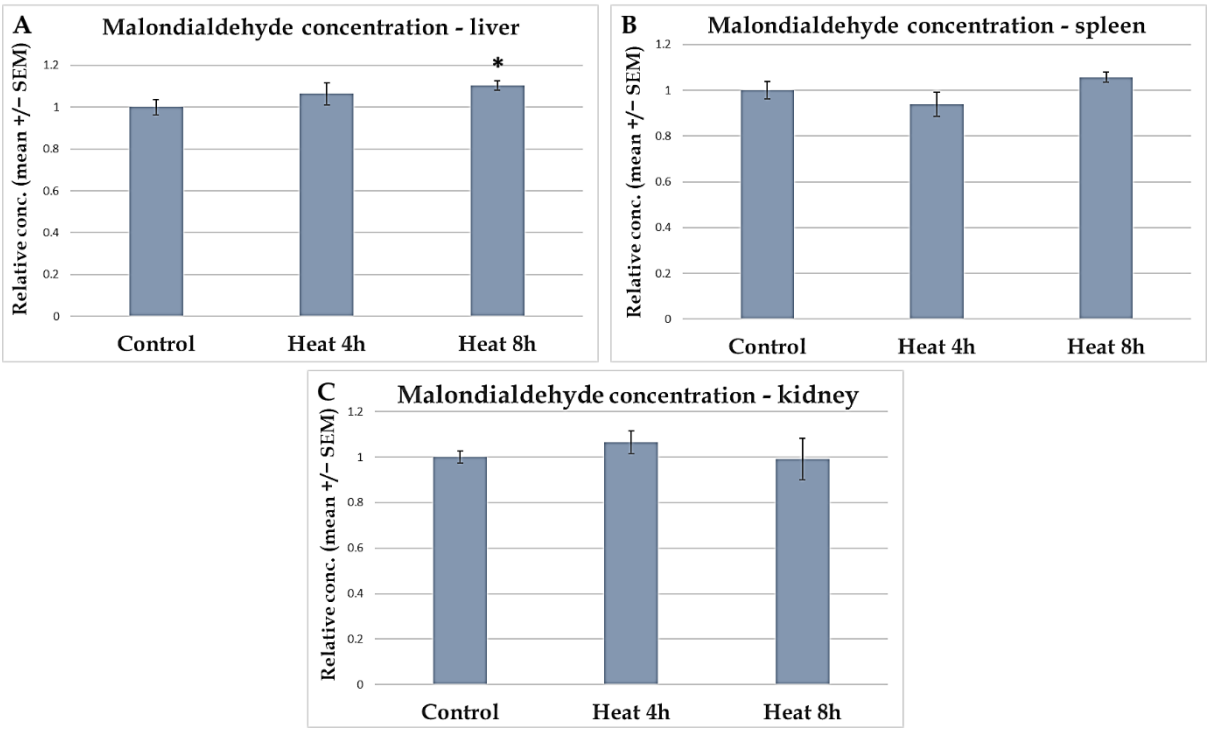


Figure 24. Malondialdehyde (MDA) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C)**. "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis. Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. *P < 0.05.

Comparing to control, heat exposure for both time durations caused decrease in GSH concentrations in the liver of chickens (**Figure 25.A**; 4 h treatment: P=0.044, 8 h treatment: P<0.001, respectively). However, no alterations were found in the spleen or kidney (**Figure 25.B-C**). There was a significant, more than 2-fold increase of glutathione peroxidase activity observed in the liver (P=0.002) after 8 h heat exposure (**Figure 26.A**). Meanwhile, after the same incubation time, decreased enzyme activity was found in the kidney (P<0.001; **Figure 26.C**). Regarding glutathione peroxidase activity in the spleen, no significant differences were observed (**Figure 26.B**).

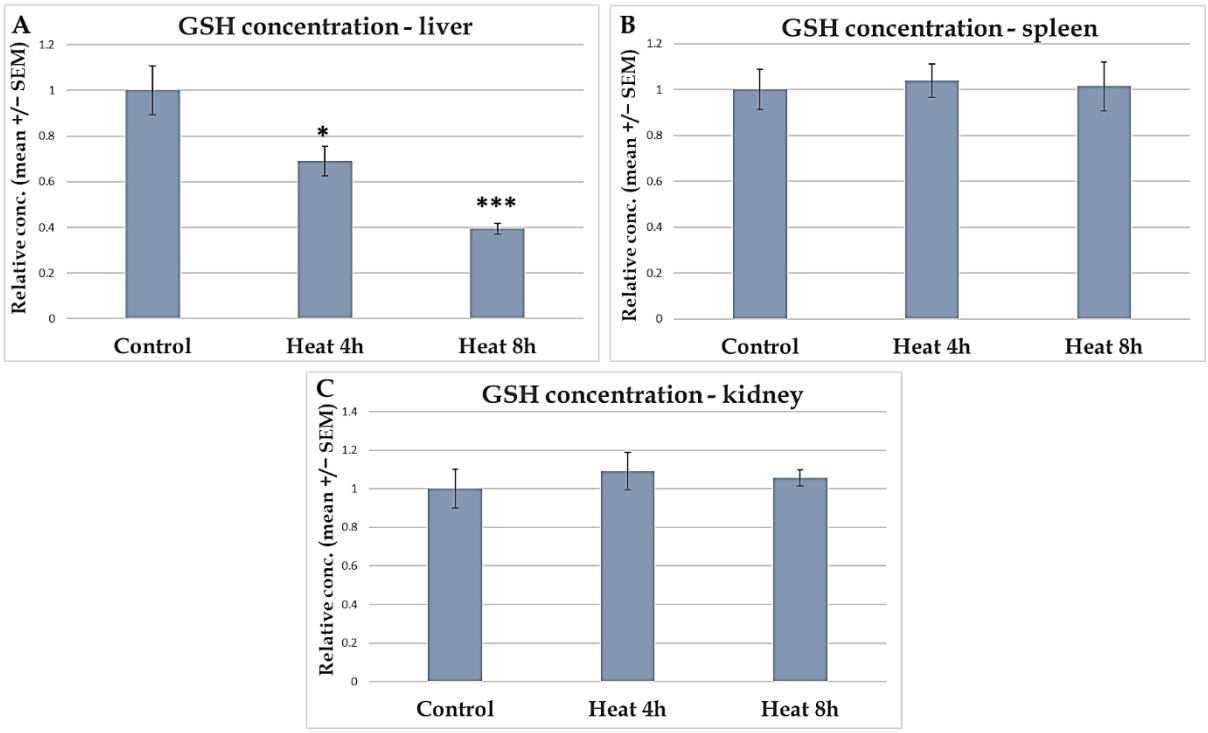


Figure 25. Reduced glutathione (GSH) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C).** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis. Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. *P < 0.05, ***P < 0.001.

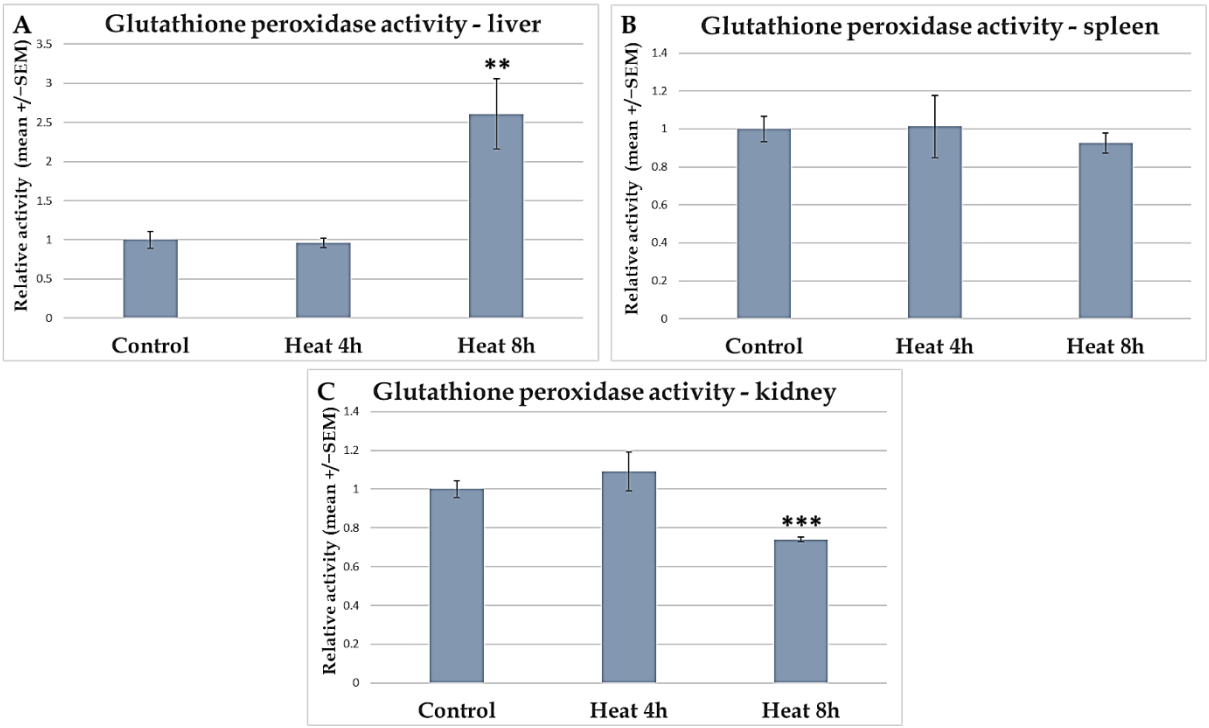


Figure 26. Glutathione peroxidase (GPx) activities of liver **(A)**, spleen **(B)**, and kidney **(C).** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis. Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. **P < 0.01, ***P < 0.001.

Hepatic protein carbonyl concentrations were decreased after both treatment periods (**Figure 27.A**; 4 h treatment: P=0.016, 8 h treatment: P<0.001, respectively); however, decreased protein carbonylation was observed in the spleen, but only in case of 8 h incubation time (P=0.049). No significant change was detected in the kidney (**Figure 27.B, 27.C**).

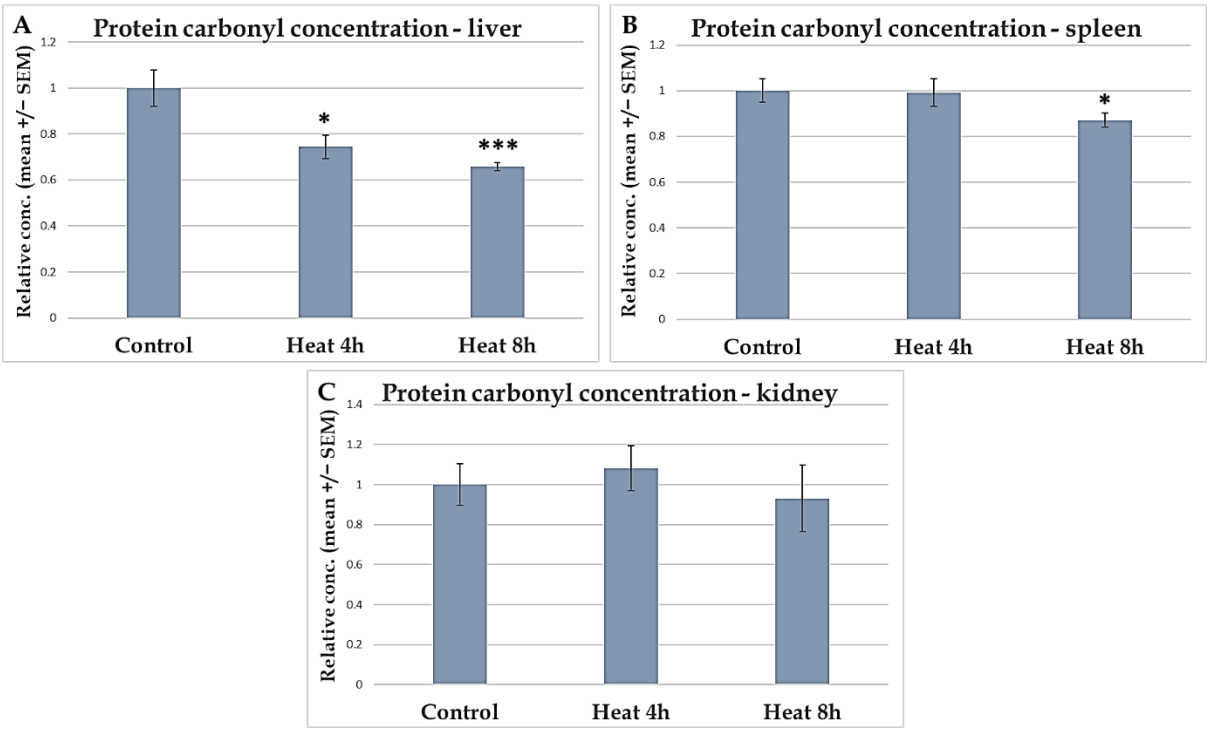


Figure 27. Protein carbonyl concentrations of liver **(A)**, spleen **(B)**, and kidney **(C).** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis. Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. *P < 0.05, ***P < 0.001.

No significant changes were found in the HSP27 concentration after any incubation time in any of the investigated organs (**Figure 28**). Relative to control, lower αA-crystallin concentrations were observed in the liver after both 4 h (P=0.040) and 8 h (P=0.005) duration of heat stress (**Figure 29.A**), while no changes were found in the other two organs (**Figure 29.B-C**). Similarly to the findings of αA-crystallin, decreased αB-crystallin concentrations were observed after both 4 h and a 8 h heat exposure in the liver, respectively (P<0.001 in both cases; **Figure 30.A**), while no changes were detected in the other two organs tested (**Figure 30.B-C**). According to the Pearson's correlation test, a significant positive correlation was found between hepatic αA-crystallin and GSH (P<0.001), and between αA-crystallin and protein carbonyl (P = 0.048) levels. A similar correlation was observed between αB-crystallin and GSH (P<0.001), and between αB-crystallin and protein carbonyl (P<0.001) concentrations in the liver (**Table 2**).

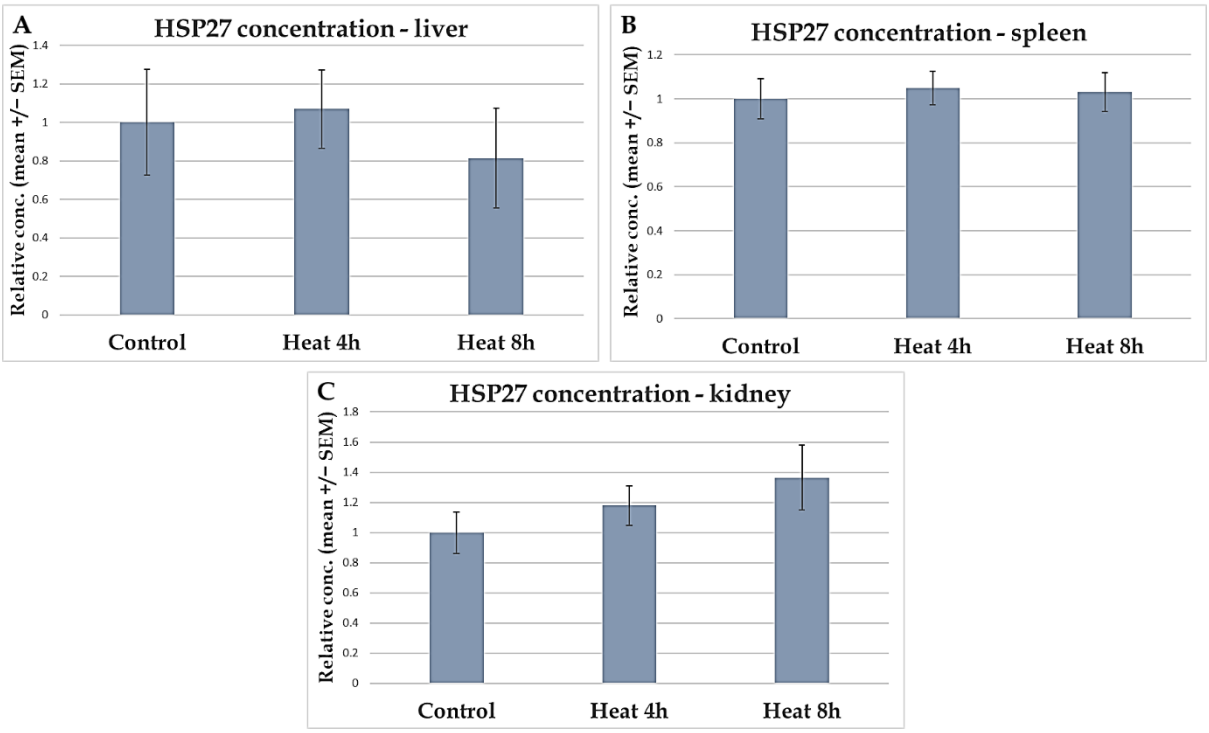


Figure 28. HSP27 (HSPB1) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C).** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis. Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups.

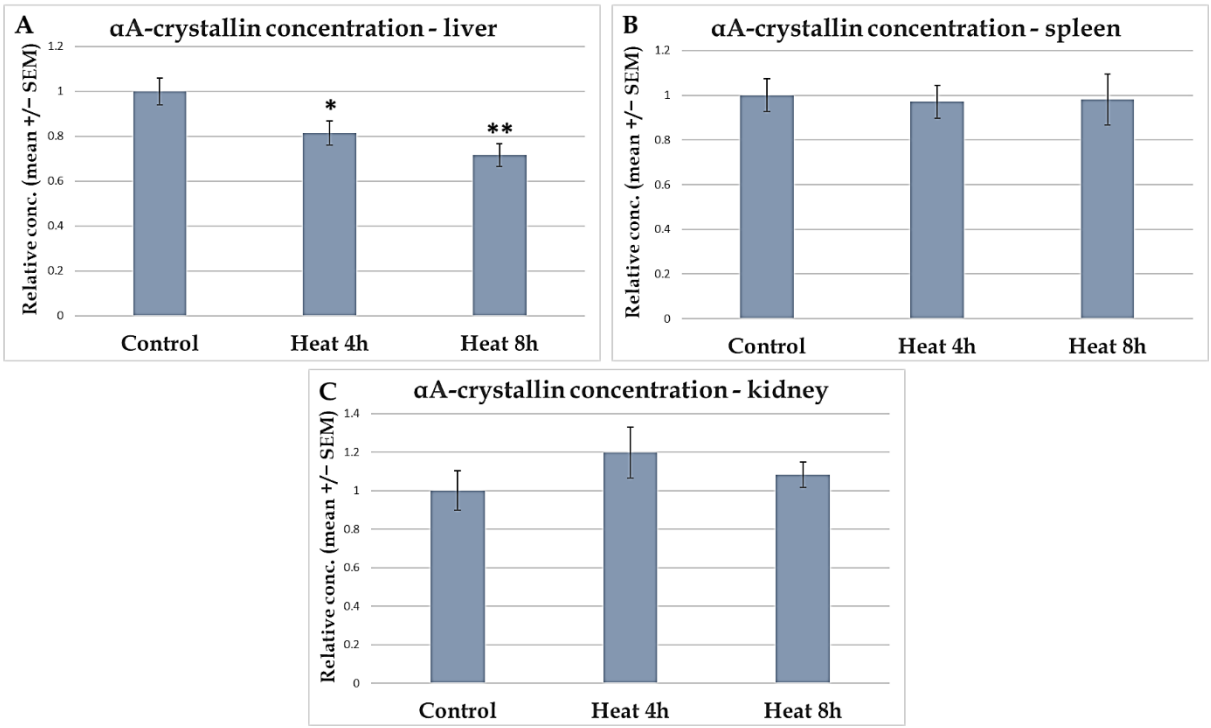


Figure 29. αA-crystallin (CRYAA; HSPB4) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C).** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Relative concentrations were calculated by considering the mean value of control group as 1. Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. *P < 0.05, **P < 0.01.

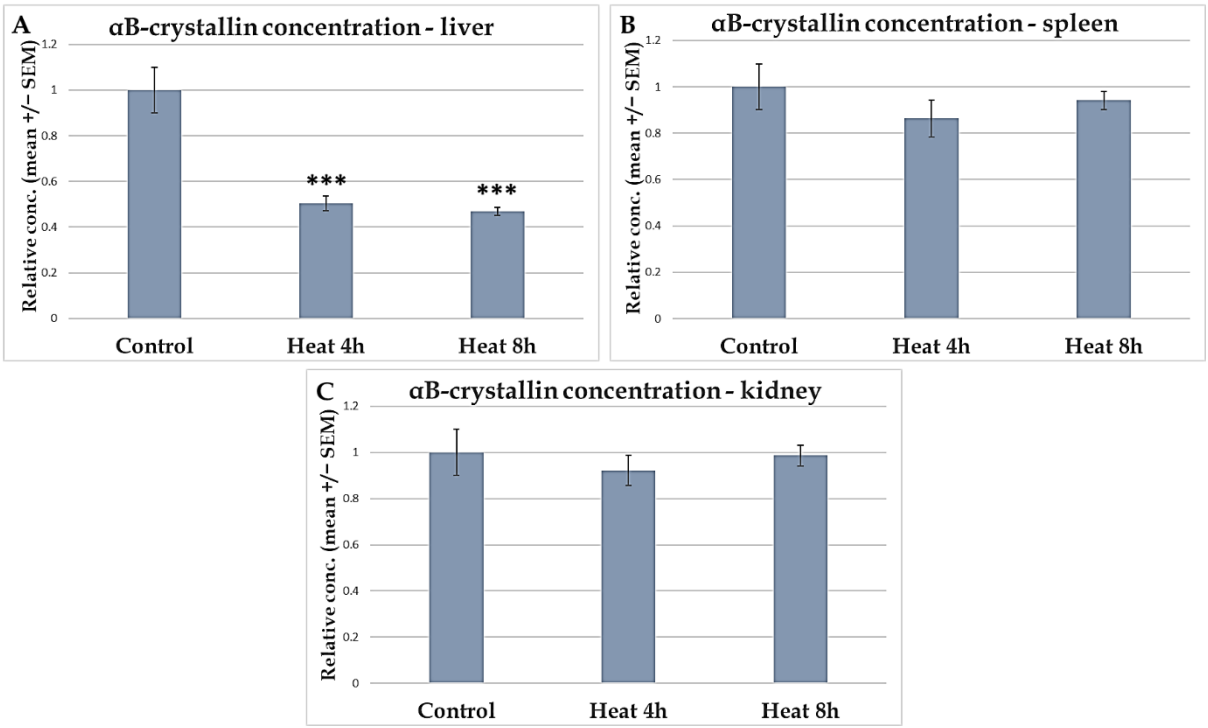


Figure 30. αB-crystallin (CRYAB; HSPB5) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C).** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity vs. 22°C for controls). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis. Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. ***P < 0.001.

Analyzed factors	Pearson correlation coefficient (r)	p-value
αB-crystallin + GSH	0.727	<0.001
αB-crystallin + carbonyl	0.698	<0.001
αA-crystallin + GSH	0.728	<0.001
αA-crystallin + carbonyl	0.423	0.048

Table 2. Correlation coefficients and P values as gained by Pearson's correlation test between hepatic α-crystallin and glutathione (GSH) concentrations, and between α-crystallin and protein carbonyl contents.

IV. Conclusions

In the frame of the project, novel primary hepatic cell culture models have been successfully established from chickens. Based on the investigation of the separated cell fractions with flow cytometry and on the immunofluorescent characterization of cultured cells, hepatocyte mono-cultures and hepatocyte – NP cell co-cultures have been prepared from chicken liver. These chicken cell cultures enable studies concerning the specific role of parenchymal and NP cells as the main liver cell fractions, and the co-culture model can mimic various inflammatory states by setting different cell type ratios. The applied ratio of 6:1 (hepatocytes to NP cells) refers to a milder hepatic inflammation with moderate intrahepatic macrophage migration (Mátis et al., 2016). On this co-culture, the interaction of the inflammatory and stress response can be studied, including molecular alterations of cell function, such as the pro- and anti-inflammatory cytokine production and the redox homeostasis of the cultured liver cells. The main advantage of these models is that they are non-tumorigenic primary cell cultures; hence the results can be better extrapolated to the *in vivo* conditions of the healthy or inflamed chicken liver.

When studying the effects of acute heat stress *in vitro*, shorter heat stress applied for 1 h could strongly influence liver cell function by significantly increasing catabolic metabolism and extracellular H_2O_2 release, and by significantly decreasing HSP70, IL-6 and IL-8 production on both cell culture models. However, all these alterations were restored after 2 h heat exposure, indicating a fast recruitment of liver cells. Hepatocyte mono-cultures and hepatocyte – NP cell co-cultures responded to heat stress on a similar manner, but the higher metabolic rate of co-cultured cells may have contributed to a better capability of inflamed liver cells for accommodation to stress conditions. The results of this study highlight the impact of short term heat stress on the liver in chickens, underline the mediatory role of oxidative stress in acute stress response and suggest a fast cellular adaptation potential in liver cells.

The established cell cultures derived from chicken were found to be proper models for short-term toxicological studies, for instance to study the specific molecular effects of acute T-2 toxin exposure. The toxin could strongly diminish the function of chicken liver cells, reflected by decreased metabolic rate, and triggered an inflammatory response by increasing pro-inflammatory cytokine and HSP70 production. However, no changes were found in the extracellular H_2O_2 levels, which can suggest that ROS production may not play a key mediatory role in the cytotoxic effects of T-2 toxin on chicken liver. In conclusion, our study provided novel data concerning the hepatic action of T-2 toxin, highlighting the molecular mechanisms and emphasizing the potential hazards of T-2 toxin in poultry farming.

As a further methodological improvement, 3D co-cultures were successfully prepared from freshly isolated chicken hepatocytes and NP cells by forming spheroids with magnetic bioprinting. The main advantage of this innovative 3D model is its suggested better extrapolability to the *in vivo* conditions based on the direct intercellular contacts, and that they can be effectively maintained for longer time compared to 2D cultures. The potential pro-inflammatory action of several pathogen-associated molecular patterns was studied on both 2D and 3D cell culture models, and remarkably differing responses were observed. Both LPS and LTA type bacterial endotoxins modulated cellular metabolic state and exerted immunomodulatory action on 3D cultures reflected by increased metabolic activity and decreased IL-8 production, respectively, while no significant alterations were found on 2D cultures. In contrast, the applied enterotoxin, PMA and Poly I:C triggered a notable response on 2D cultures, where enterotoxin stimulated, while PMA and Poly I:C depressed cellular catabolism. The viral RNA model Poly I:C evoked pro-inflammatory cytokine production by elevating IL-6 production on 3D and IL-8 release on 2D co-cultures. Based on these data, the

tested potential microbial compounds might affect hepatic metabolism, but they could only partly modulate hepatic innate immunity. The established 3D co-cultures seemed to be proper tools for testing potential pro-inflammatory molecules; however, the remarkable differences between 2D and 3D models should be addressed and further studied. These results serve as a good basis for our planned investigations in the future, targeting potential anti-inflammatory candidates.

In line with these future aims, the redox modulatory role of FWGE was tested on primary cultures of rat hepatocytes, serving as a model for further animal species. Based on our results, it can be stated that FWGE applied in appropriate concentrations did not possess cytotoxic effects in hepatocyte cultures. The FWGE effectively decreased the ROS production of cultured cells and the consecutively occurring lipid peroxidation in case of LPS triggered inflammation. Notwithstanding that an incidental pro-oxidant activity of FWGE was observed when applied in the higher (0.1%) concentration, it did not lead to oxidative distress based on assessing the activity of the glutathione antioxidant system. This observation may refer to the prooxidant activity of high dose FWGE, which is an important beneficial effect regarding tumor cells. In conclusion, FWGE as a redox modulator can provide good possibilities in alleviating inflammation associated oxidative distress, preventing cell destruction and hence improving general health condition.

Based on the *in vitro* results related to immunomodulatory stressors, the molecular effects of acute heat stress were investigated from a novel point of view in various tissues of broiler chickens in the final, *in vivo* trial. Among the examined parenchymal organs, liver was found the most susceptible to heat stress-triggered oxidative damage. However, this sensitivity was coupled to the rapid activation of hepatocellular protective mechanisms, including the distinct increase of antioxidant capacity driven by glutathione peroxidase and the excessive utilization of αA- and αB-crystallin proteins. These sHSP-s are presumed to play a key role in the acute hepatic heat stress response in chickens, while HSP27 seemed not to be strongly involved in the compensatory mechanisms. The observed heat-associated decline of protein carbonylation in the liver occurred in correlation with the highly increased utilization of αA- and αB-crystallins, resulting in an overcompensation mechanism. A similar correlation was also found between GSH and crystallin levels, suggesting further relations between the glutathione system and certain sHSP-s. The good adaptation potential of liver cells to stress conditions was also reflected by the finding that – despite of the high hepatic sensitivity to oxidative damage – mildly enhanced lipid peroxidation was detected only after the longer 8 h heat exposure. This study provided novel data regarding the heat stress response of broiler chickens, highlighting the oxidative susceptibility and effective adaptation mechanisms of the liver, and elucidating the specific role of sHSP-s in the restoration of physiological cell function under oxidative distress.

In conclusion, the established novel hepatic cell culture models provided great tools for assessing the metabolic, oxidative and inflammatory effects of several immunomodulatory stressors, such as elevated environmental temperature, mycotoxins and pathogen-associated molecular patterns on the chicken liver. Based on the results obtained, it was confirmed that the investigated environmental and nutritional factors can modulate the hepatic immune response, sensitizing the organism to concomitant diseases and possibly contributing to multifactorial health concerns in broiler chickens. The present project provided new models for studying the molecular mechanisms of the hepatic stress response, delivered relevant details to the pathomechanism of acute heat stress, T-2 toxicosis and inflammation triggered by certain microbial byproducts, and also highlighted the complex interplay of the metabolic, redox and inflammatory homeostasis in chickens.

V. List of papers published in the frame of the project

1. Papers in peer-reviewed journals:

Gábor Mátis, Anna Kulcsár, Máté Mackei, Janka Petrilla, Zsuzsanna Neogrády: ***Comparative study on the modulation of incretin and insulin homeostasis by butyrate in chicken and rabbit***, PloS ONE, 13(10): e0205512, 2018.

Ádám Kurucz, Kata Orbán, Máté Mackei, Hedvig Fébel, Zsuzsanna Neogrády, Gábor Mátis: ***Investigations on hepatic and intestinal drug-metabolizing cytochrome P450 enzymes in in wild boar compared to domestic swine***, European Journal of Wildlife Research, 66, 8., 2020.

Máté Mackei, Andor Molnár, Szabolcs Nagy, László Pál, Csaba Kővágó, Péter Gálfi, Károly Dublecz, Ferenc Husvéth, Zsuzsanna Neogrády, Gábor Mátis: ***: Effects of acute heat stress on a newly established chicken hepatocyte – non-parenchymal cell co-culture model***, Animals, 10, 409., 2020.

Máté Mackei, Kata Orbán, Andor Molnár, László Pál, Károly Dublecz, Ferenc Husvéth, Zsuzsanna Neogrády, Gábor Mátis: ***: Cellular effects of T-2 toxin on primary hepatic cell culture models of chickens***, Toxins, 12, 46., 2020

Máté Mackei, Júlia Vörösházi, Csilla Sebők, Zsuzsanna Neogrády, Gábor Mátis, Ákos Jerzsele: ***Fermented wheat germ extract as a redox modulator: alleviating endotoxin-triggered oxidative stress in primary cultured rat hepatocytes***, Oxidative Medicine and Cellular Longevity, vol. 2020, 3181202., 2020.

Máté Mackei, Gábor Mátis, Andor Molnár, László Pál, Csilla Sebők, Júlia Vörösházi, Károly Dublecz, Ferenc Husvéth, Zsuzsanna Neogrády: ***The role of small heat shock proteins in the regulation of redox homeostasis in acute heat shock response in chicken***, Journal of Thermal Biology, submitted, 2021.

Réka Fanni Barna, Máté Mackei, Erzsébet Pászti-Gere, Zsuzsanna Neogrády, Ákos Jerzsele, Gábor Mátis: ***The effects of matriptase inhibition on the inflammatory and redox homeostasis of chicken hepatic cell culture models***, Biomedicines, submitted, 2021.

2. Papers in conference proceedings:

Gábor Mátis, Anna Kulcsár, Patrícia Hatala, Máté Mackei, Dániel Balogh, Zsuzsanna Neogrády: ***Investigations on the effects of heat stress on hepatic cell culture models of chicken origin***, XVth European Poultry Conference, Dubrovnik, 2018.

Máté Mackei, Kata Orbán, Andor Molnár, Károly Dublecz, Zsuzsanna Neogrády, Gábor Mátis: ***Investigation of the cellular effects of T-2 toxin on hepatic cell culture model of chicken origin***, Poster 107: 23rd Congress of the European Society of Veterinarian and Comparative Nutrition, 2019, Torino, Italy, 2019.

Patrícia Hatala, Andrea Lajos, Kata Orbán, Gábor Mátis, Zsuzsanna Neogrády: ***: Investigating the inflammatory effect of norepinephrine in a feline primary urinary bladder cell culture***, ISFM European Feline Congress, 2019, Cavtat, Croatia, 2019.

Mackei Máté, Mátis Gábor, Molnár Andor, Kulcsár Anna, Hatala Patrícia, Nagy Szabolcs, Dublecz Károly, Husvéth Ferenc, Neogrády Zsuzsanna: ***Immunmoduláló faktorok vizsgálatára alkalmas hepatikus sejtmodellek kialakítása csirkében***, MTA Akadémiai beszámolók, 2019. január, Budapest

Mackei Máté, Mátis Gábor, Molnár Andor, Pál László, Dublecz Károly, Husvéth Ferenc, Neogrády Zsuzsanna: ***A hőstressz akut hatásainak vizsgálata csirke eredetű hepatocita mono- és hepatocita – nem-parenchimális sejt ko-kultúrán***, MTA Akadémiai beszámolók, Budapest, 2020. január

Mackei Máté, Mátis Gábor, Vörösházi Júlia, Molnár Andor, Pál László, Dublecz Károly, Husvéth Ferenc, Neogrády Zsuzsanna: ***A T-2 toxin sejtszintű hatásainak vizsgálata különböző csirke eredetű primer májmodelleken***, MTA Akadémiai beszámolók, Budapest, 2020. január

Máté Mackei, Szabolcs Nagy, Andor Molnár, Ferenc Husvéth, Károly Dublecz, Zsuzsanna Neogrády, Gábor Mátis: ***Effects of heat stress on recently established primary hepatocyte – non-parenchymal cell mono- and co-culture models of chicken origin. World's Poultry Congress, 2020,***, World's Poultry Congress, 2020, Paris, France, 2020.

Máté Mackei, Andor Molnár, Husvéth Ferenc, Károly Dublecz, Zsuzsanna Neogrády, Gábor Mátis, Hedvig Fébel: ***Investigation of the effects of T-2 toxin on primary chicken hepatocyte mono- and hepatocyte – non-parenchymal cell co-culture models***, World's Poultry Congress, 2020, Paris, France, 2020.

Máté Mackei, Andor Molnár, László Pál, Károly Dublecz, Ferenc Husvéth, Gábor Mátis, Zsuzsanna Neogrády: ***Effects of heat stress on chicken-derived primary hepatic cell culture models***, 62. Georgikon Napok, Szent István Egyetem, Georgikon Kar, Keszthely, 2020. október

Mackei Máté, Mátis Gábor, Sebők Csilla, Vörösházi Júlia, Molnár Andor, Pál László, Dublecz Károly, Husvéth Ferenc és Neogrády Zsuzsanna: ***A hőstressz redox homeosztázisra gyakorolt hatásainak vizsgálata csirkében***, MTA Akadémiai beszámolók, Budapest, 2021. január

Sebők Csilla, Vörösházi Júlia, Mackei Máté, Tráj Patrik, Szentgyörgyi Ákos, Neogrády Zsuzsanna és Mátis Gábor: ***Bakteriális sejtfalkomponensek hatásainak vizsgálata két-, illetve háromdimenziós csirke eredetű májsejttenyészeteken***, MTA Akadémiai beszámolók, Budapest, 2021. január

Vörösházi Júlia, Mackei Máté, Sebők Csilla, Neogrády Zsuzsanna, Mátis Gábor és Jerzsele Ákos: ***Fermentált búzacsíra-kivonat redox homeosztázisra gyakorolt hatásának vizsgálata patkány eredetű primer májsejttenyészeteken***, MTA Akadémiai beszámolók, Budapest, 2021. január

3. PhD theses:

Mackei Máté: Immunszuppresszív hatások vizsgálata csirke hepatocyte – Kupffer-sejt ko-kultúrán. Állatorvostudományi Doktori Iskola, Budapest

Sebők Csilla: Az antimikrobiális peptidek gyulladáscsökkentő hatásának vizsgálata csirke eredetű primer máj- és bélhámsejt tenyészeteken. Állatorvostudományi Doktori Iskola, Budapest

4. DVM theses:

Lajos Andrea: A noradrenalin gyulladáskeltő hatásának vizsgálata macska eredetű primer húgyhólyaghámsejt-tenyészeten. Budapest, 2018.

Sebők Csilla: A chicken heterophil peptide-1 gyulladáscsökkentő hatásának vizsgálata csirke eredetű májsejttenyészeteken. Budapest, 2019.

Szentgyörgyi Ákos: Bakteriális sejtfalkomponensek hatásainak összehasonlítása két-, illetve háromdimenziós csirke eredetű májsejttenyészeteken. Budapest, 2020.

Folkmann, Lars August: A hőstressz hatásainak vizsgálata újonnan kialakított csirke eredetű hepatocita – nem-parenchimális sejt ko-kultúrán. Budapest, 2020.

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Steiger, Katharina: A fermentált búzacsíra-kivonat májvédő hatásának vizsgálata patkány eredetű primer májsejttenyészeten. Budapest, 2020.

Vaktor Nelli: A T-2 toxin hatásainak vizsgálata csirke eredetű hepatikus sejtmodelleken. Budapest, 2020.

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