Theses of doctoral (PhD) dissertation

Study of immunomodulatory factors in chicken hepatic cell culture models

Máté Mackei, DVM

Supervisors: Dr. Neogrády Zsuzsanna
Dr. Mátis Gábor

UNIVERSITY OF VETERINARY MEDICINE
Veterinary Doctoral School

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Supervisors:

.....

Dr. Zsuzsanna Neogrády CSc,
Division of Biochemistry, Department of Physiology and Biochemistry
University of Veterinary Medicine, Budapest, Hungary
Supervisor

.....

Dr. Gábor Mátis PhD,
Division of Biochemistry, Department of Physiology and Biochemistry
University of Veterinary Medicine, Budapest, Hungary
Supervisor

.....

Dr. Máté Mackei

Introduction

Various environmental and nutritional factors can contribute to immunomodulation in poultry farming, resulting in negative effects on both animal health and productivity as well as affecting the cellular inflammatory and stress response. Based on results of current research projects it can be stated that by virtue of global climate change, humankind together with the whole ecosystem is facing to challenges, which have not been seen in a long time on Earth. According to recent meteorological models and already available data, by reason of human contribution and industrialization, severe alterations in weather conditions, exceptional and repeatedly occurring heat waves, unequal rainfall distribution, more intense precipitation extremes along with serious droughts are all likely to happen more frequently in the future than before. These conditions can affect our agriculture seriously, including the repeated exposure of livestock to heat stress and the more commonly occurring contamination of food and feed by molds which are able to produce harmful mycotoxins. Both heat stress and mycotoxin exposure are among the major concerns in broiler chickens, severely deteriorating animal welfare, resulting in production loss and making the animals more sensitive to complex, multifactorial diseases. Notwithstanding the fact that liver is highly susceptible to the aforementioned stressors and also plays central role in maintaining the metabolic and oxidative homeostasis, the exact, cellular effects of heat stress and mycotoxins on the chicken liver are not yet entirely elucidated.

Heat stress can lead to dysfunction in various organs including the liver, spleen, or kidney. 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. Therefore, more appropriate understanding of heat stress-related cellular consequences may help to successfully alleviate its harmful effects as well as contribute to the development of novel nutritional strategies and to the targeted application of protective agents, such as certain feed additives in broiler farming.

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. 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. 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.

As high temperature is considered to impair protein stability, maintaining the physiological conformation of proteins and preventing the aggregation of non-native proteins are especially

important. Heat-shock proteins (HSPs) 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. Among widely investigated heat shock proteins such as HSP70 and HSP90, the so-called small heat shock proteins (sHSPs) also belong to the highly relevant group of molecular chaperones, contributing to the efficient cellular adaptation to different stress conditions, but their exact role in restoring cell function in heat stressed chickens remained mostly unclear. As an immunomodulatory factor, heat stress was also reported to cause functional changes in the immune response by altering the gene expression and concentration of various pro-inflammatory cytokines in chicken.

The contamination of the feed with mycotoxins has also an exceptional importance in intensive poultry farming. The T-2 toxin as one of the most noxious members of trichothecenes can provide a serious hazard in broiler nutrition as well, threating both animal and human health by contaminating the food chain. Avian species are relatively tolerant to mycotoxins 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.

According to the majority of studies, T-2 toxin significantly increases the level of ROS and induces changes in the antioxidant status of the cells, while in other cases beside the intensive cellular damage, oxidative stress was not detected. Furthermore, some HSPs, such as HSP70, can show a correlation with the cytoprotective mechanisms against different toxic effects; however, data addressing the effects of trichothecenes on influencing HSP expression are limited.

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

Aims of the study

Summarized, the most important aims of this PhD study were:

Ad 1, to develop and characterize a novel, unique primary hepatocyte – non-parenchymal cell (predominantly Kupffer cell) co-culture from chicken origin.

Ad 2, to examine the possible metabolic, oxidative and immunomodulatory effects of acute heat stress *in vitro*, applying the established cell culture models.

Ad 3, to investigate the T-2 toxin triggered hepatic damage *in vitro*, including the monitoring of metabolic activity, interleukin (IL) production and redox homeostasis.

Ad 4, to gather information about the *in vivo* cellular consequences of acute heat exposure in parenchymal organs such as in the liver, spleen or kidney of chickens with special emphasis on oxidative stress and the importance of sHSPs.

In order to fulfil the abovementioned criteria, the below described study plan has been followed:

Study No.	Applied cell cultures/animals	Main scientific question	Laboratory analyses (measured parameters)
Study I.	Chicken primary hepatocyte mono-culture and hepatocyte – NP cell co-culture (*in vitro*)	Development and characterization of novel chicken hepatic cell culture models	Characterization with immunocytochemistry and flow cytometry
Study II.	Chicken primary hepatocyte mono-culture and hepatocyte – NP cell co-culture (*in vitro*)	The effects of acute heat stress on chicken hepatic cell cultures	Metabolic activity, extracellular LDH activity, extracellular H_2O_2, HSP70, IL-6 and IL-8 concentrations
Study III.	Chicken primary hepatocyte mono-culture and hepatocyte – NP cell co-culture (*in vitro*)	The effects of T-2 toxin on chicken hepatic cell cultures	Metabolic activity, extracellular H_2O_2, HSP70, IL-6 and IL-8 concentrations
Study IV.	Broiler chickens (*in vivo*)	The role of small heat shock proteins in chickens under acute heat stress	MDA, protein carbonyl, glutathione, HSP27, αA-crystallin, αB-crystallin concentration and glutathione-peroxidase activity (liver, spleen, kidney samples)

Materials and methods

Establishment and characterization of novel chicken-derived primary hepatocyte mono-culture and hepatocyte – NP cell co-culture models (Study I.)

In **Study I.**, liver cells were freshly isolated from three-week-old male broiler chickens of the Ross 308 strain. After decapitation, the liver was perfused via the gastropancreaticoduodenal vein of the hepatic portal system. Following multi-phase perfusion and digestion by collagenase, the organ has been excised, capsule was disrupted. In order to isolate hepatocytes and NP cells from the primary cell suspension, multi-step differential centrifugation has been carried out.

Cell cultures were prepared on cell culture dishes, previously coated with collagen type I. 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 (hepatocytes to NP cells). Hepatocyte mono-cultures were also prepared by seeding hepatocyte-enriched fraction onto cell culture 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.

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 the different cell culture models, immunocytochemical analyses were carried out using chicken specific antibodies. Albumin was detected with a chicken specific, fluorescein isothiocyanate (FITC) coupled anti-albumin antibody. Macrophages in the NP cell fraction were labelled by using a chicken macrophage specific phycoerythrin (PE) coupled antibody. Furthermore, hepatocyte enriched and NP cell containing fractions were also examined and characterized with flow cytometry.

Cellular effects of heat stress and T-2 toxin on hepatic cell cultures of chicken origin *in* vitro (Study II. and III.)

In **Study II.** and **III.** cell isolation process and culturing conditions have been carried out in accordance with the developed methodology explained in **Study I.** In **Study II.**, following 24 h culturing, confluent mono- and co-cultures were incubated at 43°C for 1 or 2 h to mimic heat stress, while control cells were incubated further at 38.5°C. In **Study III.**, cell cultures were challenged to T-2 toxin in different concentrations. Williams' medium E was supplemented with 0 (control), 10, 100, or 1000 nmol/L T-2 toxin. Treatment with toxin containing media lasted either for 8 or for 24 h, respectively.

Following treatments, samples were taken from culture media of the 6-well plates in both studies after incubation times and cells were lysed using Mammalian Protein Extraction

Reagent (M-PER). Samples were stored until further analysis at −80°C. In order to gather information about the cellular effects of the tested treatments, metabolic activity was measured by CCK-8 test and the level of necrosis has been determined using LDH leakage test. Extracellular H_2O_2 concentration was detected by Amplex Red method and HSP70, IL-6 and IL-8 levels were monitored using chicken specific sandwich ELISA tests.

Consequences of acute heat stress in parenchymal organs of chickens in vivo (Study IV.)

One-day-old male Ross 308 broiler chicks were group-housed on wheat straw litter in floor pens. Climatic conditions and housing were established according to the requirements of the Ross technology over the entire study, except the day of the treatment.

Three-phase fattening was used providing feed and drinking water *ad libitum,* in strict accordance with the instructions of the breeder. Housing and treatment of the chickens were also carried out in strict accordance with the national and international laws as well as with the institutional guidelines. 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, 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. In the control group, climatic conditions remained unchanged and corresponded completely with the breeder's recommendations (22 ± 1°C). Following treatments, 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.

Before the laboratory analyses tissue samples were thawed on ice, then homogenized in T-PER lysis buffer by Potter-Elvehjem tissue homogenizer. Homogenates were centrifuged at 5,000x g for 10 min, and supernatants were used for further measurements. Total protein concentration of homogenates was assayed by Pierce Bicinchoninic Acid (BCA) Protein Assay. Concentrations of MDA, protein carbonyls and reduced glutathione as well as glutathione peroxidase activity have been determined by specific colorimetric tests. Further, HSP27, αA-crystallin and αB-crystallin levels have been measured using chicken specific ELISA assays.

Statistics

Differences between various groups were assessed using one-way analysis of variance (ANOVA) and Dunnett's post hoc tests for pairwise comparisons. Relationships between different variables were assessed using Pearson's correlation test. Differences were considered significant at $P < 0.05$.

Results and discussion

Establishment and characterization of novel chicken-derived primary hepatocyte mono-culture and hepatocyte – NP cell co-culture models (Study I.)

In the present study novel primary hepatic cell culture models have been successfully established and applied from chicken origin (**Study I.**). Based on the investigations 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. As justified by immunocytochemistry, the NP cell fraction comprised of mainly macrophages, predominantly Kupffer cells as the resident liver macrophages, and presumably also circulation-derived macrophages. However, the presence of other NP cell types, such as stellate cells or biliary endothelial cells can be also suggested.

According to our recent knowledge, no similar liver model has yet been prepared of chicken origin. Therefore, the newly prepared cell culture models enable to carry out unique studies concerning the specific role of parenchymal and NP cells as the main liver cell fractions. Further, the hepatocyte – NP cell 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. On the 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.

Cellular effects of heat stress on hepatic cell cultures of chicken origin *in vitro* (Study II.)

The investigation of the effects of acute intense heat stress have been successfully carried out applying the established cell culture models. Short term (1 h) intense heat stress greatly influenced liver cell functions by increasing metabolism and extracellular H_2O_2 release, and by decreasing HSP70, IL-6 and IL-8 production. However, all these alterations were restored after 2 h of heat exposure, indicating a fast adaptation and recruitment of liver cells. These data highlight the impact of short-term heat stress on the functions of chicken liver cells as well as underline the mediatory role of oxidative stress in acute stress response and imply a fast cellular adaptation potential of liver cells.

Effects of T-2 toxin on hepatic cell cultures of chicken origin *in vitro* (Study III.)

Based on our results, the established primary hepatocyte mono-cultures and hepatocyte – NP cell co-cultures derived from chickens were found to be appliable models to study the specific molecular effects of T-2 toxin. 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 – in line with several abovementioned studies – can suggest that ROS production may not play a key mediatory role in the cytotoxic effects of T-2 toxin on chicken liver; however, further studies – investigating and monitoring further redox parameters and applying longer incubation times – would be essential to completely discover the molecular background of this question in the future. In conclusion, the present investigations 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.

Consequences of acute heat stress in parenchymal organs of chickens in vivo (Study IV.)

In the last phase of the work, molecular effects of acute heat stress in various tissues of broiler chickens were investigated from a novel point of view. Among the examined parenchymal organs, liver was found to be 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 sHSPs are presumed to play a key role in the acute hepatic heat stress response in chickens, while HSP27 seemed to not 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 reduced glutathione (GSH) and crystallin levels, suggesting further relationship between the glutathione system and certain sHSPs. 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 – only mildly enhanced lipid peroxidation was detected and just after the longer 8 h heat exposure. In conclusion, the present 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 sHSPs in the restoration of physiological cell function under oxidative distress.

Conclusions

Our results regarding the immunomodulatory action of the investigated factors and their effects on the redox homeostasis may be of great importance for the agriculture in the future. Further, in some aspects, specific role of HSP70 and sHSPs in the maintenance of cellular homeostasis and physiological redox balance during heat stress or following mycotoxin exposure have been also described along with the importance of oxidative stress induced increased ROS production. With the monitoring of different cytokines, such as IL-6 and -8, additional knowledge was also gained in correlation with the direct immunomodulatory effects of the investigated stressors. Finally, applying the newly established co-culture models, valuable information has been also achieved regarding the role of macrophage containing NP cell fraction in the hepatic stress response.

Our results serve with novel aspects for the better understanding of the harmful cellular consequences induced by heat stress and T-2 toxin exposure. The present study may serve with valuable information for the establishment of advanced solutions against the investigated immunomodulatory factors and for the aimed application of specific feed additives or further protective agents in the future.

New scientific results

Ad 1,

Novel primary hepatocyte mono-cultures and hepatocyte – NP cell co-cultures from chicken origin have been successfully established and characterized by flow cytometry and immunocytochemistry. These cell cultures can serve as proper tools for studying the hepatic inflammatory and stress response triggered by immunomodulatory factors.

Ad 2,

Shorter term heat stress influence hepatic function by significantly increasing catabolic metabolism and extracellular H_2O_2 release, and by intensely 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 sufficient cellular adaptation potential of liver cells.

Ad 3,

The established cell cultures were found to be proper models for short-term toxicological studies involving T-2 toxin. Physiological function of liver cells was strongly diminished by T-2 toxin, reflected by decreased metabolic rate, elevated HSP70 concentration, and T-2 toxin-triggered inflammatory response resulting in increased pro-inflammatory cytokine production. On the other hand, no changes were found in the extracellular H_2O_2 levels, which may suggest that ROS production does not necessarily play a key mediatory role in the cytotoxic effects of T-2 toxin in the liver of chickens.

Ad 4,

According to the *in vivo* experiments, liver was found susceptible to heat stress-triggered oxidative damage, indicated by enhanced lipid peroxidation and rapid activation of protective pathways, including the definite increase of glutathione peroxidase activity and the excessive utilization of αA- and αB-crystallin proteins. Heat-associated decline of protein carbonylation and GSH content was observed in the liver in correlation with the increased involvement of αA- and αB-crystallins in cellular defense, resulting supposedly in an overcompensation mechanism.

Own scientific publications

Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

Mackei, M., Mátis, G., Molnár, A., Sebők, C., Vörösházi, J., Pál, L., Dublecz, K., Husvéth, F. & Neogrády, Z.: **The relationship between small heat shock proteins and redox homeostasis during acute heat stress in chickens**. *Journal of Thermal Biology*, 103040, 2021. **Impact factor: 2.902**

Mackei, M., Molnár, A., Nagy, S., Pál, L., Kővágó, C., Gálfi, P., Dublecz, K., Husvéth, F., Neogrády, Z. & Mátis, G.: **Effects of Acute Heat Stress on a Newly Established Chicken Hepatocyte—Nonparenchymal Cell Co-Culture Model**. *Animals*, *10*(3), 409, 2020. **Impact factor: 2.752**

Mackei, M., Orbán, K., Molnár, A., Pál, L., Dublecz, K., Husvéth, F., Neogrády, Z. & Mátis, G.: **Cellular effects of T-2 toxin on primary hepatic cell culture models of chickens**. *Toxins*, *12*(1), 46, 2020. **Impact factor: 4.546**

Mackei, M., Matis, G., & Neogrady, Z.: **The effects of T-2 toxin on animal health, focusing especially on poultry: literature review**. *Magyar Állatorvosok Lapja*, *140*, 475-483, 2018. **Impact factor: 0.143**

Oral and poster presentations on international conferences

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, Paris, France, 2022 August (delayed).

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, 2022 August (delayed).

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** 23rd Congress of the European Society of Veterinary and Comparative Nutrition, Turin, Italy, 2019

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

Oral presentations on Hungarian national conferences

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Effects of heat stress on chicken-derived primary hepatic cell culture models, 62. Georgikon Napok, Szent István Egyetem, Georgikon Kar, Keszthely, 2020.

Mackei Máté, Mátis Gábor, Sebők Csilla, Vörösházi Júlia, Molnár Andor, Pál László, Dublecz Károly, Husvéth Ferenc, Neogrády Zsuzsanna

A hőstressz redox homeosztázisra gyakorolt hatásainak vizsgálata csirkében

MTA Akadémiai Beszámolók, Budapest, Hungary, 2021.

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, Hungary, 2020.

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A T-2 toxin sejtszintű hatásainak vizsgálata különböző csirke eredetű primer májmodelleken

MTA Akadémiai Beszámolók, Budapest, Hungary, 2020.

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, Budapest, Hungary, 2019.

Mátis Gábor, Kulcsár Anna, Hatala Patrícia, Tóth Adrienn, Mackei Máté, Neogrády Zsuzsanna **A T-2 toxin sejtkárosító hatásainak összehasonlító vizsgálata csirke primer bélhámsejt- és májsejttenyészeten**

MTA Akadémiai Beszámolók, Budapest, Hungary, 2018.

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A hőstressz sejtszintű hatásainak vizsgálata csirke májsejt – Kupffer-sejt ko-kultúrán

MTA Akadémiai Beszámolók, Budapest, Hungary, 2017.

Publications not related to the topic of the present dissertation

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Sebők, C., Tráj, P., Vörösházi, J., Mackei, M., Papp, M., Gálfi, P., Neogrády Z. & Mátis, G. **Two Sides to Every Question: Attempts to Activate Chicken Innate Immunity in 2D and 3D Hepatic Cell Cultures**. *Cells*, 10(8), 1910, 2021. **Impact factor: 6.600**

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