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Study of immunomodulatory factors in chicken hepatic cell culture models

Ph.D. thesis

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List of abbreviations

$^1O_2$	singlet oxygen
AME_n	nitrogen-corrected apparent metabolizable energy
ANOVA	analysis of variance
AP-1	Activator protein-1
ATP	adenosine triphosphate
aw	water activity
BCA	bicinchoninic acid
BSA	bovine serum albumin
CCK-8	Cell Counting Kit-8
CEF	chicken embryonic fibroblast
CRYAA; HSPB4	αA-crystallin
CRYAB; HSPB5	αB-crystallin
CYP	cytochrome P450
DAPI	4',6-diamidino-2-phenylindole
DDGS	distillers dried grains with solubles
DFD	dark, firm, dry
DNA	deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DON	deoxynivalenol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol bis (2-aminoethyl ether) tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FAO	Food and Agriculture Organization
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
FS	forward scatter
gp80	80 kD membrane glycoprotein
gp130	130 kD membrane glycoprotein
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSG	glutathione disulfide
GDP	guanosine monophosphate
GRP78	78-kDa glucose-regulated protein

GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HBSS	Hanks' balanced salt solution
HOCl	hypochlorous acid
HPA axis	hypothalamic–pituitary–adrenal axis
HRP	horseradish peroxidase
HSP	heat shock protein
HSP70	70 kilodalton heat shock protein
HSPB1	Heat shock protein beta-1=HSP27
HSR	heat shock response
IgA	immunoglobulin A
IgE	immunoglobulin E
IL	interleukin
IL-8RA and B	interleukin-8 receptor A and B
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase
LO•	alkoxyl radical
LOO•	peroxyl radical
LOOH	lipid hydroperoxide
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
M-PER	Mammalian Protein Extraction Reagent
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS	nitric oxide synthase
NP cell	non-parenchymal cell
$O_2•^-$	superoxide anion radical
O_3	ozone
OH•	hydroxyl radical
ONOO-	peroxynitrite
PBS	phosphate buffered saline
PE	phycoerythrin
PI3K	phosphatidylinositol 3-kinase
PSE	pale, soft, exsudative

PUFA	polyunsaturated fatty acid
RCS	reactive chlorine species
RH	relative humidity
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROM	reactive oxygen metabolites
ROS	reactive oxygen species
SAM axis	sympathetic–adrenal medullar axis
SEM	standard error of the mean
sHSP	small heat shock protein
SOD	superoxide dismutase
SS	side scatter
STAT	signal transducer and activator of transcription
TBA	thiobarbituric acid
THI	temperature humidity index
TMI	transition metal ions
TNF	tumor necrosis factor
T-PER	Tissue Protein Extraction Reagent

1. Summary

Heat stress and mycotoxin exposure are among the most important environmental immunomodulatory factors in agriculture impairing animal health, productivity and leading to serious animal welfare issues. On cellular level, excess heat exposure can trigger a specific defense mechanism, called heat shock response acting for the restoration of cell homeostasis by several mechanisms, such as affecting heat shock protein synthesis, redox homeostasis and pro-inflammatory cytokine production. The first aim of the study therefore was to establish a novel avian hepatocyte – non-parenchymal (NP) cell (predominantly Kupffer cells) co-culture as an applicable model for investigating the cellular effects of heat stress and its interaction with inflammation in chicken liver. On the other hand, T-2 toxin as one of the most noxious members of the trichothecene mycotoxin group has been shown to be a serious hazard for general food and feed safety and in consequence, for human and animal health. As liver is one of the key organs in its metabolism, beyond the investigation of heat stress, the other aim of the PhD study was to monitor the immunomodulatory and cytotoxic effects of T-2 toxin applying the mentioned primary hepatocyte mono-culture and hepatocyte – NP cell co-culture models of chicken origin. Further, another aim of the study was to monitor the *in vivo* effects of single time occurring acute heat exposure in broiler chickens with special emphasis on parenchymal organs such as the liver, the kidney and the spleen.

Cell fractions were isolated by differential centrifugation from freshly perfused chicken liver, and hepatocyte mono-cultures as well as hepatocyte – NP cell co-cultures (with cell ratio 6:1, hepatocytes to NP cells, mimicking a milder hepatic inflammation) were prepared. Isolated and cultured cells were characterized by flow cytometry and immunocytochemistry applying hepatocyte- and macrophage-specific antibodies. In the *in vitro* heat stress study, confluent cell cultures were exposed to 43°C temperature for 1 or 2 h, while controls were cultured at 38.5°C. The metabolic activity, lactate dehydrogenase (LDH) enzyme activity, extracellular hydrogen peroxide (H_2O_2) production, concentrations of heat shock protein 70 (HSP70) and that of the pro-inflammatory cytokines interleukin (IL-)6 and IL-8 were assessed. Shorter heat stress applied for 1 h has strongly influenced liver cell function by significantly increasing catabolic metabolism and H_2O_2 release, and by 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 rapid cellular adaptation potential in liver cells.

In the mycotoxin-related study, cultures were exposed to 10 (T10), 100 (T100) and 1000 (T1000) nmol/L T-2 toxin treatment for 8 or 24 h. Similarly to the mentioned *in vitro* investigations, alterations of cellular metabolic activity, the concentration of extracellular H_2O_2, HSP70 and the production of IL-6 and IL-8 were investigated. Metabolic activity was intensely decreased by T-2 toxin administration in both cell culture models, in every applied concentration and after every incubation time. Concentrations of HSP70 and IL-8 were significantly increased in hepatocyte mono-cultures exposed to higher T-2 toxin levels (both in T100 and T1000 groups for HSP70 and in T1000 group for IL-8, respectively) compared to controls after 24 h incubation. Similarly, IL-6 levels were also significantly elevated in the T100 and T1000 groups in both of mono- and co-cultures, but only after 8 h of incubation time. In spite of the general harmful effects of T-2 toxin treatment, no significant differences were observed in H_2O_2 production. Furthermore, the two cell culture models showed different levels of H_2O_2, HSP70 and IL-8 concentrations independently of T-2 toxin supplementation suggesting the specific role of Kupffer cells in the toxin mediated effects. In conclusion, the established new primary cell cultures derived from chicken proved to be proper models to study the specific molecular effects caused by T-2 toxin. Metabolic activity and immune status of the examined cell cultures were intensively affected; however, no changes were found in H_2O_2 levels.

Along with the *in vitro* experiments, in order to investigate the *in vivo* effects of acute heat exposure, 32-day-old Ross 308 broiler chickens were subjected to 37°C environmental temperature combined with 50% relative humidity for 4 or 8 hours respectively. Following sampling, redox parameters such as malondialdehyde (MDA), reduced glutathione (GSH), protein carbonyl levels as well as glutathione peroxidase (GPx) activity were assessed in liver, spleen, and kidney homogenates. The concentrations of small heat shock proteins (sHSPs) HSP27, αA- and αB-crystallin were also investigated. Underlying the significance of our *in vitro* results, among the investigated organs, liver was found the most susceptible to heat-provoked oxidative stress, indicated by enhanced lipid peroxidation and rapid activation of protective pathways, including the definite increase of GPx 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, supposedly leading to an overcompensation mechanism. These latter results also underline the hepatic sensitivity to acute heat shock, potential adaptation mechanisms, and the specific role of sHSPs in the restoration of physiologic cell function.

According to our results, the molecular consequences of important environmental stressors such as the heat stress and T-2 toxin exposure have been successfully described and

characterized under both *in vitro* and *in vivo* circumstances. The newly established primary hepatic cell culture models proved to serve as proper tools for monitoring the deleterious effects of the abovementioned immunomodulatory factors, while the results and significance of the heat stress related investigations on cell cultures have been also supported and highlighted by the *in vivo* experiments. Information provided by the present study contribute to the better understanding of the cellular effects of acute heat stress and T-2 toxin in broiler chickens and may serve with beneficial knowledge for the development of novel solutions for the aimed application of protective agents such as specific feed additives in the future.

1. Összefoglalás

Mind a hőstressznek való fokozott kitettség, mind a takarmányok mikotoxinokkal való szennyezettsége a legfontosabb környezeti eredetű immunmoduláns faktorok közé tartoznak, melyek nagyban befolyásolhatják az állatok egészségi állapotát és termelékenységét, valamint súlyos állatjólléti problémákhoz is vezethetnek. A hőstressz megjelenése olyan specifikus, sejtszintű következményekkel járhat, mint például az úgynevezett hősokk-válasz kialakulása, melynek során a hősokkfehérjék aktiválódnak, illetve a redox-homeosztázisban és egyes pro-inflammatorikus citokinek termelődésében is változások következnek be. A hősokk-válasz révén kialakuló változások hozzájárulnak a sejtek homeosztázisának helyreállításához. Jelen munka elsődleges célja ezzel összefüggésben olyan újonnan kialakított, csirke eredetű hepatocita – nem-parenchimális (NP) sejt (túlnyomórészt Kupffer-sejt) ko-kultúrák létrehozása volt, mely modellek alkalmasak lehetnek a hőstressz hatásainak behatóbb tanulmányozására csirkék májában.

A T-2 toxin, mint a trichotecén vázas mikotoxinok egyik legjelentősebb képviselője, takarmány- illetve élelmiszerbiztonsági szempontok szerint is komoly veszélyt jelent napjainkban. Mivel a toxin lebontásában fontos szerepet játszó méregtelenítő folyamatok fő helyszíne a máj, így a hőstressz vizsgálatán túlmenően a PhD munka másik fő célja a T-2 toxin immunmoduláns és citotoxikus hatásainak vizsgálata volt a már említett, csirke eredetű primer hepatocita mono- és hepatocita – NP-sejt ko-kultúrák (6:1 sejtarány; hepatociták:NP sejtek) felhasználásával. Ez utóbbi sejttenyészet, a makrofágok meghatározott arányú jelenléte miatt, enyhe fokú gyulladásos folyamatok vizsgálatára szolgáló májmodellként is alkalmazható. Célunk volt továbbá az *in vitro* vizsgálatok elvégzésén túl az egyszeri, akut hőstressz *in vivo* hatásainak tanulmányozása is brojlercsirkékben, különös tekintettel olyan parenchimás szervekre, mint a máj, a lép és a vese.

Az egyes sejtfrakciókat a frissen perfundált májból többlépcső perfúzióval nyertük, és differenciáló centrifugálás segítségével különítettük el. Az izolált és tenyésztett sejtek jellemzését áramlási citormetriai, valamint immuncitokémiai módszerekkel, hepatocita és makrofág specifikus ellenanyagok felhasználásával végeztük. A hőstresszel kapcsolatos *in vitro* kísérletben a sejttenyészetek 43°C-nak voltak kitéve 1, illetve 2 órán keresztül, míg a kontroll tenyészetek esetében az inkubálási hőmérséklet 38,5°C volt. Ezt követően a sejttenyészetek metabolikus aktivitását, a laktát-dehidrogenáz (LDH) enzim aktivitását, valamint az extracelluláris hidrogén peroxid (H_2O_2), a 70kDa tömegű hősokkfehérje (HSP70), az interleukin (IL-)6 és IL-8 koncentrációját határoztuk meg. A rövidebb ideig, 1 órán át tartó hőstressz erőteljesen befolyásolta a májsejtek metabolikus aktivitását, valamint a H_2O_2 felszabadulását, és csökkentette a HSP70, IL-6 és IL-8 termelődését mindkét sejtmodellen.

Ezzel szemben a hosszabb ideig tartó, 2 órás hőstresszt követően az említett változások nem voltak megfigyelhetőek, ami a májsejtek hőstresszel szembeni gyors alkalmazkodására enged következtetni. A hepatocita mono- és hepatocita – NP-sejt ko-kultúrák hasonló módon reagáltak a kezelésre, azonban a ko-kultúrák esetében megfigyelhető magasabb metabolikus aktivitás az enyhébb gyulladásban lévő máj hatékonyabb adaptációs képességére is utalhat. Eredményeink segíthetik a rövidtávú hőstressz során a májban végbemenő sejtszintű folyamatok pontosabb megértését, valamint kiemelik az oxidatív stressz szerepét az akut hőstressz kiváltotta sejtszintű folyamatokban, és jól tükrözik a májsejtek hatékony alkalmazkodási képességét is.

A második kísérlet során a sejttenyészeteket 8 vagy 24 órán keresztül, 10 (T10), 100 (T100), illetve 1000 (T1000) nmol/l koncentrációban kezeltük T-2 toxinnal. A már említett *in vitro* kísérlethez hasonlóan a kezelés és mintavétel után ebben az esetben is a tenyészetek metabolikus aktivitásának, valamint a H_2O_2, HSP70, IL-6 és IL-8 koncentrációjának meghatározását végeztük el. A toxin intenzíven csökkentette a metabolikus aktivitást minden alkalmazott koncentrációban, minden inkubációs időt követően és mindkét sejttenyészet esetében. A HSP70 és IL-8 mennyisége szignifikánsan magasabb volt a hepatocita mono-kultúrákban a nagyobb koncentrációjú toxinkezelést kapott csoportokban (HSP70: T100 és T1000; IL-8: T1000) a kontroll sejtekhez képest 24 órás inkubációt követően. Az IL-6 koncentrációja hasonlóképp magasabbnak bizonyult mindkét sejttenyészetben a 8 órás kezelést követően a T100 és T1000 csoportokban. Másrészről azonban, annak ellenére, hogy a T-2 toxin egyértelműen káros hatással volt a sejttenyészetekre, nem volt kimutatható változás a H_2O_2 koncentrációjában. Továbbá, a két különböző sejtmodellt összehasonlítva eltérő H_2O_2, HSP70 és IL-8 koncentrációk voltak megfigyelhetők, ami a Kupffer-sejtek specifikus szerepére utalhat a toxin kiváltotta reakciók kialakításában. Összefoglalva elmondhatjuk, hogy az általunk újonnan létrehozott primer sejkultúrák alkalmas modellnek bizonyultak a T-2 toxin molekuláris hatásainak vizsgálatára. A toxin a sejttenyészetek metabolikus aktivitását és immunstátuszát is nagymértékben befolyásolta, miközben nem okozott változásokat a H_2O_2 koncentrációjában.

Az *in vitro* kísérletek elvégzésén túl célunk volt továbbá az akut hőstressz *in vivo* vizsgálata is, mely munkához 32 napos Ross 308 brojlercsirkék kerültek felhasználásra. Az állatokat 50%-os relatív páratartalom mellett tettük ki 37°C-os teremhőmérsékletnek 4 és 8 órán keresztül. A mintavételt követően a máj-, lép- és vesehomogenizátumokat felhasználva különböző redox-paramétereket, így a malondialdehid (MDA), a redukált glutation (GSH) és a protein-karbonilok koncentrációját, illetve a glutation-peroxidáz (GPx) enzim aktivitását, határoztuk meg. Ezen túlmenően, nyomon követtük a kistömegű hősokkfehérjék (sHSP-k) csoportjába tartozó HSP27, αA- és αB-crystallinok koncentrációjában bekövetkező változásokat is. Eredményeink alapján, *in vitro* vizsgálataink jelentőségét is alátámasztva, a

hőstressz káros hatásaival szemben legérzékenyebb szervnek a máj bizonyult. Ennek megfelelően a hőstressznek kitett csoportok májában fokozott mértékű lipidperoxidáció és egyes protektív folyamatok, így a glutation rendszer és a crystallin fehérjék által kialakított védekezőmechanizmusok gyors aktiválódása volt megfigyelhető. A hőstressz hatására bekövetkező protein-karbonil és GSH koncentráció-csökkenés valószínűleg az αA- és αB-crystallinok által közvetített sejtszintű válaszreakcióból fakadó túlkompenzációs mechanizmussal lehet összefüggésben. Ez utóbbi eredményeink is alátámasztják a máj akut hőstresszel szembeni kitettségét, valamint segíthetnek a jövőben az sHSP-k mediálta védő mechanizmusok lefolyásának pontosabb megértésében.

Eredményeink alapján kijelenthetjük, hogy sikeresen vizsgáltuk a hőstressz és a T-2 toxin, mint nagy jelentőségű, környezeti eredetű immunmoduláns faktorok, molekuláris szintű hatásait mind *in vitro*, mind *in vivo* körülmények között. Az újonnan kialakított primer hepatikus sejtmodelljeink jól alkalmazható és megbízható eszközöknek bizonyultak a fent említett vizsgálatok elvégzésére. A kutatás során nyert eredmények nagymértékben hozzájárulhatnak az akut hőstressz és a T-2 toxin által kiváltott sejtszintű folyamatok megértéséhez, mely előrelépés nagyban segítheti a jövőben új típusú megoldások kidolgozását, valamint protektív hatású anyagok és takarmánykiegészítők alkalmazását a probléma enyhítése céljából.

2. 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 (Frame et al., 2020). 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 (Medina et al., 2017; Surai, 2015). 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 (Lan et al., 2016).

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 and King, 2015). Heat stress can lead to dysfunction in muscles and in parenchymal organs alike, including the liver, spleen, or kidney (Gan 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 (Slimen et al., 2016). 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 (Altan et al., 2003).

Excessively high temperatures initiate a specific defence 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 especially important (Horowitz and Robinson, 2007). 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 (Yadav et al., 2020). 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 (Singh et al., 2017), 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 of pro-inflammatory cytokines, such as increasing splenic interleukin IL-4 and IL-12 concentrations in chicken (Ohtsu et al., 2015). Further, the cellular immune system may also get diminished by heat stress, reflected by decreased total white blood cell count and disturbed 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. According to recent findings, the prevalence of trichothecene mycotoxins (including T-2 toxin) can be in some regions, including Europe, more than 50% considering all feed samples, serving with serious issues also for broiler industry (Leite et al., 2021).

The T-2 toxin as one of the most noxious members of mycotoxins can provide a serious hazard in broiler nutrition as well, threating both animal and human health by contaminating the food chain (Milićević 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 (Awad et al., 2008). 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 signaling and inflammatory pathways (Wu et al., 2014b). In most cases, T-2 toxin significantly increases the level of 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 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 (El Golli-Bennour and Bacha, 2011). Elevated HSP70 concentration 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., 2006a), 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 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).

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 novel data on the pathomechanisms of stress-associated multifactorial diseases, highlighting new ways of improving animal health and productivity.

3. Literature overview

3.1. Effects of heat stress

Among numerous harmful environmental factors such as overstocking, excess dust, wet litter, improper or contaminated feed, heat stress is an outstanding issue in intensive poultry production, having increasing significance due to combination of events including increasing temperatures and more frequent heat waves caused by global climate change (Bazaz et al., 2018; Mottet and Tempio, 2017). Commercial broiler chicken breeds are rapidly-growing animals with a high feed consumption and relatively high metabolic heat production compared to their body size, which also affects thermoregulation. Furthermore, feathers impede heat release while the absence of sweat glands also exacerbate the problem of thermoregulation during periods of heat stress (Møller, 2015). Elevated environmental temperatures, especially combined with higher relative humidity (RH), can cause suffering and remarkably deteriorates the health, welfare and growth performance of broilers (Surai and Kochish, 2017). Slower growth rates, decreased fertility, uneven carcass composition and quality as well as animal welfare issues and elevated veterinary costs can be all associated with heat stress in the livestock sector (Gonzalez-Rivas et al., 2020).

Homoeothermic species, such as avian or mammalian farm animals, have a thermoneutral zone where no extra energy is required for thermoregulation beyond that is needed for maintenance of normal cellular metabolic processes (Kingma et al., 2014). As the ambient temperature rises beyond the thermoneutral zone limits, the core body temperature is getting increased. In this case, evaporation by sweat (if possible) and panting become the only viable route of significant heat loss. Further, body temperature and mitochondrial heat output also increase under these circumstances, and in severe cases animals unavoidably fall prey to hyperthermia and heat stress (Renaudeau et al., 2012). Avian species are hatched with poikilotherm attributes while they show homeotherm characteristics from the age of 4–5 days, whenceforth, elevated environmental temperature becomes a more important issue to these animals. The core body temperature in birds ranges between 34 and 44°C, depending on several factors such as body size or style of living. On the other hand, mammalian core temperatures vary between 30 and 40°C (McCafferty et al., 2015).

Heat exposure causes accelerated respiration rate and panting in birds, as well as increased cloacal temperature, decreased movements, and wing elevation (Shakeri et al., 2018). Further, air sacs are crucially important in avian species for the facilitation of heat exchange between the body and the environment. Air sacs are especially useful during panting because they facilitate air circulation on big surfaces, increasing gas exchange and evaporative heat loss (Fedde, 1998). However, increased panting during heat stress causes

elevated carbon dioxide levels resulting in subsequently higher blood pH and alkalosis, which reduces blood bicarbonate supply while blood calcium level also lowers. This process is critical in breeders and laying hens because in consequence, eggshell mineralization can be also highly disturbed (Lara and Rostagno, 2013).

On cellular level, a specific stress response pathway, called heat shock response is initiated by heat stress acting for the restoration of cell homeostasis by complex alterations of several signaling and metabolic pathways (Richter et al., 2010). Heat shock proteins are primarily involved in the successful cellular adaptation to stress conditions, while the synthesis of most other proteins gets discontinued during HSR (Gupta et al., 2010). Further, HSR is usually linked to enhanced oxidative stress, reflected by elevated level of prooxidants (such as ROS) and inadequate level of antioxidants (Surai and Kochish, 2017). Heat-triggered oxidative stress of broilers resulted in enhanced lipid peroxidation in the liver and in the heart muscle (Lin et al., 2006), and increased oxidative damage of muscular proteins as well as reduced antioxidant capacity due to the disfunction of the respiratory chain (Huang et al., 2015; Punchihewage et al., 2018).

Elevated temperatures can lead to severe harmful effects in numerous organs. A significant increase of environmental temperature induces intense adrenal catecholamine release (Calefi et al., 2017), which influences the cardiovascular system and results in accelerated heart- and respiratory rates via stimulation of the parabrachial complex (Davern, 2014). Hepato-splanchnic vasoconstriction and decreased splanchnic blood flow also occur as a result of changes in the cardiorespiratory system (Epstein and Roberts, 2011). When these conditions are paired with elevated hepatocellular metabolic demands, extreme hypoxia will develop quickly, exposing the liver to the detrimental effects of the heat shock response (Hall et al., 2001). Furthermore, heat exposure can highly increase the permeability of the gastrointestinal tract, allowing endotoxins to enter the bloodstream even in healthy animals (Le et al., 2020). A decrease in portal blood flow combined with a reduction in hepatic function severely decreases the detoxifying capacity in the event of a heat stroke (Epstein and Roberts, 2011). Among various tissues, liver was found highly susceptible to heat-provoked oxidative stress in broilers. The reason of the hepatic sensitivity is presumably the high concentration of polyunsaturated fatty acids (PUFA) in the liver, particularly when compared to the proportion of potential antioxidants. These PUFA molecules are especially prone to oxidation and therefore to oxidative damage caused by heat stress, enhancing the organ's exposure to the harmful effects of elevated ambient temperatures (Lin et al., 2006).

It has been also shown that macrophages, monocytes, lymphocytes, and granulocytes express specific receptors for many neuroendocrine products of the disturbed hypothalamic–pituitary–adrenal (HPA) and sympathetic–adrenal medullar (SAM) axes, such as cortisol and catecholamines, which can affect various metabolic processes, cell proliferation, cytokine

secretion, antibody production and cytolytic activity (Marketon and Glaser, 2008). In laying hens subjected to heat stress lower relative weights of thymus and spleen has been found (Lara and Rostagno 2013), while in broilers under heat stress lower lymphoid organ weights were also observed (Niu et al., 2009). In another study, reduced liver weights were observed in laying hens exposed to chronic heat stress conditions (Felver-Gant et al., 2012). Heat stress was reported to cause functional changes in the immune response by altering the gene expression of pro-inflammatory cytokines, such as increasing IL-4 and IL-12 concentrations in the blood plasma of chickens (Ohtsu, 2015). Further, the cellular immune system may also get diminished by heat stress, reflected by decreased total white blood cell count (Mashaly et al., 2004), and macrophage activity (Bartlett and Smith, 2003). Heat stress also strongly affected the immune response by decreasing the splenic IL-6 and IL-12, and further, caecal IL-1β and IL-10 gene expression in *Salmonella* Enteritidis infected chickens (Quinteiro-Filho et al., 2017).

Based on the aforementioned data, besides the detrimental effects observed in many of the organs, heat-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 **(Figure 1.)**. Besides hepatocytes, Kupffer cells, as the resident liver macrophages, together with further circulation-derived macrophage cells, are predominantly involved in hepatic inflammatory and stress response (Kolios et al., 2006). Further, these cells also play key role in the regulation of metabolic processes, serving as a link between inflammation and metabolism (Baffy, 2009). Therefore, monitoring the function of hepatic macrophages in the complex regulation of inflammation and stress response could highlight new ways of improving animal health and productivity.

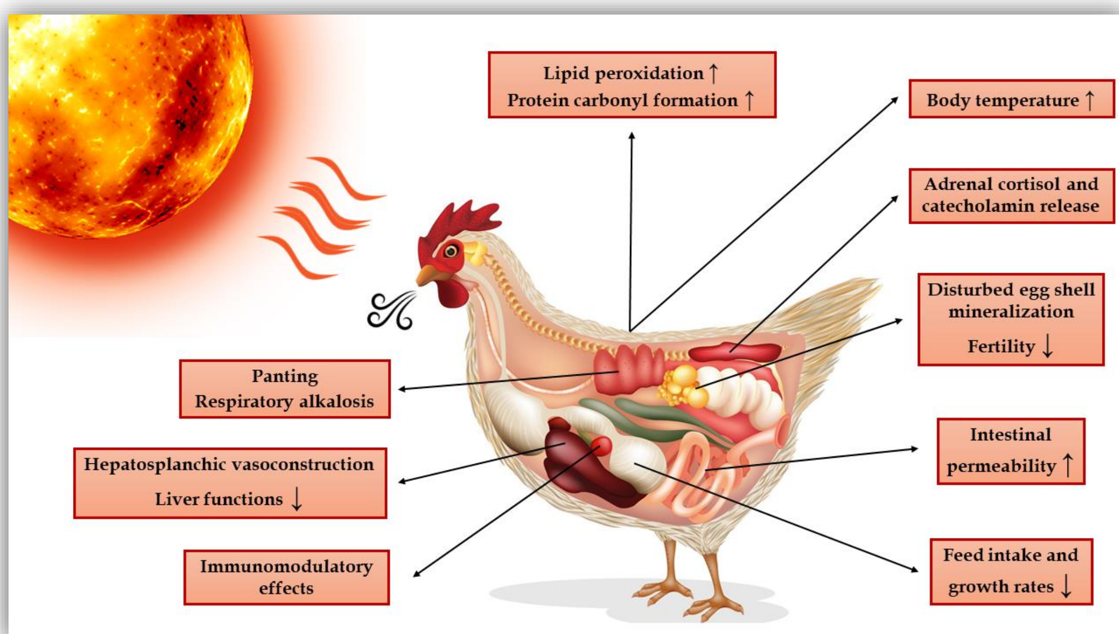

Figure 1. Schematic overview of the negative consequences of heat stress in poultry
(chicken picture: www.vectorstock.com)

3.2. Mycotoxins

3.2.1. General summary of mycotoxins

The contamination of feed and food by molds is a serious issue worldwide nowadays. As secondary metabolites, these fungi can produce molecules which – although they are not essential for their survival – have a major and often indirect impact on their physiological state. Such metabolites might be for example growth factors that act on plants, various compounds produced against bacteria, certain pigments or pre-vitamins such as ergosterol, and mycotoxins. The latter ones are toxic substances that can intensely affect the cellular processes of eukaryotes, in particular protein and nucleic acid synthesis, and other metabolic pathways in different cell components. Current knowledge suggests that there are hundreds of harmful fungal products; however, the number of mycotoxins having significant impact in animal nutrition is much lower (da Rocha et al., 2014; Moss, 2002).

Intensive and detailed research in correlation to the harmful effects of mycotoxins and to their exact mechanism of action have been initiated in the 1960s. In this period, a mysterious and unknown disease, called turkey X disease – which led to the death of more than 100,000 turkeys in the United Kingdom – was discovered to be caused by high levels of aflatoxin contamination of the peanut meal in the feed (Benkerroum, 2019).

It is clear that molds are of significant problem in crop and food production as well as in animal farming worldwide, causing enormous economic damage. This is supported by the FAO's assessment that in the 2000s, at least a quarter of the world's total cereal crop was contaminated with mycotoxins above threshold (Bhat et al., 2010). On the other hand, based on recent results we can state that the problem is getting even more serious nowadays because of the severe effects of climate change. Unequal rainfall distribution, more intense precipitation extremes along with serious droughts are all weakening the host plants and support the appearance and the spread of harmful mycotoxin producing mold species (Frame et al., 2020; Moretti et al., 2019). In some regions, also including Europe, more than 50% of all feed may be contaminated with mycotoxins (Leite et al., 2021). Only a fraction of this amount, which poses a serious risk, is destroyed. Notwithstanding, the remainder is often being used as food or feed. The importance of mycotoxins in food hygiene and food safety is also highlighted by the fact that some toxins, such as aflatoxin, can accumulate in animal tissues and for this reason enter the food chain (Yang et al., 2020). Therefore, mycotoxins can be also harmful to humans or other species indirectly, through ingestion of food of animal origin, also underlying the relevance of mycotoxins in correlation with the One Health concept. It is also important to note that mold contamination does not always mean the presence of mycotoxins, as the intensity of their production can be influenced by a number of external factors. These may include various climatic features such as temperature and humidity, pH conditions, the

exact species of fungi infecting the feed, the time of infection or the degree of grain damage. It is also worth to mention that depending on the environmental factors, the same fungal species may produce completely different types of mycotoxins (Devreese et al., 2013).

Fungi contaminating crops can be classified to the field (hygrophilic), storage (xerophilic) and intermediate fungi categories. Prior to harvesting, field fungi – such as *Fusarium* and *Alternaria* species – might colonize the ripening grains on standing crops already in the field. The majority of field fungal species do not infect crops following harvest, but they do release mycotoxins prior to or shortly after reaping (Tola and Kebede, 2016). Storage fungi can be present only in limited numbers before harvesting and multiply during storage period, once environmental factors promote their growth over that of other species. The most prominent members of the genera are *Aspergillus* and *Penicillium* fungi (Magan et al., 2003). Fungi in the intermediate class are hygrophilic and mesohilic species, which continue to grow during storage if water activity remains relatively high (Fleurat-Lessard, 2017). Interactions with host plants, genotype, the type of soil, and biological factors all play a role in mold contamination during the preharvest period. On the other hand, substrate status (grain damage and nutritional constituents), environmental circumstances (temperature and moisture), and biotic factors such as the higher prevalence of certain microorganism or insect pests all influence fungal growth and production after harvest (Sanchis and Magan, 2004).

In Europe at present, in contrast to the less frequent acute mycotoxicoses, there is a much higher incidence of lower level but long-term mycotoxin contamination, which can lead to chronic and less specific symptoms in the animals. The immunosuppressive effects of these toxins have also been described in numerous cases. For example, the presence of subacute toxin doses might result in a higher susceptibility of animals to further complex multifactorial diseases (Farkas et al., 2013). Nowadays, in addition to the normal, intact form of the mycotoxins, more and more attention is being paid to the so-called masked and hidden mycotoxins. Based on the currently accepted classification, mycotoxins can be structurally divided into three different groups. These are the free toxins, the matrix-associated toxins (also known as masked mycotoxins), which can be covalently or non-covalently attached to certain molecules, and the mycotoxins which have been structurally modified in some ways. Members of the latter groups are often highly toxic also in their modified forms; however, they can be converted in several detoxification processes, and by the metabolic activity of intestinal microbes back to the original toxins or to further molecules, which might be even more dangerous than the initial one. An additional risk is that usually only certain types of free mycotoxins are detected in the feed using current analytical methods, while the identification of matrix-associated and structurally modified toxins is often not achievable.
Consequently, these modified derivatives are not considered in the estimation of food and feed mycotoxin exposure (Berthiller et al., 2013; Medina et al., 2017).

3.2.2. The effects of T-2 toxin

In temperate climatic zones, field fungi of the *Fusarium* genus, including plant pathogenic and saprobiont species, are widespread and can cause significant economic damage on several levels of agriculture (Rampersad, 2020). Trichothecenes, produced by the genus, are relatively small, sesquiterpene molecules of amphipathic character, which are able to passively cross the cell membrane, thus can be readily absorbed from the gastrointestinal tract and after inhalation, from the lungs as well as from the surface of the skin (Chaudhary et al., 2015). There are about 200 fungal toxins belonging to the group of trichothecenes, which can be classified according to their structure (trichothecenes A, B, C and D). They share the common feature of a 12,13-epoxide ring as a basic skeleton and various further substitution groups (Cope, 2018). Several fungal species other than *Fusarium* are capable of producing trichothecene mycotoxins, such as *Cephalosporium, Stachybotrys, Trichoderma, Trichotecium, Myrothecium* species, although they have lesser importance (Zhu et al., 2020). According to the mentioned classification, T-2 toxin is categorized as a non-macrocyclic type A trichothecene. It is an extremely stable molecule, highly resistant to heat exposure and UV radiation. Therefore, it is not degraded during the food and feed production process or following autoclaving: at least 30-40 minutes at approx. 200°C are needed to inactivate the toxin (Moss, 1996).

After absorption, liver is the primary organ of the toxin's metabolism. The exact cellular mechanism of the degradation process is an area of intensive research nowadays; however, it is important to mention, that in different animal species sometimes different enzymes might play a central role in the detoxification process. In the complex degradation process HT-2 toxin and thereafter neosolaniol, T-2 triol and tetraol, as well as a number of further intermediates can be synthesized from T-2 toxin and the end products are mainly excreted via bile from the body (Yang et al., 2017). Major metabolic pathways of T-2 toxin are hydrolysis, hydroxylation, and conjugation. De-epoxidation is also a crucial step in the detoxification of trichothecenes (Yang et al., 2013). There is a wide variation in the toxicity of the different metabolites. HT-2 is considered a highly toxic compound, similar to the T-2 toxin, while others are mainly much less harmful molecules (Wu et al., 2013). Half-life of T-2 toxin in blood plasma is mostly short, and elimination is usually completed within 48 h, depending on the mode of application, on the ingested amount, and on species-specific characteristics. Compared to other mycotoxins such as aflatoxin, the ingested toxin does not accumulate to a significant quantity in the different organs (Kuca et al., 2008; Li et al., 2011). In addition to the mentioned effects, xenobiotic-transforming capacity of liver cells can be also altered by the toxin, which can supposedly occur due to the inhibition of the microsomal monooxygenase enzyme system including cytochrome P450 (CYP) enzymes, also affecting physiological drug metabolism and causing

changes in the withdrawal period of animal products following medication (Osselaere et al., 2013).

T-2 toxin has significant harmful effects on different organs and tissues. Several studies have shown that the toxin also has negative impact on other fungal species, plants, insects and in some extent on all animals. 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. This decreased sensitivity is based probably on the moderate absorption after oral exposure, the extensive metabolism and rapid elimination of trichothecenes in birds (Awad et al., 2008).

In avian species, as well as in mammals, the toxin can be genotoxic, cytotoxic, teratogenic and it has intense immunomodulatory effects as well (Rezar et al., 2004). T-2 toxin causes damage in the digestive system, in the liver and in other parenchymal organs, but also affects the nervous system and the integument (Nayakwadi et al., 2020). The toxin impairs DNA, RNA and protein synthesis, acting mainly on the 60S ribosomal subunit (Chaudhari et al., 2009). Unlike other trichothecenes, T-2 toxin diminishes the initiatory steps of polypeptide synthesis, while other molecules of the same group, such as deoxynivalenol (DON), inhibit the elongation and termination phases (Shifrin and Anderson, 1999). It is also able to inhibit the mitochondrial electron transport, affects the physiological cell cycle and induces apoptosis *in vivo* and *in vitro* alike (Guo et al., 2018). That is the reason why actively dividing cells, such as cells of the digestive tract, spleen, liver, bone marrow or Bursa Fabricii, are generally more sensitive to T-2 toxin. Certain MAPK (mitogen-activated protein kinase) cascades, such as the JNK (c-Jun N-terminal kinase) and p38 protein kinase pathways, are involved in the induction of cell death, but the exact molecular background of this process is not yet fully understood. It is also important to note that through MAPK and JNK signaling pathways, the expression levels of genes, involved in various metabolic processes, are also significantly affected by the toxin (Wu et al., 2014a; Fang et al., 2012). Further results showed, that in T-2 toxin induced apoptosis, the caspase-2 pathway plays a key role, and the process is not exclusively mediated by the mitochondrial pathway but by the caspase-8 and caspase-3 mediated cascades (**Figure 2.**; Huang et al., 2007).

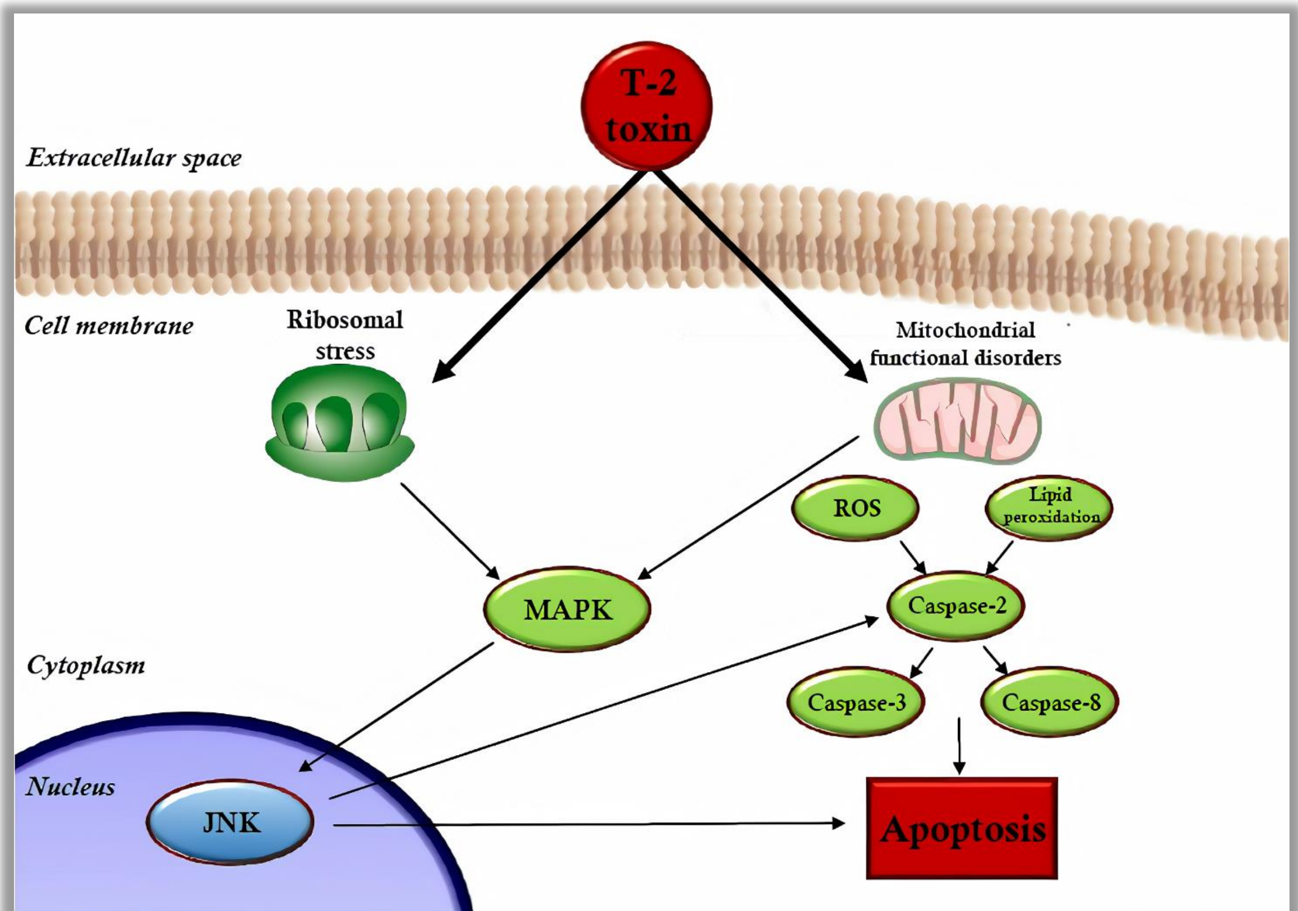

Figure 2. Effects of T-2 toxin on ribosomal and mitochondrial function and various apoptosis signalling pathways.

In several cases, the toxin has been found to have an immunosuppressive effect on the organism; however, some studies have reported an immunostimulatory effect (Pestka et al., 2004). The observed dissimilarity may be related to species-specific differences, to the method of uptake, the amount of toxin and the duration of exposure. Following severe intoxication, the degeneration and atrophy of the bone marrow, spleen, and lymphatic organs, as well as lymphocyte depletion can be observed (Kamalavenkatesh et al., 2005). It is also very important that the presence of T-2 toxin can affect the effectiveness of regular vaccinations. In these cases, reduced antibody titers might be seen following vaccination against diseases of high economic importance such as avian influenza virus or Marek's disease (Kamalavenkatesh et al., 2005; Kufuor-Mensah et al., 2015). On the contrary, other researcher groups have reported that prolonged exposure to lower concentrations of T-2 toxin substantially increases serum immunoglobulin (Ig)A and IgE concentrations, which may be due to a transient activation of gene sequences involved in the inflammatory response and in the functioning of certain immune processes (Pestka et al., 2004).

Regarding the effects of T-2 toxin on cytokines and on other inflammatory mediators, inconsistent results can be found in the literature, too. *In vitro* studies described reduced IL-1β and TNF-α concentrations in primary porcine macrophages (Seeboth et al., 2012), while other experiments suggested, that together with lipopolysaccharides, the toxin synergistically activated IL-1β and IL-18 mediated inflammatory response in human macrophages (Kankkunen et al., 2009).

Another highly important non-ribosomal effect of T-2 toxin is the intensive free radical generation and the oxidative stress associated harmful effects, such as nuclear and mitochondrial DNA damage, elevated lipid peroxidation and disturbances in the cell signaling pathways (Wu et al., 2014b; Chaudhary and Rao, 2010). Furthermore, on cellular level HSP70 is one of the most important member of the heat shock proteins and the expression of this protein shows correlation with the cytoprotective mechanisms against different toxic effects (El Golli-Bennour and Bacha, 2011). Data regarding the effects of trichothecenes on influencing HSP70 gene as well as protein concentration are limited. Elevated HSP70 gene expression caused by T-2 toxin was observed in the placenta of pregnant rats *in vivo* (Sehata et al., 2005) and similarly, T-2 toxin induced HSP70 protein production in Vero cells *in vitro* (El Golli et al., 2006a). In contrary to the foregoing, it has been observed in several cases that although trichothecenes clearly damaged the cells and tissues tested *in vivo* and *in vitro*, there were no signs of increased oxidative stress (such as elevated ROS, or lipid peroxidation levels) or altered concentrations of HSP70. This may be due to methodological differences between studies and differences in the doses used (Frankic et al., 2006; Rezar et al., 2007).

It is also important to note; however, that many investigations on this topic have so far been carried out only in laboratory animals. 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.

3.3. Free radicals and oxidative stress

Free radicals are molecules which contain one or more unpaired electrons in the outermost shells. For this reason, they are of highly reactive character and are capable to attack biomolecules such as cellular proteins, lipids, carbohydrates as well as the DNA content, stealing one electron from the neighboring molecule, while generating another product, containing an additional unpaired electron and becoming the next free radical itself (Riley, 1994). Based on structure and origin, the group of free radicals can be subdivided into three main further classes: reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS). Regarding significance and cellular impact, the group of ROS molecules is often defined as the most relevant one, including molecules such as superoxide anion radical ($O_2•^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), singlet oxygen ($^1O_2$), peroxyl radical (LOO•), alkoxyl radical (LO•), peroxynitrite ($ONOO^-$), lipid hydroperoxide (LOOH), hypochlorous acid (HOCl), and ozone (O_3). It is important to note that numerous ROS molecules do not contain any unpaired electrons, and because of this, in their normal form, they are not genuine free radicals (Glasauer and Chandel, 2013). However, based on their high reactivity, these molecules can serve as potential source of free radicals in the organism, which feature assigns a reason, why they are also included in this groups of molecules. ROS is a broadly used expression to describe oxygen-containing reactive species; however, other alternative terms exist in the literature as well, including reactive oxygen intermediates (ROIs), reactive oxygen metabolites (ROMs), or oxygen radicals (Li et al., 2016). Constant cellular production of ROS is physiological; however, in case it becomes uncontrolled, free radical induced chain reactions may happen resulting in cellular damage followed by apoptosis or necrosis (Slimen et al., 2014). The two main sources of ROS produced under physiological conditions are mitochondria and the NADPH oxidase enzyme family. In the mitochondria molecular oxygen is used as the final acceptor of electrons from the respiratory chain, and beside oxide ion and water formation, in a smaller amount, $O_2•^-$ can be also produced. This does not result under normal, controlled circumstances in negative consequences, what is more, it might play a useful role in the maintenance of oxidative eustress conditions. On the other hand, following exposure to harmful factors, such as thermal stress, toxic substances or radiation, the production of free radicals can quickly increase, the preserved equilibrium of pro- and antioxidant agents can be disturbed, leading to detrimental oxidative distress (Robb et al., 2018; Sies, 2017).

The mentioned factors such as heat stress, were also found to induce iron release from ferritin proteins and to increase the amount of further transition metal ions (TMI), which can have a role in the transformation of oxygen to $O_2•^-$. Furthermore, there was a positive correlation found between plasma iron levels and xanthine oxidase enzyme activity, which is involved in the purine degradation, producing H_2O_2, and indirectly $O_2•^-$ as a byproduct. On the other hand, through the Fenton reaction especially reactive OH• can be also produced (Slimen et al., 2014; Kirkinezos and Moraes, 2001). Similarly to the reactions catalyzed by xanthine oxidase, H_2O_2 can be also produced in further metabolic processes, such as by the action of cytochrome P450 enzymes (Rhee et al., 2017).

Heat stress has also been linked to reduced activity of the antioxidant enzyme SOD which has the function to catalyze the transformation of $O_2•^-$ to H_2O_2. In the next phase, hydrogen peroxide is broken down to water by the enzymes catalase and GPx (Ighodaro and Akinloye, 2018). Under physiological conditions, there is a balance between ROS production and antioxidant levels. Cells possess a wide arsenal of antioxidants, among other enzymes like the already mentioned SOD, catalase and GPx as well as smaller molecules like Vitamin E and C, glutathione, certain heat shock proteins and flavonoids (McGreal et al., 2012; Sies, 2018). If the scale is tipped in the direction of free radicals, oxidative distress with consequent cellular damage occurs. The activity of GPx depends on the amount of reduced glutathione presented as a hydrogen donor. Reduced glutathione is the primary thiol redox buffer in cells, and is synthesized in the cytosol from glycine, L-cysteine and L-glutamate. GSH is involved in a wide variety of detoxifying reactions producing glutathione disulfide (GSSG), which is converted back to GSH by the glutathione reductase enzyme (Flohé, 2013).

Elevated temperature increases the activity of the nitric oxide sythase (NOS) enzyme, producing nitric oxide (NO), a molecule causing vasodilation, from the breakdown of the amino acid arginine to citrulline (Tatoyan and Giulivi, 1998). NO may bind to the haem part of cytochromes in the electron transport chain, disturbing their function and leading to increased levels of $O_2•^-$ (Poderoso et al., 1996). NO and $O_2•^-$ may also react with each other producing peroxynitrite ($ONOO^-$), which is also a potent oxidant molecule (**Figure 3.**; Radi et al., 2002).

ROS are involved in the normal function of the immune system, in particular by acting against harmful agents such as bacteria, following phagocytosis (Dupré-Crochet et al., 2013). Although most studies have focused on the detrimental effects of free radicals and oxidative distress, ROS play an essential role in physiological cellular signal mechanisms or in other metabolic processes, since they can serve as messenger molecules or might modify the cellular redox status even if they are produced in physiological concentration under beneficial oxidative eustress conditions (Reid, 2001; Sen, 2001). Further, ROS were also identified to be involved in the activation of certain enzymes, detoxification processes, or in the facilitation of glycogen repletion (Finaud et al., 2006). It is also important to mention, that inhibition of ROS

production results in decreased contractile force of muscular fibers, while their increased concentrations lead to enhanced contraction strength indicating that ROS is playing a vital role in physiological muscle contraction. On the other hand, after exceeding a certain ROS concentration, among other factors they play a role in the development of muscle fatigue (Reid, 2001).

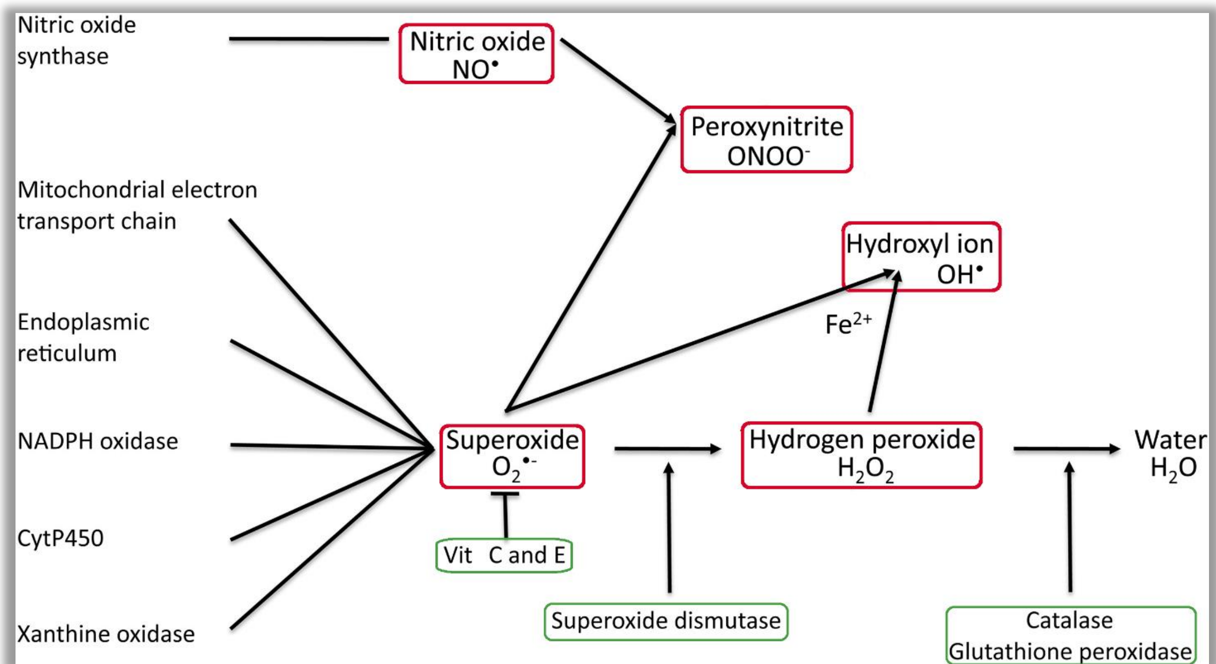

Figure 3. The main sources and degradation of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) under physiological circumstances

In spite of the mentioned beneficial features, excessive ROS exposure results in numerous harmful cellular effects, such as the induction of lipid, protein or DNA oxidation. Free radicals, especially OH• are highly prone to react with PUFA molecules, presented in high concentration in the cellular membranes, resulting in lipid peroxidation and in increasing MDA concentrations, as well as damaged membrane integrity (Halliwell and Gutteridge, 2015). Free amino acids as well as polypeptides are also targets of oxidative damage. Direct oxidation of the side chains leads to the formation of carbonyl groups (aldehydes or ketones). Proline, threonine, arginine and lysine are especially vulnerable to this kind of oxidative effects (Dalle-Donne et al., 2003). Intense ROS formation can also modify several enzymes, protein-like transcription factors, structural proteins and ion channels by the modulation of cysteine residues in the allosteric and active sites of the proteins. Furthermore, ROS mediated activation of redox-sensitive transcription factors, such as p53, activator protein-1 (AP-1) and NF-κB10 regulate cell differentiation and apoptosis or the synthesis of certain pro-inflammatory cytokines (Bretón-Romero and Lamas 2014).

Mycotoxins such as trichothecenes can also cause severe cellular oxidative damage. In most studies it was reported that T-2 toxin significantly increased the level of ROS and induced changes in the antioxidant status of the cells (Bócsai et al., 2016; Chaudhari et al., 2009), while in other studies beside the intensive cellular damage, oxidative stress was not detected, as mentioned before (Bensassi et al., 2009; Rezar et al., 2007). As the result of the oxidative consequences, membrane integrity can be also diminished following mycotoxin exposure, resulting in elevated MDA concentration due to the enhanced level of lipid peroxidation (Bócsai et al., 2016).

3.4. Heat shock proteins

Heat shock proteins are conserved molecules, produced in some form in all the living organisms. This group of molecules was indirectly and accidentally discovered by Ferruccio Ritossa in the 1962, as a set of genes whose expression had been increased in *Drosophila melanogaster* following heat shock (Ritossa, 1962). A decade later, these regions were found to code HSPs (Tissiéres et al., 1974). Initially it was hypothesized that HSPs only protect the organism against heat stress, but it has later been shown that their synthesis is provoked by several kinds of stressors including heat, ischemia, nutrient deprivation, infections, irradiation, heavy metals and other toxic substances, hence, their name is at first glance misleading (Gasch et al., 2000). HSP upregulation caused by these factors facilitates cell survival in the face of endogenous or exogenous threats that might cause cell damage or death (Finka et al., 2015). Furthermore, one kind of stress can provide tolerance to another type of stressor, such as heat exposure to heavy metals or vica versa. The phenomenon is referred as crossprotection (Sung et al., 2018). It was revealed by several research studies, that 50–200 genes are substantially induced by stressors in a wide variety of organisms ranging from archaea to human cells (Schumann, 2017).

In the wider sense, these stress-inducible proteins are categorized into seven classes based on their function (**Table 1.**). The first class is mostly referred as "molecular chaperones", which is the most prevalent group across all organisms (Richter et al., 2010). Their main role is to refold proteins that have denatured and lost their stability as a result of different stressors. In the narrow sense, numerous studies refer only the group of molecular chaperones as classical HSP-s. Components of a special proteolytic system, which are used to degrade irreversibly aggregated as well as misfolded proteins from the cell, belong to the second class. Members of the third category are responsible for the restoration of non-physiological alterations of nucleic acids such as heat-triggered methylation of ribosomal RNAs. These molecules include different RNA- and DNA-modifying enzymes, which are used to repair detrimental DNA modifications and processing failures that might occur during stress conditions (Jantschitsch et al., 2003). A group of specific metabolic enzymes, responsible for the regulation and stabilization of cellular energy supply make up the fourth HSP class (Malmendal et al., 2006). The fifth class of proteins contains transcription factors and kinases, both of which are needed to further trigger and stimulate stress response pathways or suppress expression cascades, such as ribosome assembly pathways (Refaii and Alix, 2009). Proteins required for the maintenance of the physiological structure of subcellular elements such as the cytoskeleton belong to the sixth class. The seventh group includes various detoxifying, transport and membrane-modulating proteins needed for the preservation of normal membrane function as well as stability (Richter et al., 2010).

Table 1. Classification of heat shock proteins

Class I.	Molecular chaperones (classical HSP-s)
Class II.	Specific proteolytic enzymes
Class III.	RNA- and DNA-repairing enzymes
Class IV.	Enzymes playing role in the stabilization of the cellular energy supply
Class V.	Transcription factors and kinases involved in the activation the stress response pathways
Class VI.	Proteins required for the maintenance of the physiological structure of subcellular elements
Class VII.	Detoxifying, transport and membrane-modulating proteins responsible for the preservation of normal membrane stability

Despite the fact that genes from these seven protein classes are upregulated in all the living creatures in response to heat stress, the exact members of the classes and the number of genes presented in each group of molecules can sometimes highly differ depending on the species. In contrast to archaea and prokaryotes, eukaryotic cells are hypothesized to express more chaperones and regulatory proteins, while metabolic, nucleic acid repair, and detoxifying gene products are of higher importance in the lower-level organisms. Further, this distinction may also be linked to the transition from unicellular to multicellular species (Richter et al., 2010).

Interestingly, based on the comparison of the stress induced response mechanisms of different organisms, it can be stated that the conservation of specific genes is considerably lower outside the class of molecular chaperones. This might be attributed to the fact that the particular role of individual proteins within the proteome varies greatly between different organisms across all the kingdoms of life resulting from their various biochemical lifestyles. As a consequence, it comes as no surprise that a unique, species-specific set of proteins involved in the repair of cellular damage has been developed beyond the conserved HSPs (Chen et al., 2018; Zininga et al., 2018). Individual heat-inducible gene expression changes are transient and very complex in the heat shock response, occurring quickly after exposure. As an example, *Saccharomyces cerevisiae* has been particularly well characterized in this regard. As yeast cells are transferred from physiological conditions (25°C) to elevated ambient temperature (37°C), the expression of most HSPs rapidly increases, peaking after 10–15 min (Gasch et al., 2000). However, some genes reach their expression maximum later (after 20 min), while others

do not increase even until after 2 h of incubation (Eisen et al., 1998). Genes involved in the maintenance of normal cell organization, DNA/RNA repair, and certain metabolic processes, exhibit a delay in expression. Although the reactions in the first phases refer to processes that quickly reverse the detrimental effects of thermal shock, the subsequent mechanisms might play a role in correlation with processes of adaptation or recovery following heat-induced cellular damage. Molecular chaperone genes are clearly among the most significantly upregulated HSPs, not only in yeast but also in other organisms, in terms of expression levels (Tiroli-Cepeda and Ramos, 2011).

Under physiological conditions, most molecular chaperones are already present at elevated levels. Moreover, the fact that unlike enzymes, chaperones are required in stoichiometric ratios to restore the normal structure of damaged and unfolded proteins also explains their enormous demand in the cells (Calderwood, 2018). As a result, they might become a significant component of overall cytosolic protein content in response to stress (Phipps et al., 1991; Pockley, 2003).

Molecular chaperones, as one of the most important group of HSPs (belonging to Class I.), are divided into six families:

- HSP100s
- HSP90s
- HSP70s
- HSP60s
- HSP40s (J-domain-containing proteins)
- HSP20s (sHSPs)

As the stability of cellular polypeptides is relatively low and aggregation can occur at all times. Even during physiological conditions, there is a persistent need for chaperones in the refolding of damaged proteins, as well as during *de novo* protein synthesis (Ahmad et al., 2015; Mayer, 2010). Molecular chaperones identify and react nonspecifically with a broad range of partially or globally unfolded polypeptides based on the recognition of structural irregularities. Such a detectable feature of unfolded proteins can be the increased exposure of hydrophobic amino acids, forming additional apolar parts in the molecule, or specific peptide sequences (Ciechanover and Kwon, 2017; Zhang et al., 2014). Molecular chaperones avoid unintended intermolecular interactions rather than provide exact structural information for folding. The mode of action is based on the change in affinity to the nonnative proteins, resulting in controlled binding and release mechanisms, coupled with ATP hydrolysis to cover the energy demand of the reaction. ATP consumption is characteristic for almost all heat shock protein families, except sHSPs (Jeng et al., 2015; Mogk and Bukai, 2017). The latter group is representing the first line of defense in several points of view, having a role in the initial capture

of the unfolded proteins. That is the reason why sHSPs are called as holdases, while other molecular chaperones like HSP70 and HSP90 received the foldase designation. Furthermore, while foldases are constantly presented in the cells and expressed in constitutively or stress-induced structure forms, holdases are sometimes only expressed in a higher concentration upon stress reaction, but the exact background and species-specific differences are not completely cleared yet (Borchel et al., 2018; de Graff et al., 2020).

Eukaryotic HSP70 has more than 50% amino acid sequence similarity compared to its prokaryotic version, called DnaK highlighting the fact, that this can be considered as one of the most conserved HSPs (Evans et al., 2010; Lopez et al., 2016). HSP70 can be involved in the normal protein folding under physiological conditions; however, under stress it successfully helps to impede unwished aggregation of unfolded proteins as well as plays a key role in the refolding of already aggregated proteins (Wruck et al., 2018). HSP70 is composed of two subdomains, which are the ATPase and the protein-binding domains. HSP70 activity is assisted by a variety of co-chaperons, such as the HSP40s, called also as J-domain-containing proteins, which has a prominent role in accelerating the ATP hydrolysis by HSP70 (Liu et al., 2020). Simultaneously, further molecules belonging also in the HSP40 class might play a role in the binding and delivering of the misfolded polypeptides to HSP70 (Alderson et al., 2016). The other important cochaperone of HSP70 are the so called nucleotide-exchange factors which are responsible for the facilitation of the HSP70 chaperone cycle, boosting the speed of ADP/ATP exchange after ATP hydrolysis (Bracher and Verghese, 2015). In addition, according to the most recent studies, HSP90 is suggested to have an important role in the assistance of HSP70 machinery (Morán Luengo et al., 2018). Significance of nucleotide-exchange inhibitor molecules in protein homeostasis has been also discovered through the regulation of HSP70 (Liang et Zhou, 2020).

In the category of molecular chaperones, sHSPs are prevalent and complex members, presented across all kingdoms of life (Basha et al., 2012). The number of various sHSP species correlates to the complexity and the developmental level of the organism. For this reason, in prokaryotes and unicellular eukaryotic cells one or two cytosolic sHSP species can be found, while the diversity of sHSPs increases in multicellular eukaryotes. The exact number of various sHSPs are not yet completely cleared in many of the animal species; however, it was already recorded, that in humans, 10 different sHSPs can be detected, while for example in *Caenorhabditis elegans* this number is 16. In case of plants, up to 50 different proteins belonging in this group of molecular chaperones can be identified (Mogk et al., 2019). More than 400 different proteins belong to the family, which is named after on their relatively low molecular weight (12-43 kDA) and include in most of the cases an α-crystallin domain which is considered to be a unique and characteristic element of these molecules (Basha et al., 2012).

Some of the most prominent members of the family are HSP27 (HSPB1), αA-crystallin (CRYAA; HSPB4), and αB-crystallin (CRYAB; HSPB5) (Haslbeck and Vierling, 2015; Singh et al., 2017; Skouri-Panet et al., 2012).

Small heat shock proteins typically form large spherical oligomers, often consisting of 24 subunits (Zhang et al., 2015). sHSPs are primarily ATP-independent chaperones and prevent the aggregation of unfolded proteins generated as a consequence of various harmful stressors such as heat stress (Mchaourab et al., 2009). According to recent findings, sHSPs may serve as storage depots for unfolded proteins, promoting the process of refolding induced by other molecular chaperones, such as HSP70, HSP90 and HSP100 (**Figure 4.**; Haslbeck and Vierling, 2015; Richter et al., 2010).

As major cytoskeletal chaperone proteins, sHSP-s have also received an increased attention as key biomarkers and potential therapeutic targets for several human and animal diseases, including cancer, neuropathies, and cataracts (Arrigo and Gibert, 2014; Huang et al., 2010). However, it should be also noted, that the exact interactions of the mentioned factors have not been fully investigated at the molecular level, especially in poultry.

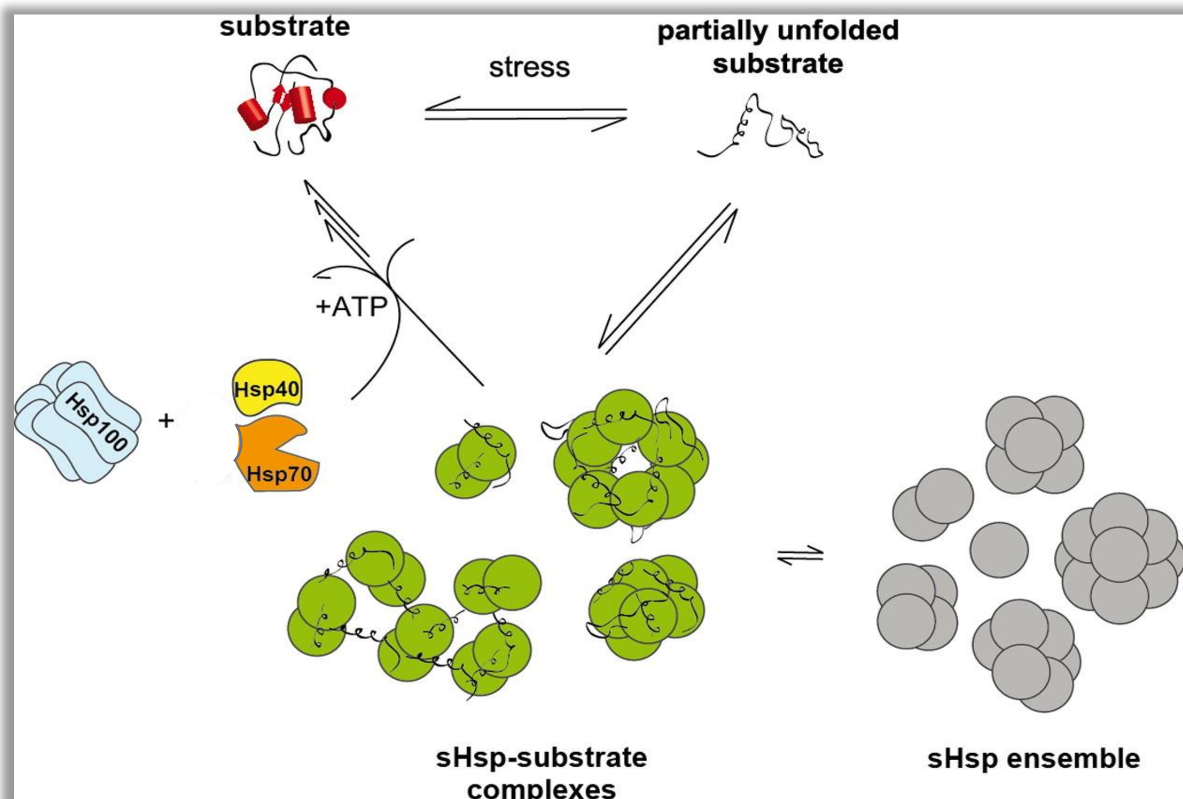

Figure 4. Mechanism of action and role of small heat shock proteins (sHSP) in protein folding and refolding (Haslbeck and Vierling, 2015). Following stress events, physiological protein structure may be disturbed resulting in the change of affinity of HSPs to the nonnative proteins. In the initial phase, sHSPs as major holdase-type HSPs capture the unfolded proteins without ATP demand. Holdases provide sufficient environment for the binding of foldase-type HSPs, such as HSP70 and HSP100, which are responsible for the ATP-dependent protein refolding.

3.5. Inflammatory cytokines with special emphasis on IL-6 and IL-8

Cytokines are small polypeptides produced by various cell types and have a significant impact on cell-cell interactions and communication. Cytokine is a general expression; however, this group of proteins may have other denominations such as monokines (cytokines produced by monocytes), lymphokines (cytokines made by lymphocytes), interleukins (cytokines produced by various cell types and acting mainly on leukocytes), and chemokines (cytokines with chemotactic activities; Zhang and An, 2007). Cytokines might have the ability to act directly on the cells that released them (autocrine action), on nearby cells (paracrine action), or even on distant cells (endocrine action; Papanicolau, 2000). It is typical for multiple cell types to produce the same cytokine or for a single cytokine to act on various types of cells at the same time. Further, their redundant activity means that similar cellular reactions can be stimulated by different cytokines (Tanaka and Kishimoto, 2014). They are playing an important role as parts of cascade mechanisms, as one cytokine induces its target cells to release additional cytokines. Cytokines can be synthesized by different cell populations including parenchymal cell types such as hepatocytes; however, the predominant producers are mainly macrophages and helper T cells (Zhang and An, 2007).

Different cytokines may act synergistically or antagonistically. According to the actual classification, the two major groups of cytokines are the pro-inflammatory (e.g., IL-1β, IL-6, IL-8 and TNF-α) and the anti-inflammatory cytokines (e.g. IL-4, IL-10, IL-11). The first group is involved in the up-regulation of inflammatory reactions, while the latter contains mostly immunoregulatory proteins that control and modulate the pro-inflammatory cytokine response. How a specific cytokine acts on its target cells depends on the characteristics of the molecule, the producing and responding cell type and abundance of the interleukin itself (Wojdasiewicz et al., 2014). Interleukins are produced in high concentrations during immune response, and the result of an inflammatory reaction depends on the balance of the released cytokines. IL-6 and -8 both have long been recognized as important pro-inflammatory cytokines whose expression is associated with numerous stressors including environmental or feedborne factors, such as heat stress and mycotoxin induced detrimental effects (Hammerich and Tacke, 2014).

Serum levels of IL-6 increase rapidly following inflammatory response, and can be therefore properly used as diagnostic marker to detect inflammatory conditions, such as liver injuries (Jekarl et al., 2013). IL-6 – as predominant member of IL-6-type cytokine family – binds directly to the cells by interacting with the 80 kD membrane glycoprotein (gp80) that complexes with the signal-transducing transmembrane molecule gp130. Dimerization of the intracellular domain of two gp130 molecules induces Janus kinase (JAK) and tyrosine kinase enzymes,

which reactions result in the phosphorylation of gp130. Following phosphorylation, signal transducer and activator of transcription proteins (STAT; mainly STAT-3) and Ras/MAPK become activated and trigger numerous downstream effects caused by IL-6 (Tacke et al., 2009).

On the other hand, IL-8 has been identified as a member of the CXC chemokine family. IL-8 acts via two related receptors: IL-8RA (CXCR1) and IL-8RB (CXCR2). Binding of IL-8 results in the activation of the coupled G-protein by the exchange of bound GDP to GTP. Subsequently, the trimeric G-protein dissociates, activating signal transduction pathways. Activated phosphatidylinositol 3-kinase (PI3K) generates phosphatidylinositol (3,4,5) triphosphate, which leads to the induction of phospholipase D and subsequently results in the accumulation of phosphatidic acid. At the same time, IL-8 also activates phospholipase C, which transforms phosphatidylinositol (4,5) bisphosphate to inositol (1,4,5) trisphosphate and diacylglycerol. Inositol (1,4,5) trisphosphate increases cytosolic Ca^{2+} concentration, which together with elevated diacylglycerol content leads to the activation of protein kinase C and the transcription factor NF-kB (Akdis et al., 2011).

Heat stress has been shown to have intense immunomodulatory effects and therefore a strong impact on interleukin levels. Heat shock response inhibited the expression of various cytokines, such as TNF-α, IL-1, IL-12, IL-10 and IL-18 (Wang et al., 2002). An additional study showed, that elevated ambient temperatures strongly affected the immune response by decreasing the splenic IL-6 and IL-12, and caecal IL-1β and IL-10 gene expression in chickens (Quinteiro-Filho et al., 2017). On the contrary, heat stress increased IL-1 and TNF-α expression in the brain and plasma of heat-stroked rats (Leon et al., 2006; Lin et al., 1995) and intensified IL-6 production in the gut mucosa *in vivo* (Wang et al., 2000) and in Caco-2 cells *in vitro* (Parikh et al., 1998). Further, the cellular immune system may also get diminished by heat exposure, reflected by decreased total white blood cell count (Mashaly et al., 2004) and macrophage activity (Bartlett and Smith, 2003).

Similarly to heat exposure, partly inconsistent data can be found in the literature regarding the effects of T-2 toxin on inflammatory cytokines and other mediators, *In vitro* studies showed decreased IL-1β and TNF-α concentrations in primary macrophages of porcine origin (Seeboth et al., 2012), while on the contrary, together with lipopolysaccharides, the toxin synergistically activated IL-1β and IL-18 mediated inflammatory response in human macrophages (Kankkunen et al., 2009). Furthermore, in mouse peritoneal macrophages, DON as another trichothecene mycotoxin of great importance, induced 78-kDa glucose-regulated protein (GRP78) degradation. GRP78 is known as a key regulator of endoplasmic reticulum (ER) and therefore an adequate marker of ER stress, which contributed to induced IL-6 expression (Shi et al., 2009). Similarly to the latter findings, T-2 toxin induced significantly higher IL-6 and IL-8 production in intestinal IPEC-J2 cell cultures (Pomothy et al., 2020).

In summary, current results are often conflicting how heat stress and trichothecene exposure affect interleukin production, and whether they exert immunosuppression or act as immunostimulant effectors. The outcome of such events might depend on several factors, such as the species of the affected animals, the severity and length of the exposure or the investigated cell types.

3.6. Importance of *in vitro* cell culture models

Cell culture studies provide a valuable complement to *in vivo* experiments and are especially useful for enhancing our understanding of tissue and cell morphology as well as cell biology and metabolic processes. The method also provides in-depth knowledge about the development and pathomechanism of various diseases and concerning the mode of action of certain drugs or drug candidates in pre-clinical and clinical trials (Kapałczyńska et al., 2018).

The first cell cultures were established by Harrison in 1907 in a research related to the origin and function of nerve fibers (Harrison et al., 1907). Thereafter, the procedure has been constantly developed and was used to observe cell growth and differentiation in the beginning (Harrison, 1959). At the present time, *in vitro* experiments can be carried out using primary cells isolated directly from donors' organ or already existing immortalized cell lines preserved in cell banks (Burdall et al., 2003). Primary cultures are deriving from living organisms and typically include populations of various cell types present in the source tissue. This is the reason why it is crucial to apply a proper isolation protocol to separate the required cell types (LeCluyse et al., 2005). It is also worth to mention, that another characteristic feature of primary cell cultures, besides the difficulties with isolation is their shorter life span in comparison with cell lines. However, they closely mimic genotypic and phenotypic profile of *in vivo* cells and thus make it possible to perform more specific and accurate experiments in many aspects (Dutton et al., 2019).

On the contrary, although cell lines consist of a more uniform population of cells, they are often directly or indirectly genetically modified, what can highly alter their phenotype, native functions and metabolic profile. Further, constant passage of cell lines can cause genotypic and phenotypic alterations over an extended period as well, which genetic drift might result in heterogeneity of subcultures after a certain time. For this reason, cell lines may not always adequately represent *in vivo* circumstances and may provide different or in some cases, even misleading results (Kaur and Dufour, 2012). Furthermore, there are also other major disadvantages of cell lines in comparison to primary cell cultures, such as the contamination by other cell lines or *Mycoplasma* infection. Cross-contamination of cell lines either inter- or intraspecies was first investigated in the early 1970s. According to the contemporary results of wide-range screening studies, the majority of cell lines distributed by cell banks and used worldwide at that time point, were contaminated with HeLa cells (Nelson-Rees et al., 1981). When such contamination of another, rapidly proliferating cell line occurs, the culture can be entirely taken over by the invading cell line even after a few passages. Furthermore, *Mycoplasma* infection can persist invisibly and undetectably in cell cultures for a longer time period while causing important alterations in gene expression and subsequently in cell metabolism (Armstrong et al., 2010). *Mycoplasma* contamination was found to increase the

phosphorylation of NF-κB and MAPK signal pathways as well as results in altered gene expression and synthesis of certain inflammatory factors, leading to altered inflammatory response and decreased sensitivity to lipopolysaccharide stimulation *in vitro* (Feng et al., 2019). Because of all the mentioned factors, great care must be taken when applying cell lines in research, and primary cell cultures should be included and preferred over cell lines in experiments where key findings have to be confirmed.

Culturing of the cells can be carried out under adherent conditions where they are directly attached to the cell culture plate forming a 2D monolayer, or in a suspension culture, which in some cases (e.g. blood-derived lymphocytes) resembles more accurately to the original circumstances of the living organism (Tsang et al., 2017). Another possibility is the establishment of 3D cell cultures, which have been gaining attention nowadays due to their further benefits mimicking in some aspects better the original *in vivo* conditions (Pampaloni et al., 2007). The benefits of 2D models include easy and low-cost cell culture maintenance as well as the ability to perform quickly and conveniently several functional tests. Unfortunately, adherent cultures also have some limitations such as they do not perfectly mimic the natural structures of tissues due to the altered cell-cell and cell-extracellular environment interactions which factors may affect also cell differentiation, proliferation and the expression of certain genes and proteins (Hickman et al., 2014).

On the other hand – considering the classification based on cell type composition of the applied cell cultures – these models can be classified into the group of mono-cultures and co-cultures. Mono-culturing is the methodology, in which only one type of cells is included in the culture, while in case of co-cultures investigation of multiple, distinct cell types is possible within the same culture environment (Paschos et al., 2015). Co-culture systems were first created in the 1980s as a system for studying cell–cell communication (Lawrence et al., 1978). The methods of co-culture preparation varied with the time, but in general it can be stated that this technique has been performed mostly for the purpose of analyzing the specific role of certain cell types in the investigated processes and to observe the different cell-cell interactions and their relationship with each other (Mamidi et al., 2011; Sharma et al., 2004). Three types of cell–cell interactions can be observed in co-culture models, which are the cell–cell adhesion, cell–extracellular matrix connection, and paracrine signaling through soluble molecules. All three types of interactions can have a concurrent effect within the cell cultures, or each type can exist individually too, depending on the culture environment (Paschos et al., 2015). Direct co-cultures can be prepared in simple cell culture dishes, for instance by layering two cell types onto each other, or mixing the different cell suspensions prior seeding. In some cases; however, direct physical contact may be associated with numerous technical difficulties. In case of such an issue, preparation of an indirect co-culture is advantageous, where the

insertion of a porous membrane is also necessary to keep the co-cultivated cell types separated (Zou et al., 2018).

Regarding the significance of cell culture systems and the extrapolability of results achieved applying *in vitro* models a valuable example is a study carried out by Chen et al., (2015). In this project, the effects of heat stress in rat myocardial cells were compared *in vivo* and *in vitro* to confirm the reliability of the results achieved in cell culture experiments. The level of cellular damage was measured by the detection of creatine kinase isoenzyme, creatine kinase-MB and lactate dehydrogenase activity, while HSP70 concentrations were also monitored. The results revealed similar trends in the parameters *in vivo* and *in vitro*; however, there were smaller differences detected in correlation with time dependency (Chen et al., 2015). These results highlight the great potential of cell culture models, especially taking animal welfare issues as well as the 3R principle into consideration, but it also suggests the limitations of these methods.

Primary cultures of hepatocytes serve as advantageous tools that have been extensively applied in the research of physiological liver function, as well as in the investigation of liver diseases, pathophysiology, pharmacology, xenobiotic biotransformation as well as inflammatory response (Shen et al., 2012). Nevertheless, it is recommended to incubate hepatocytes with defined amount of NP cells (predominantly Kupffer cells) in appropriate concentration, for proper investigation of hepatic inflammatory processes. Further, with the proper adjustment of hepatocyte - Kupffer cell ratios, different phases and severity of hepatic inflammation can be modelled. For instance, with the ratio of 6:1 (hepatocytes to Kupffer cells) milder, while with 2:1 ratio severe hepatic inflammation can be successfully mimicked (Mátis et al., 2017). Such unique cell culture systems have been already developed of rat (Bhatia et al., 1999 and porcine origin (Mátis et al., 2017); however, according to our recent knowledge, no similar model exists in case of avian cell cultures. The importance and relevance of the establishment of such a co-culture is supported by the fact that Kupffer cells play a key role in the mediation of hepatic inflammatory response, and have an important role in physiological metabolic processes as well (Nguyen-Lefebvre and Horuzsko, 2015).

4. Significance and aims of the study

Heat stress and mycotoxin contamination both belong to the most important immunomodulatory factors in intensive broiler farming impairing animal health and productivity as well as resulting in animal welfare issues. On cellular level, excess heat exposure can trigger heat shock response acting for the restoration of cell homeostasis by several mechanisms, such as affecting heat shock protein synthesis, redox homeostasis and pro-inflammatory cytokine production.

Our research group has already been established and characterized a primary co-culture comprised of hepatocytes and NP cells (predominantly resident macrophages, Kupffer cells) of pig origin, which can serve as proper tool for investigations on the cellular inflammatory and stress response. Since no similar avian liver cell culture models have been prepared before, 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 of hepatic cells in birds compared to mammals, our existing cell isolation procedures had to be adapted to chickens, and separated cell fractions needed to be characterized by specific methods, such as immunocytochemistry and flow cytometry (**Study I.**).

Furthermore, the molecular effects of a shorter (1 h) and a longer (2 h) heat exposure on the metabolic activity, oxidative status (H_2O_2), HSP70 and pro-inflammatory cytokine production (IL-6 and IL-8) were aimed to be assessed on the newly established primary liver cell cultures mimicking acute heat stress *in vitro*. Applying mono-cultures of hepatocytes and co-cultures of parenchymal and NP cells may highlight the role of different cell types in stress response. The established co-cultures as inflammatory models can presumably contribute to understand the link between hepatic inflammation and distress conditions (**Study II.**).

A further aim of the present PhD study was to investigate the effects of T-2 toxin on the applied hepatic cell culture models. As playing a crucial role in the detoxification processes, and as one of the main targets of trichothecene mycotoxins, liver is especially exposed to the harmful effects of T-2 toxin. Further, as other studies provided different results depending on the investigated organs and cell types, another goal of the trial was to compare the effects of T-2 toxin on the mono- and co-cultures, monitoring cell type specific differences. According to existing studies as well as the initial hypothesis of our study, T-2 toxin similarly to heat stress, may alter metabolic activity, redox status and pro-inflammatory cytokine production of the examined cell culture models (**Study III.**).

Finally, in order to extend our knowledge about the acute heat shock response, the main aim of the *in vivo* study was to investigate the impact of acute heat stress on cellular oxidative status and sHSP abundance in parenchymal organs, such as in the spleen, kidney or liver. To gain a comprehensive understanding of the redox state of the animals, MDA, GSH, and protein carbonyl concentrations, as well as GPx enzyme activity, were examined. To assess the function and role of sHSPs in the HSR of chickens, HSP27, αA-crystallin, and αB-crystallin protein concentrations were determined in the investigated organs. This study may reveal novel data concerning the complex interplay of sHSPs and heat-provoked oxidative damage in different tissues of chickens, while also reflecting key points for alleviating the adverse effects of heat stress (**Study IV.**).

Table 2. Overview of the performed studies

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 in liver, spleen and kidney tissue samples

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 cells) 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 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.

5. Materials and methods

5.1. Ethic statement

In **Study I.**, in order to setting up the cell isolation and separation procedure, some preliminary measurements were carried out using one broiler in each trial (eight totally). For the characterization of cell fractions gained by the finally established method (with immunocytochemistry and flow cytometry) and to study the cellular effects of heat stress (**Study II.**) and T-2 toxin (**Study III.**), liver cells had to be isolated from the same single chicken in order to ensure the homogeneity of the prepared primary cell cultures. All experimental procedures as well as housing, feeding, and treatment of the animals were carried out in strict 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, approval date: 27 April 2015). Experimental procedures of **Study IV.** 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).

5.2. Chemicals and reagents

All reagents were purchased from Merck KGaA (Darmstadt, Germany), except when otherwise specified.

5.3. Study I.

5.3.1. Cell isolation and culturing conditions

In **Study I.**, 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 (Aviagen 308, 2018), and obtained from Gallus Ltd. (Devecser, Hungary).

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°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 (**Figure 5.**).

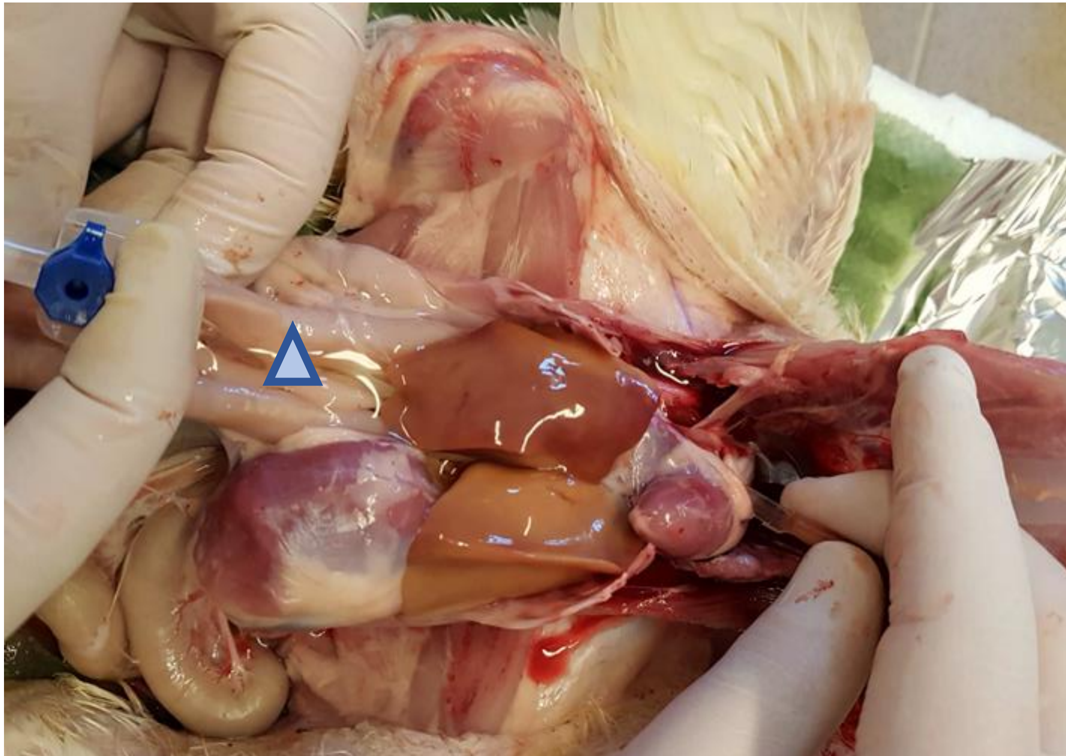

Figure 5. Cannulation of the hepatic portal system via the gastropancreaticoduodenal vein (marked with blue triangle).

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 unwished cell aggregate formation. Thereafter, the cell suspension was centrifuged three times at 100× g for 3 min, and the hepatocyte-enriched sediment was resuspended in Williams' Medium E, supplemented with 0.22% $NaHCO_3$, 50 mg/L gentamycin, 2 mM glutamine, 4 μg/L dexamethasone, 20 IU/L insulin, and 5% foetal bovine serum (FBS).

The hepatic NP cell fraction (containing mostly Kupffer cells as resident macrophages) was separated from the supernatants gained in the low-speed (100× g) centrifugation steps. The supernatants were centrifuged at 350× g for 10 min to sediment the remaining hepatocytes, cell detritus and red blood cells, and the newly gained supernatant was centrifuged again at 800× g 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 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: 8.5×10^5 cells/mL hepatocytes; 1.5×10^5 cells/mL NP cells).

Cell cultures were prepared on 6-well Advanced TC plates (Greiner Bio-One, Frickenhausen, Germany) and Lumox x-well (Sarstedt, Nümbrecht, Germany) cell culture dishes, previously coated with collagen type I 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 (hepatocytes 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 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.

5.3.2. Characterization of cell cultures with Giemsa staining and immunocytochemistry

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 on the Lumox-x well plates, immunocytochemical analyses were carried out using chicken specific antibodies. Albumin was detected with a chicken specific, fluorescein isothiocyanate (FITC) coupled anti-albumin antibody (Cedarlane, Burlington, Canada). Macrophages in the NP cell fraction were labelled by using a chicken macrophage specific phycoerythrin (PE) coupled antibody (Southern Biotech, Uden, The Netherlands).

Following 48 h incubation, cultures 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 (Olympus Corporation, Tokyo, Japan) and a Canon EOS 1100D camera (Canon Inc., Tokyo, Japan).

5.3.3. Characterization of cell cultures with flow cytometry

Hepatocyte enriched and NP cell containing fractions were examined with flow cytometry. Cell suspensions with approximately 1×10^6/mL cell concentration were filtered prior to data acquisition 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 (Beckman Coulter Inc., Brea, CA, USA). 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. Isolated cells have been also characterized applying the abovementioned FITC coupled anti-albumin and PE conjugated macrophage specific antibodies. Data gained by flow cytometry were analyzed 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.

5.4. Study II. and III.

5.4.1. Cell Isolation and culturing conditions

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.** Following multi-step perfusion of the liver, hepatocytes and NP cells were isolated by differential centrifugation. Both hepatocyte mono- and hepatocyte – NP cell co-cultures were prepared in 6-well and 96-well Advanced TC (Greiner Bio-One, Frickenhausen, Germany) type I collagen coated cell culture dishes. Cultures were incubated at 38.5°C in 90-100% relative humidity along with 5% CO_2. Culture media were changed 4 h following seeding, and confluent monolayers were gained after 24 h culturing as previously described.

5.4.2. Treatments of the cell cultures

In **Study II.**, following 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 heat stress, while control cells were incubated further at 38.5°C. The incubation conditions were set based on literature data and on our pilot studies, considering that birds have generally higher physiological body temperature than mammals; however, cells isolated from avian species are often cultured at temperatures similar to those of the mammalian cells. Normal incubation temperatures of avian cell cultures are ranging from 37 to 41.5°C, and temperatures mimicking heat stress are varied between 40 and 45°C (Ibtisham et al., 2018; Slawinska et al., 2016; Sung et al., 2018; Xu et al., 2017), hence, as a compromise, 38.5°C was chosen for maintaining control cells, and 43°C (lasting for 1 or 2 h) was applied to study the *in vitro* effects of acute heat stress.

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, in both the applied cell culture models.

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; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA) and 1% ethylene diamine tetraacetic acid (EDTA). Samples were stored until further analysis at −80°C.

5.4.3. Measurements

5.4.3.1. Cellular metabolic activity

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 (Thermo Fisher Scientific, Waltham, MA, USA) after 2 h incubation at 38.5°C.

5.4.3.2. LDH leakage test

In order to monitor cytotoxicity, extracellular 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 a 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.

5.4.3.3. Extracellular H_2O_2 production

Extracellular H_2O_2 concentration of cell supernatants was assessed with the Amplex Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, MA, 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 a working solution at room temperature, fluorescence was detected with a Victor X2 2030 fluorometer (λex = 560 nm; λem = 590 nm).

5.4.3.4. Extracellular HSP70, IL-6 and IL-8 concentrations

The concentrations of HSP70, IL-6, and IL-8 were measured in the culture media of 6-well dishes by chicken specific sandwich 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 Pierce Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Waltham, MA, 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.

5.4.4. Statistical analysis

All the data analysis was performed using R 3.5.3. software (GNU General Public License, Free Software Foundation, Boston, MA, USA). On 96-well plates, six wells were included in one treatment group, while cells were examined in triplicates on 6-well plates. Normal distribution and homogeneity of variance were checked by Shapiro-Wilk test and Levene's test, respectively. Differences between various groups were assessed using one-way analysis of variance (ANOVA) and post-hoc tests for pairwise comparisons. All data were standardized to protein concentrations measured from the cell lysates. Results were assessed as the mean ± standard error of the mean (SEM). Differences were assumed significant at $P < 0.05$.

5.5. Study IV.

5.5.1. Animals, treatments and samplings

Raising and housing of the animals was carried out in the Department of Animal Sciences, Georgikon Faculty, Hungarian University of Agriculture and Life Sciences, Keszthely, Hungary. One-day-old male 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 over the entire study, except the day of the treatment.

Three-phase fattening was used providing feed and drinking water *ad libitum*, applying starter (day 0-10; mash), grower (day 11-24; pelleted feed) and finisher (from day 25; pelleted feed) diets. The experimental diet was based on corn and wheat with sunflower meal, DDGS (distillers dried grains with solubles) and soybean meal as main protein sources. Vitamins and trace elements, according to nutritional recommendations, were provided via a commercial, phase-specific premix. The composition and the calculated nutrient content of diets as well as premix composition are shown in **Tables 3-5.** The growth performance of the animals (with 1062 ± 10.1 g body weight at the end of the grower phase) matched the parameters described in the Broiler Management Handbook for Ross 308 broilers (Aviagen 308, 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, as stated above.

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). Following selection process, body weight of the animals has been also measured in order to confirm random

distribution. 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 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.

Table 3. Composition of experimental diets (g/kg feed). DDGS: distillers dried grains with solubles

Ingredient	Starter (day 0-10)	Grower (day 11-24)	Finisher (day 25-32)
Corn	375	406	430
Wheat	100	100	100
Soybean meal, 46%	345	239	215
Sunflower meal, 34%	50.0	100	100
DDGS Corn	30.0	50.0	50.0
Sunflower oil	54.2	64.9	68.3
Monocalcium phosphate	10.9	8.27	7.20
Limestone	18.3	15.8	15.1
L-Lysine	3.82	4.45	3.78
DL-Methionine	3.14	2.33	2.02
L-Threonine	0.89	0.73	0.54
L-Valine	0.63	0.27	0.00
L-Isoleucine	0.00	0.00	0.00
Premix	5.00	5.00	5.00
Salt	2.85	2.68	2.67
Sodium bicarbonate	0.61	0.57	0.59
Phytase	0.10	0.10	0.10
Xylanase	0.10	0.10	0.10

Table 4. Calculated nutrient content of the diets (%).
AME_n: nitrogen-corrected apparent metabolizable energy

	Starter (day 0-10)	**Grower (day 11-24)**	**Finisher (day 25-32)**
Crude protein	23.0	20.7	19.6
AME_n (MJ/kg)	12.65	13.0	13.2
Ca	1.05	0.90	0.85
Available P	0.35	0.30	0.27
Crude fibre	4.17	4.86	4.78
Na	0.16	0.16	0.16
Cl	0.23	0.23	0.23
Lys	1.39	1.20	1.10
Met	0.65	0.56	0.52
Cys	0.36	0.34	0.31
Met+Cys	1.01	0.90	0.84
Thr	0.93	0.82	0.77
Trp	0.28	0.25	0.23
Val	1.12	0.98	0.91
Ile	0.96	0.84	0.80
Arg	1.53	1.36	1.29
Gly	1.10	0.99	0.94
Ser	1.09	0.96	0.91

Table 5. Premix composition (per kg premix)

Nutrient content		
Dry matter	%	96.5
Crude ash	%	58.4
Ca	%	14.0
Total P	%	0.01
Available P	%	0.01
$NaHCO_3$	%	0.20
Zn	mg	22,000
Cu	mg	3,000
Fe	mg	15,000
Mn	mg	22,000
I	mg	400
Se	mg	80.0
Vit A	IU	3,200,000
Vit D3	IU	1,160,000
Vit E	mg	20,000
Vit K3	mg	1000
Thiamine	mg	800
Riboflavin	mg	2,000
Pyridoxine	mg	1220
Vit B12	mg	10.0
Nicotinic acid	mg	15,400
Pantothenic acid	mg	4,800
Folic acid	mg	540
Biotin	mg	48.0
Cholin chloride	mg	90,000
Betain	mg	50,000

5.5.2. Sample processing

Before the laboratory analyses tissue samples were 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 (Thermo Fisher Scientific, Waltham, USA) 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 (Thermo Fisher Scientific, Waltham, USA) using bovine serum albumin (BSA) as standard. Absorbance values were recorded at 562 nm after 30 min incubation at 37°C using a Multiskan GO 3.2 reader.

5.5.3. Measurements

5.5.3.1. Malondialdehyde concentration

As a marker of lipid peroxidation, MDA concentration was measured by a specific colorimetric test. According to the protocol, 300 µL freshly prepared thiobarbituric acid (TBA) stock solution was mixed with 100 µL diluted tissue homogenate supernatant. 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.

5.5.3.2. Reduced glutathione concentration

As a prominent member of the antioxidant defense system, 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 37°C 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 37°C. Absorbance was measured at 412 nm.

5.5.3.3. Glutathione peroxidase activity

Glutathione peroxidase enzyme activity 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.

5.5.3.4. Protein carbonyl concentration

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 13,000x g, supernatant was removed, and freshly formed pellet was dissolved and incubated at -20°C in 500 µL ice-cold acetone for 5 min. After the second centrifugation (13,000x 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.

5.5.3.5. HSP27, αA-crystallin and αB-crystallin concentration

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). In case of the first two parameters, competitive ELISA, while for the analysis of HSP27 concentration, sandwich ELISA tests were applied. Absorbance values were detected at 450 nm using a Multiskan GO 3.2 microplate reader.

5.5.4. Statistical analysis

Data analysis was performed using R 3.5.3. software (GNU General Public License, Free Software Foundation, Boston, MA, USA). Normal distribution and homogeneity of variance were checked by Shapiro-Wilk test and Levene's test, respectively. 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. Results were expressed as the mean ± standard error of the mean (SEM). Differences were considered significant at $P < 0.05$.

6. Results

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

6.1.1. Giemsa staining and immunocytochemistry

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 6.**).

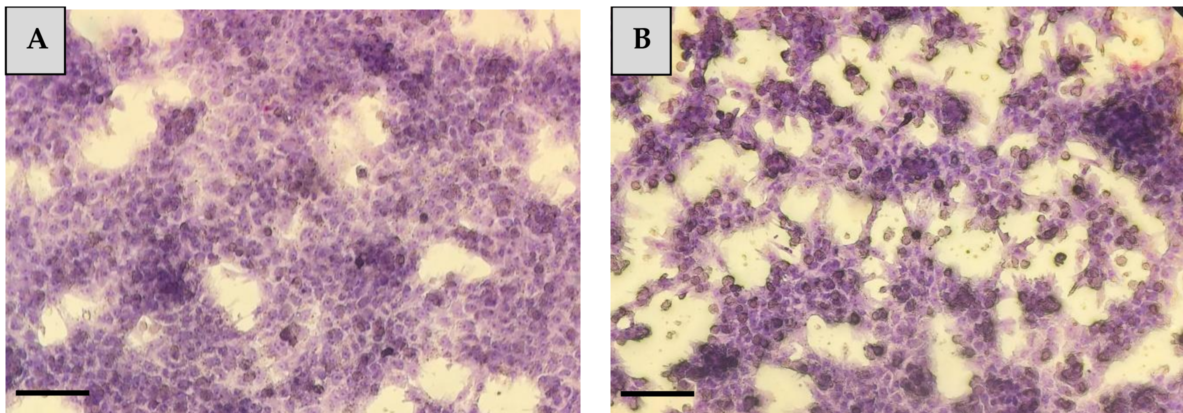

Figure 6. Giemsa staining of A. hepatocyte mono-cultures and B. hepatocyte – non-parenchymal cell co-cultures after 48 h culturing (200x magnification, bar = 100 µm).

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 7.**). 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 8.**).

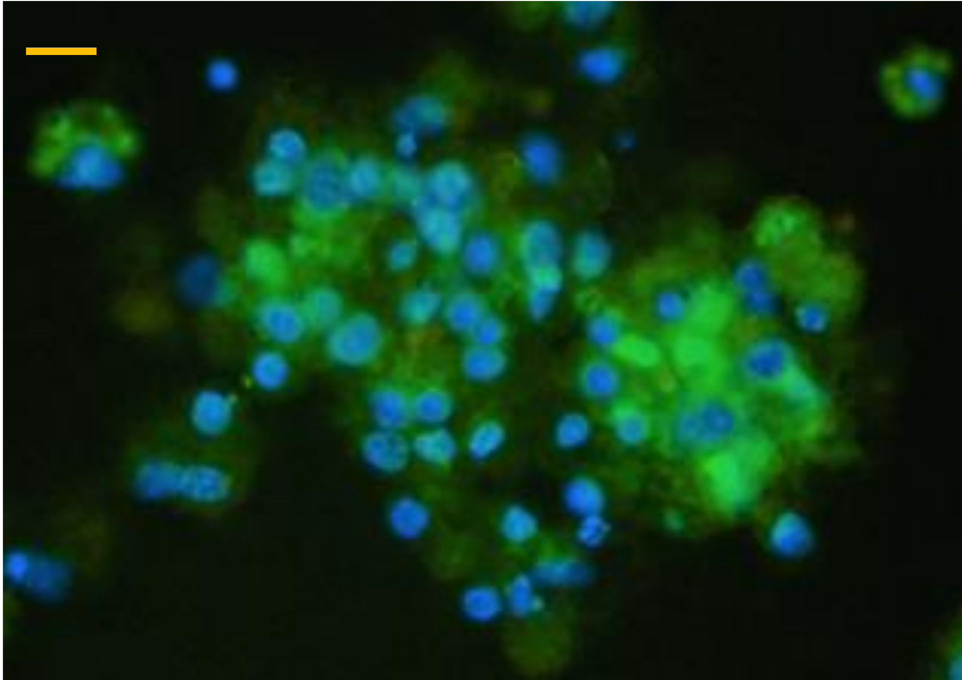

Figure 7. Immunofluorescent detection of hepatocytes in a hepatocyte – non-parenchymal cell co-culture after 48 h culturing with a chicken specific, fluorescein isothiocyanate (FITC) coupled anti-albumin antibody (400x magnification, bar = 40 μm). Blue color indicates cell nuclei with DAPI staining, while green color refers to hepatocytes detected with the FITC conjugated antibody.

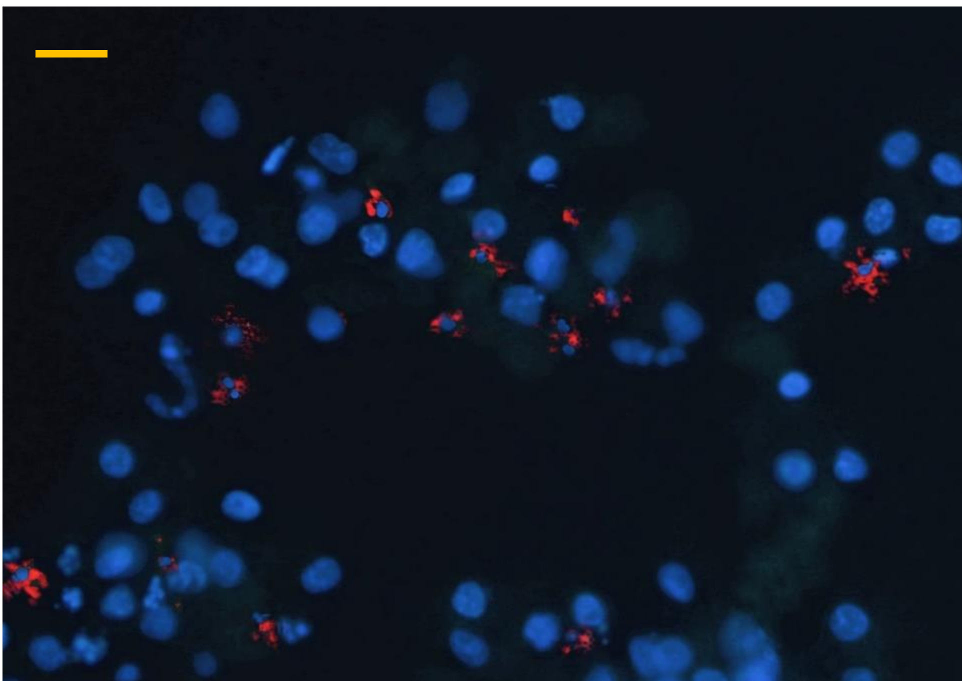

Figure 8. Immunofluorescent detection of macrophages in a hepatocyte – non-parenchymal cell co-culture after 48 h culturing with a phycoerythrin (PE) coupled chicken macrophage specific antibody (400x magnification, bar = 40 μm). Blue color indicates cell nuclei with DAPI staining, while red color refers to macrophages detected with the PE conjugated antibody.

6.1.2. Characterizing cell fractions with flow cytometry

Applying scatter profiles to monitor the characteristics and homogeneity of the separated cell suspensions, two well-defined, reproducible fractions were isolated (**Figure 9.**). The type of the comprising cells in 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.

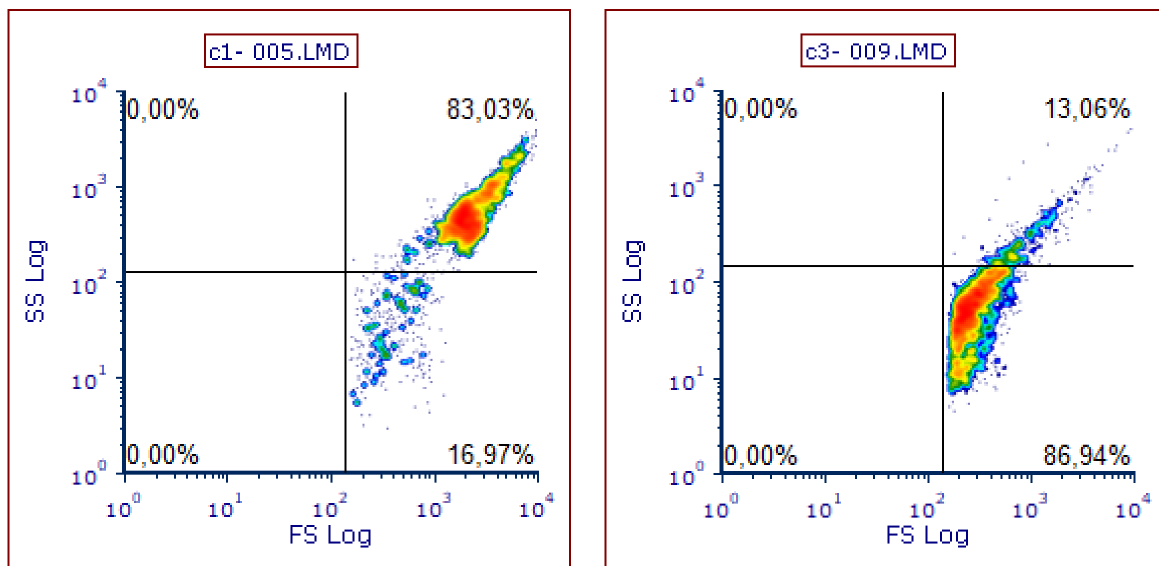

Figure 9. A representative density plot figure showing the forward (FS) and side (SS) scatter profiles of hepatocytes (left panel) and non-parenchymal cells (right panel).

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

6.2.1. Cellular metabolic activity

The metabolic activity of cultured cells, monitored with the CCK-8 assay (**Figure 10.**), 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 ($P = 0.004$).

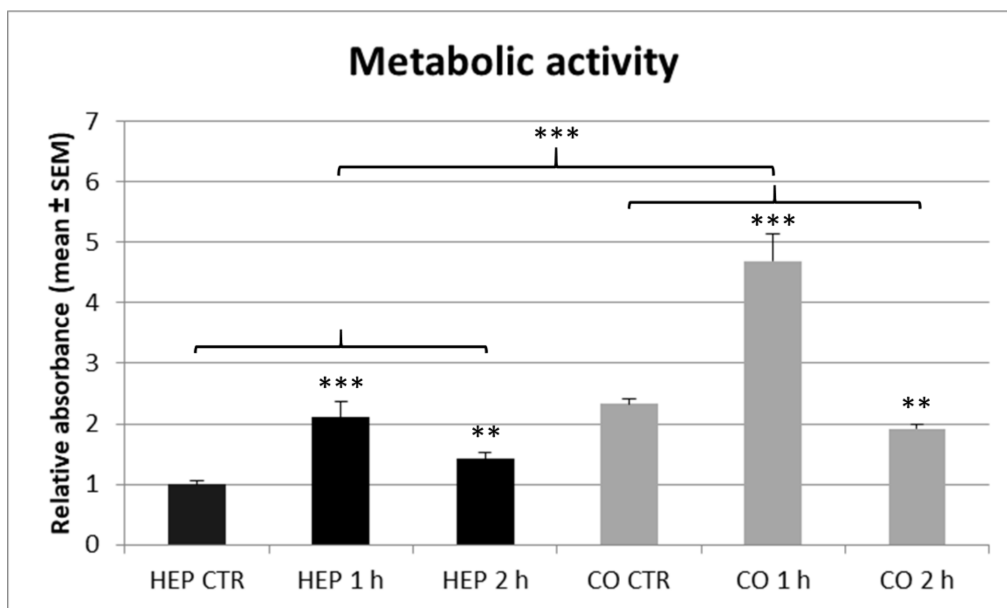

Figure 10. The metabolic activity of hepatocyte mono-cultures (“HEP”) and hepatocyte – non-parenchymal cell co-cultures (“CO”) as indicated by the CCK-8 assay. The “CTR” refers to control cells with no heat exposure, while “1 h” and “2 h” indicate incubation of cell cultures at 43ºC for 1 h or 2 h, respectively. 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. **$P < 0.01$, ***$P < 0.001$.

6.2.2. LDH leakage test

The amount of necrotic cells was monitored by measuring the extracellular LDH activity (**Figure 11.**). 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.

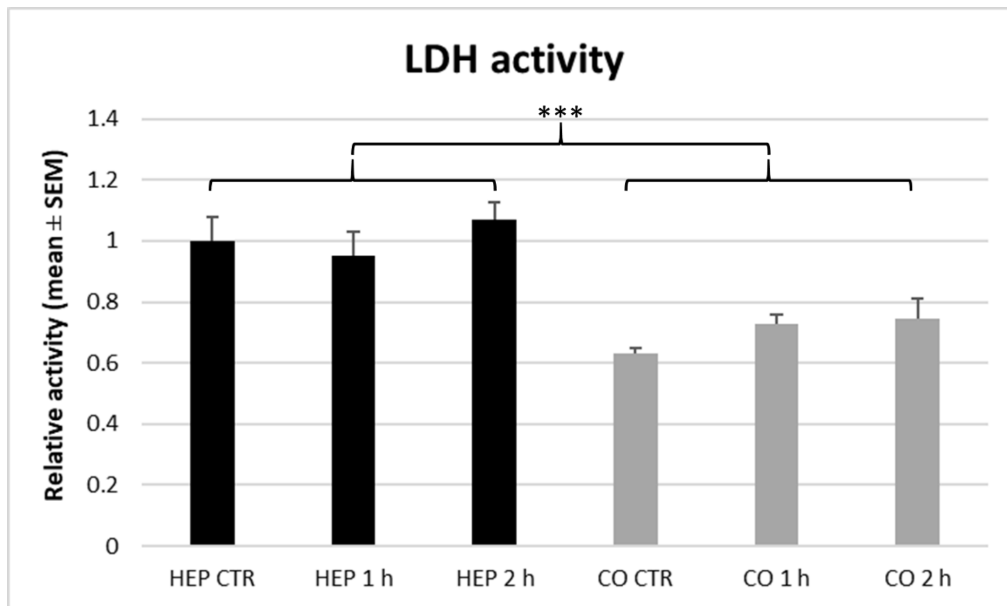

Figure 11. The LDH activity of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") as indicated by specific photometric assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43°C for 1 h or 2 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. ***$P < 0.001$.

6.2.3. Extracellular H_2O_2 production

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 12.**). 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.

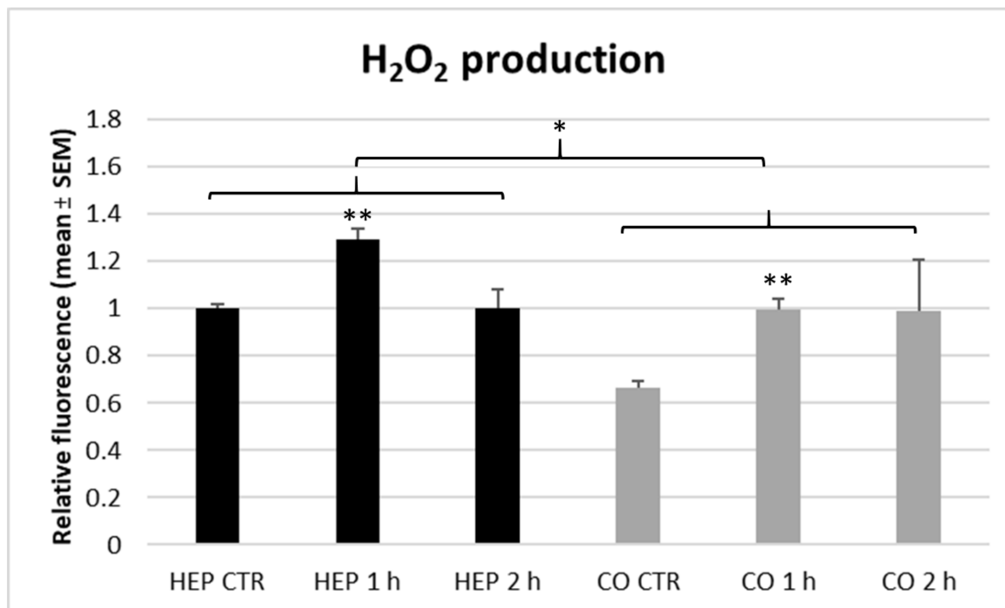

Figure 12. The hydrogen peroxide (H_2O_2) production of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") as indicated by the Amplex Red assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43°C for 1 h or 2 h, respectively. Relative fluorescence values 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.

6.2.4. Extracellular HSP70 concentration

The HSP70 concentration of cell culture supernatants (**Figure 13.**), 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 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.

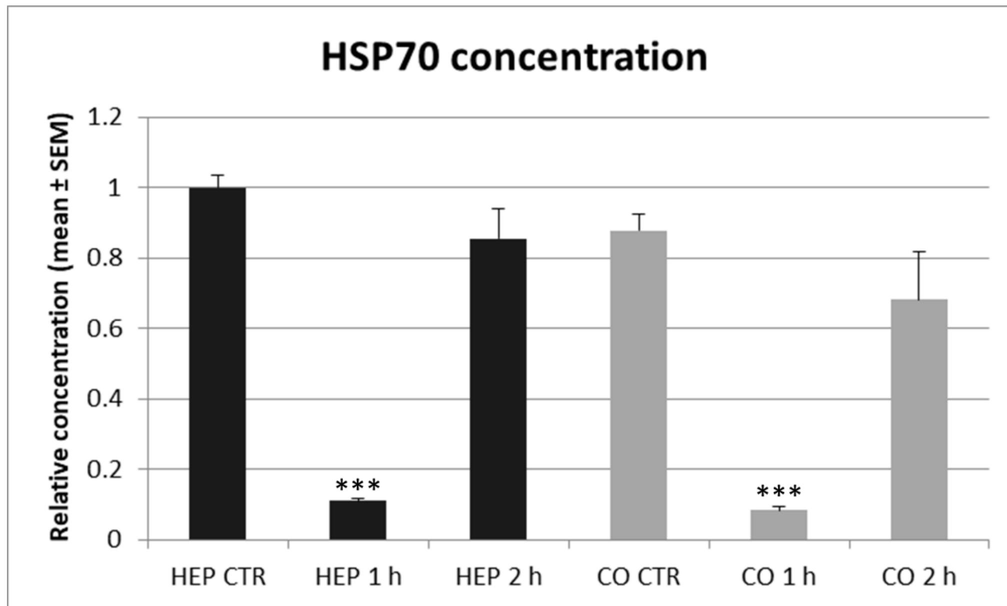

Figure 13. The heat shock protein 70 (HSP70) concentration in culture media of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") as measured by a chicken specific ELISA assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43ºC for 1 h or 2 h, respectively. Relative concentrations were calculated by considering the mean value of control hepatocyte mono-cultures as 1 (4.17 mg/mL). 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.

6.2.5. Extracellular IL-6 and IL-8 concentration

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 14.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.

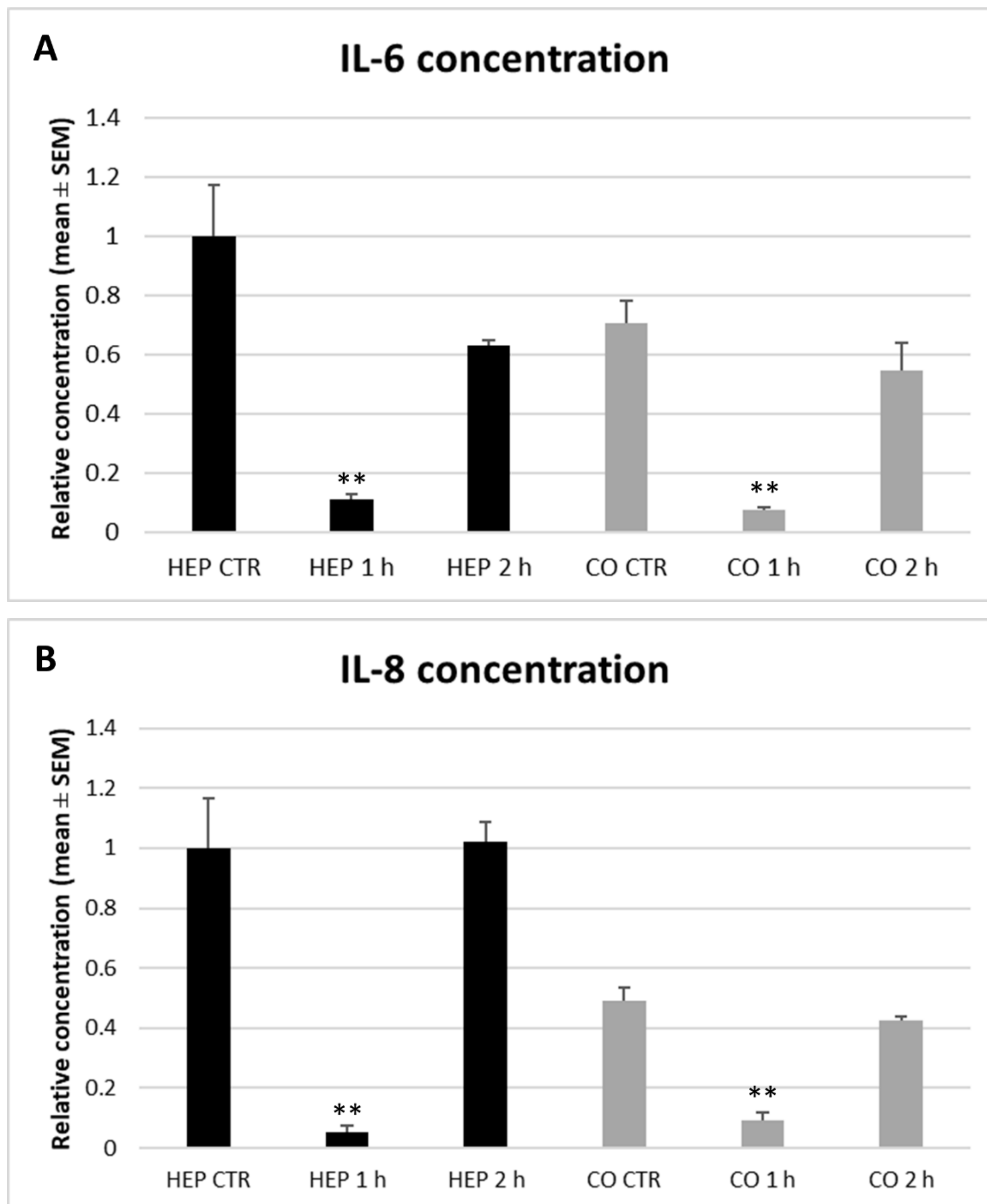

Figure 14. Interleukin-6 (IL-6; **A**) and interleukin-8 (IL-8; **B**) concentration in culture media of hepatocyte mono-cultures ("HEP") and hepatocyte – non-parenchymal cell co-cultures ("CO") detected by a chicken specific ELISA assay. The "CTR" refers to control cells with no heat exposure, while "1 h" and "2 h" indicate incubation of cell cultures at 43ºC for 1 h or 2 h, respectively. Relative concentrations were calculated by considering the mean value of control hepatocyte mono-cultures as 1 (11.52 and 1.38 ng/mL, respectively). 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. **$P < 0.01$.

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

6.3.1. Cellular metabolic activity

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 15.A.**) and 24 h (**Figure 15.B.**) incubation times (8 h incubation of hepatocyte mono-culture: $P = 0.003$; $P = 0.011$; $P = 0.027$ for 10, 100 and 1000 nmol/L T-2 toxin, respectively; 8 h incubation of co-culture: $P = 0.045$; $P = 0.002$; $P = 0.002$ for 10, 100 and 1000 nmol/L T-2 toxin, respectively, 24 h incubation of hepatocyte mono-culture: $P = 0.002$; $P < 0.001$; $P = 0.002$ for 10, 100 and 1000 nmol/L T-2 toxin, respectively, 24 h incubation of co-culture: $P < 0.001$; $P = 0.043$; $P < 0.001$ for 10, 100 and 1000 nmol/L T-2 toxin, respectively).

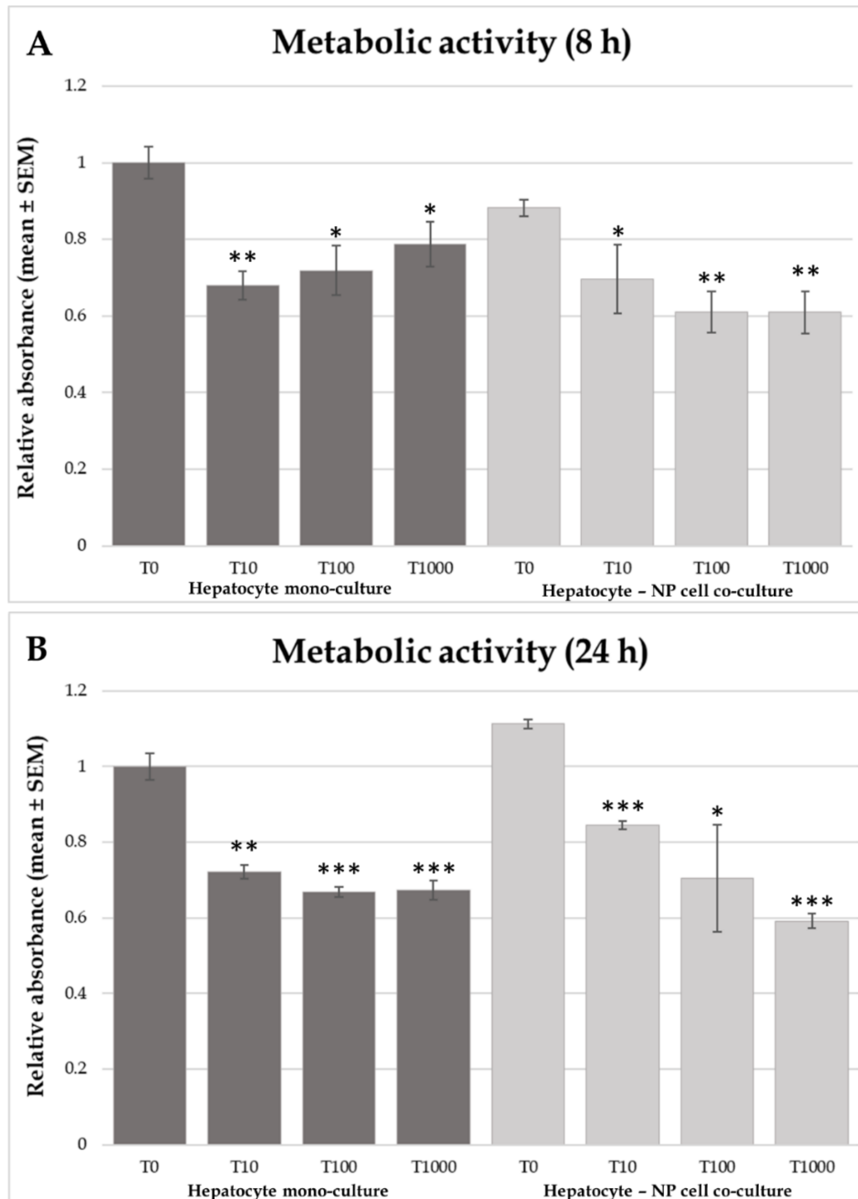

Figure 15. Effects of T-2 toxin treatment on cellular metabolic activity of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures assessed by CCK-8 test. Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h **(A)** or 24 h **(B)**. "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. Relative absorbances were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1. Results are expressed as mean ± standard error of the mean (SEM), n = 3/group. Significant differences between experimental groups are indicated with asterisks on the bars. *P < 0.05; **P < 0.01; ***P < 0.001.

6.3.2. Extracellular H_2O_2 production

According the present results, H_2O_2 production was not affected by T-2 toxin treatment using 8 h or 24 h incubation in any of the applied cell culture models (**Figure 16.**). 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 16.A.**; $P < 0.001$), while no difference was detected following 24 h treatment (**Figure 16.B.**).

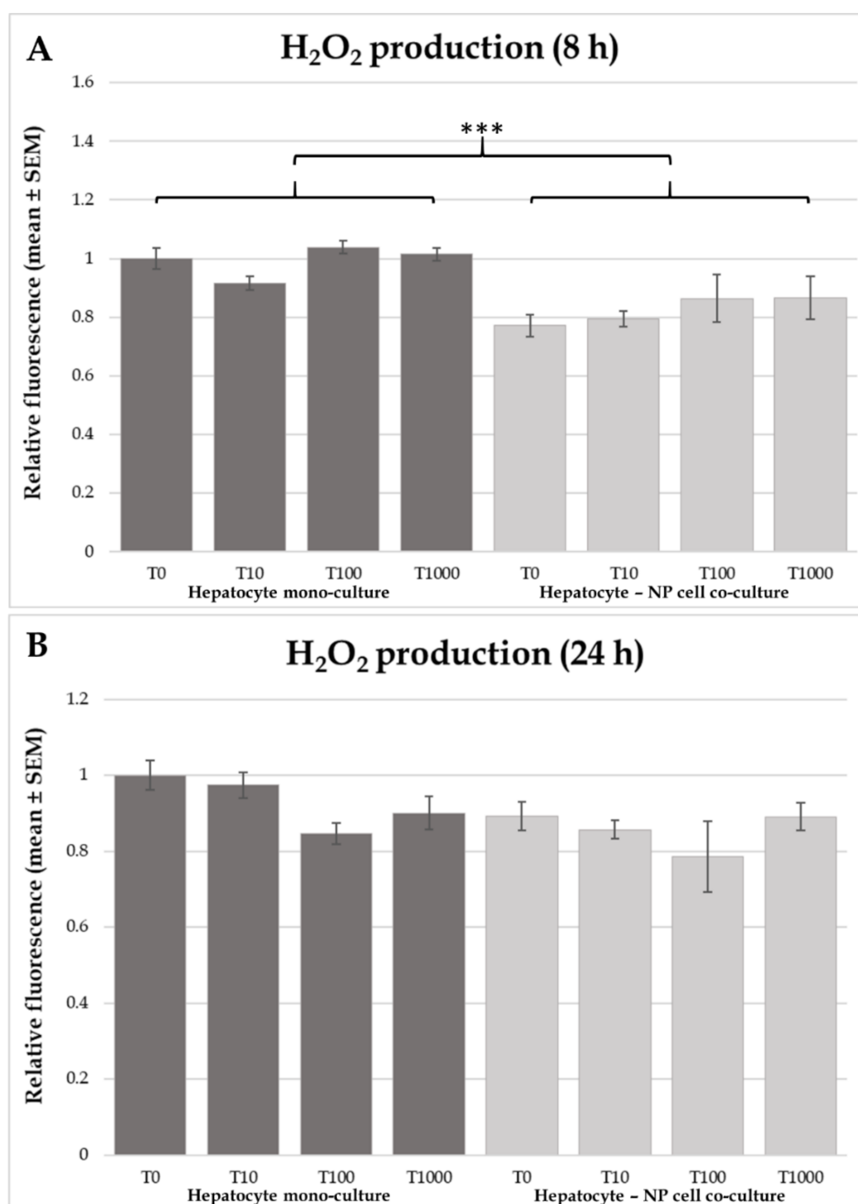

Figure 16. Effects of T-2 toxin treatment on H_2O_2 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures assessed by Amplex Red method. Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h **(A)** or 24 h **(B)**. "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. Relative fluorescences were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1. Results are expressed as mean ± standard error of the mean (SEM), n = 3/group. Significant differences between experimental groups are indicated with asterisks on the bars. ***$P < 0.001$.

6.3.3. Extracellular HSP70 concentration

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 17.**). Due to methodological difficulties, no analyzable data is available for HSP70 after 8 h incubation. Addition of T-2 toxin at 100 or 1000 nmol/L elevated HSP70 concentrations (P = 0.039 and = 0.044) in the hepatocyte mono-cultures after 24 h incubation. However, no differences were detected in the co-cultures (**Figure 17.**).

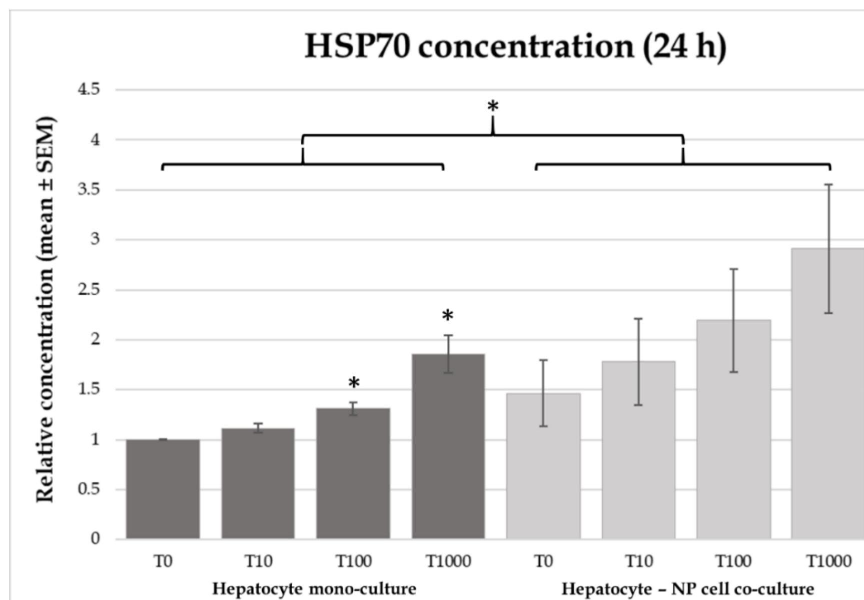

Figure 17. Effects of T-2 toxin treatment on HSP70 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures assessed by chicken specific ELISA tests. Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h or 24 h. "T0" 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. Relative concentrations were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1 (2.615 mg/ml). Results are expressed as mean ± standard error of the mean (SEM), n = 3/group. Significant differences between experimental groups are indicated with asterisks on the bars. *P < 0.05.

6.3.4. Extracellular IL-6 and IL-8 concentration

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 = 0.008; P = 0.006 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 18.**).

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 = 0.048), No such significant effect was found in the hepatocyte mono-culture model (**Figure 19. 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 19. B.**). Concerning the different models, higher IL-8 production (P = 0.007) was shown in the hepatocyte mono-cultures after 8 h incubation but not after 24 h (**Figure. 19.**).

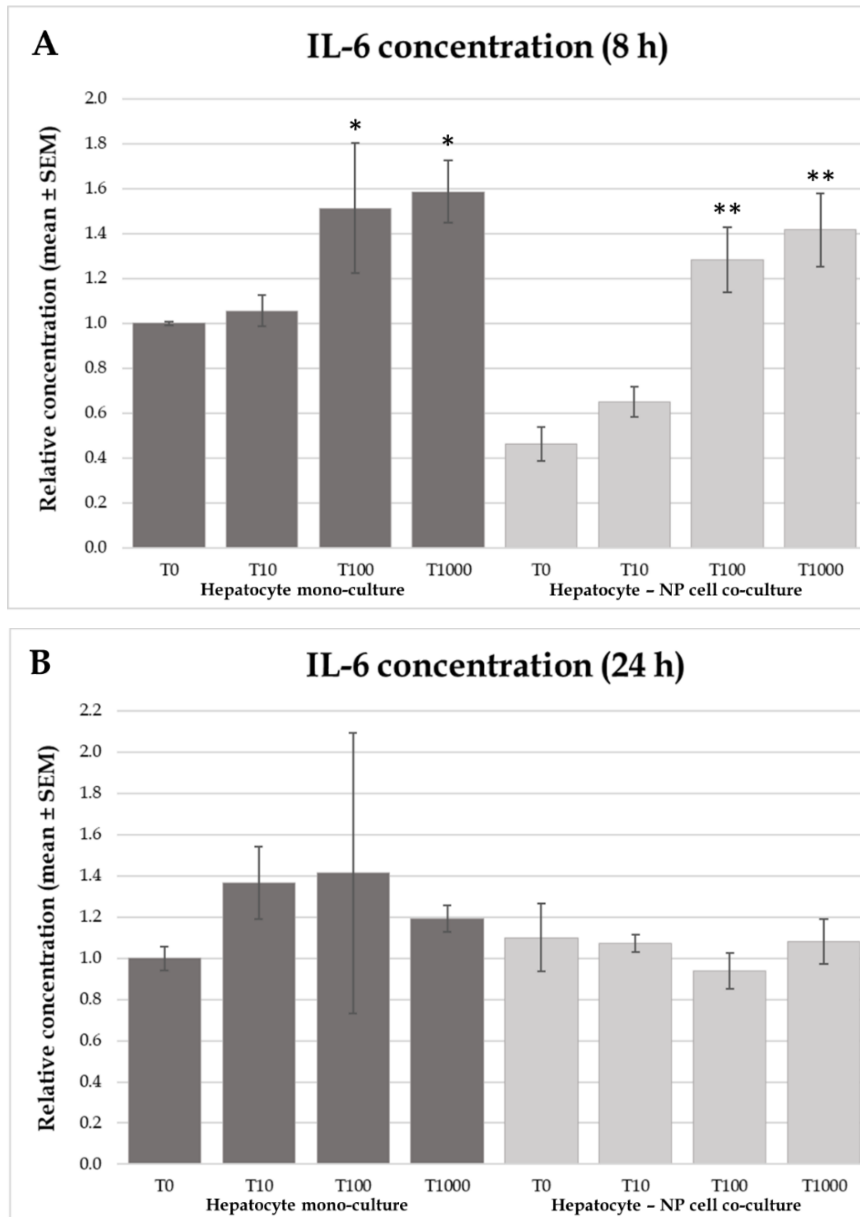

Figure 18. Effects of T-2 toxin treatment on IL-6 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures assessed by chicken specific ELISA tests. Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h **(A)** or 24 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. Relative concentrations were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1 (9.37 and 4.06 ng/mL, respectively). Results are expressed as mean ± standard error of the mean (SEM), n = 3/group. Significant differences between experimental groups are indicated with asterisks on the bars. *$P < 0.05$; **$P < 0.01$.

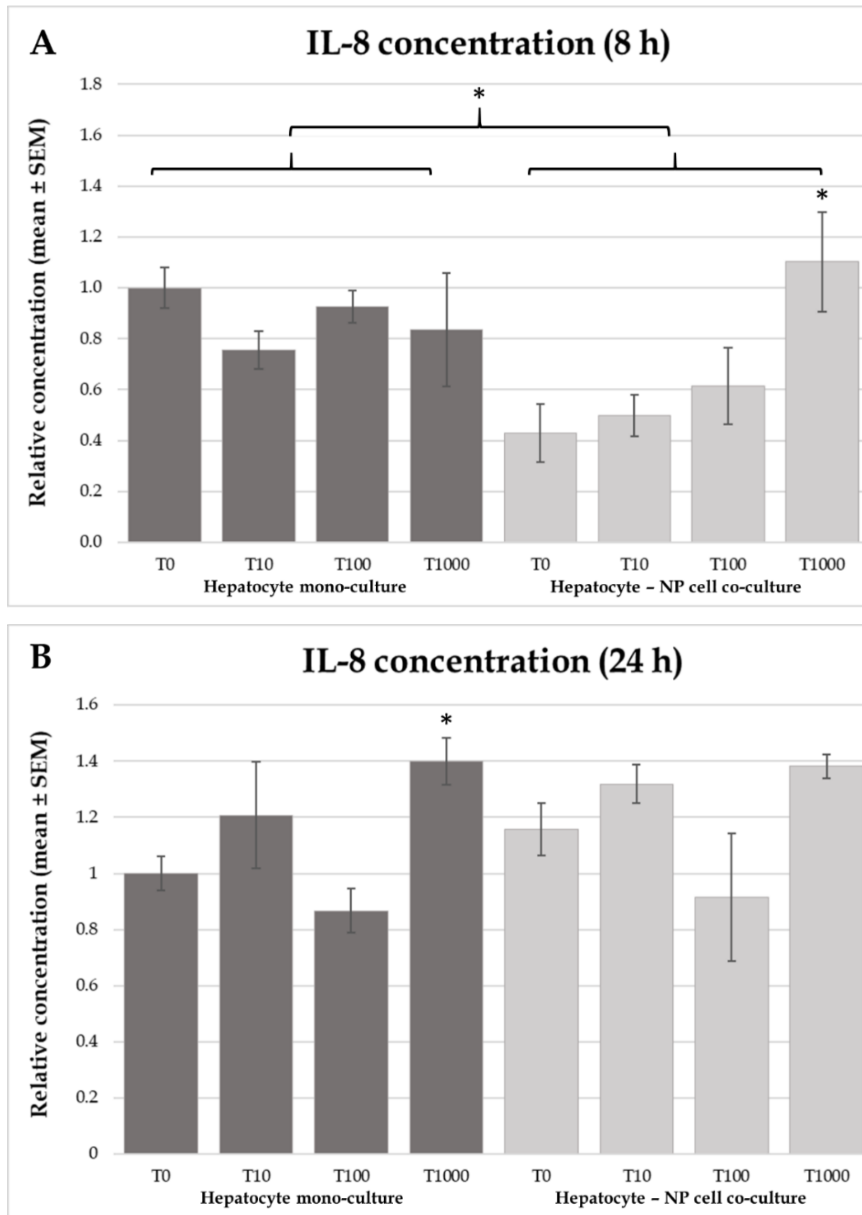

Figure 19. Effects of T-2 toxin treatment on IL-8 production of chicken derived primary hepatocyte mono- and hepatocyte – non-parenchymal cell co-cultures assessed by chicken specific ELISA tests. Cells after 24 h culturing were treated with different concentrations of T-2 toxin for 8 h **(A)** or 24 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. Relative concentrations were calculated by considering the mean value of T0 hepatocyte mono-cultures as 1 (2.615 and 0.858 ng/mL, respectively). Results are expressed as mean ± standard error of the mean (SEM), n = 3/group. Significant differences between experimental groups are indicated with asterisks on the bars. *P < 0.05.

6.4. Study IV. Consequences of acute heat stress in parenchymal organs of chickens *in vivo*

6.4.1. Cloacal temperature

Cloacal temperatures were elevated after both 4 h and 8 h heat exposure times ($P < 0.001$ in both groups, respectively) compared to controls (**Figure 20.**).

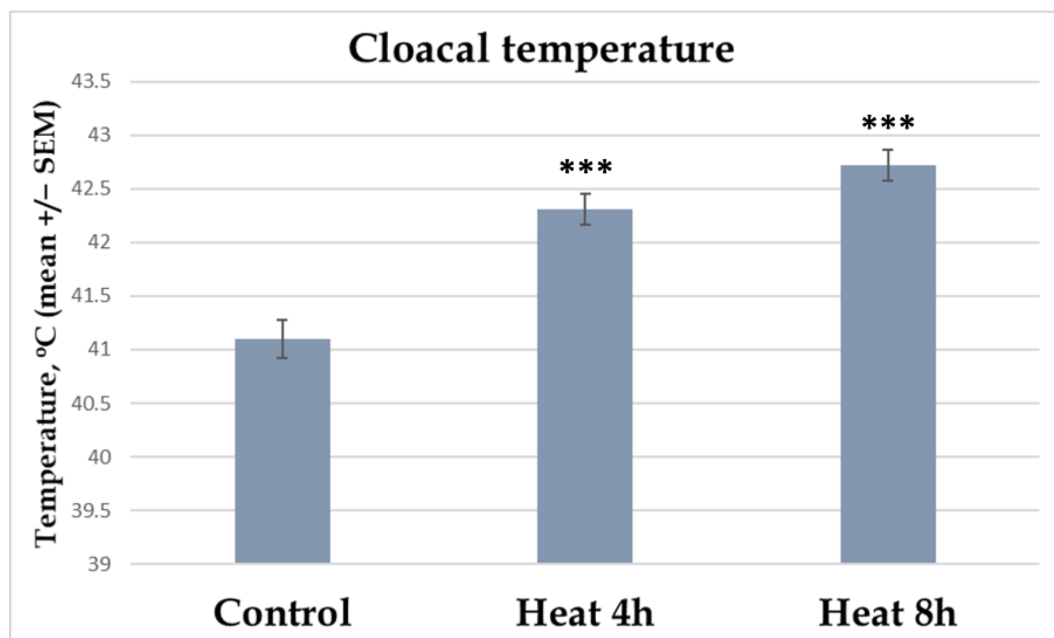

Figure 20. Cloacal temperatures registered following treatments; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37ºC, 50% relative humidity). Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups. Significant differences between experimental groups (heat stressed vs. control animals) are indicated with asterisks on the clamps. ***$P < 0.001$.

6.4.2. Malondialdehyde concentration

Hepatic MDA concentration was higher (P = 0.021) 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 21/A**). No significant effect was either detected in correlation with any of the heat stressed and control groups in the spleen and kidney (**Figure 21/B, 21/C**).

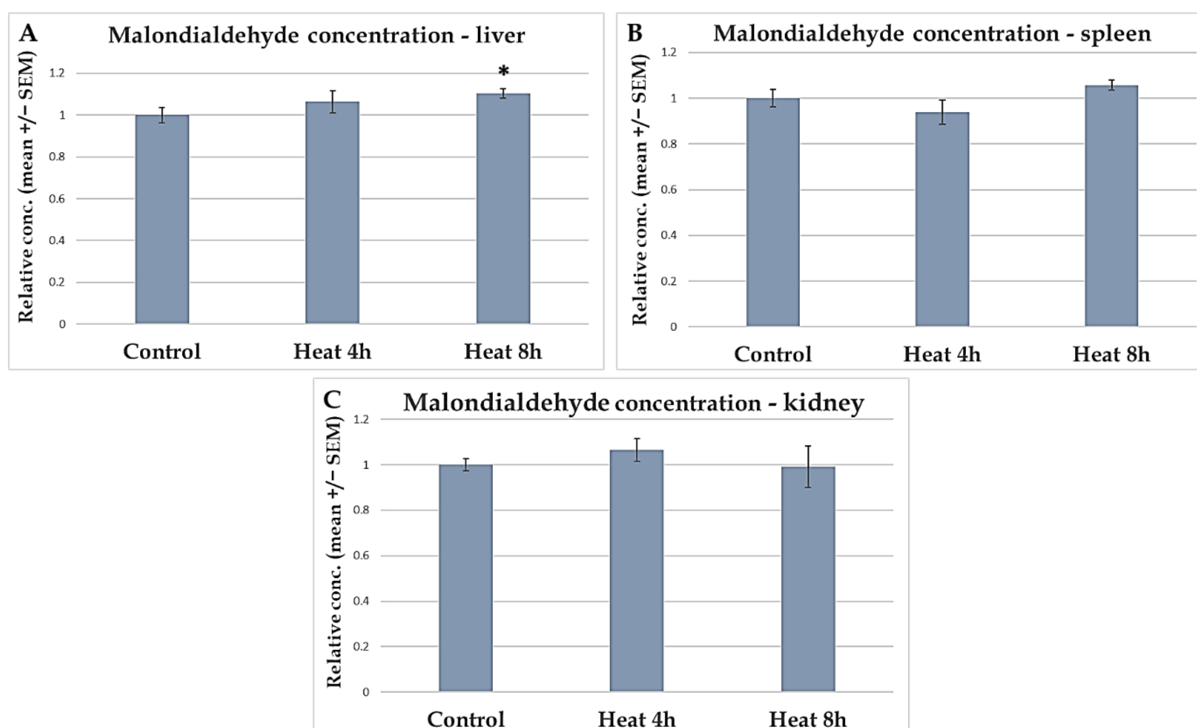

Figure 21. Malondialdehyde (MDA) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C)** assessed by chicken specific colorimetric tests. "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis (44.65; 11.35 and 9.74 pmol/mL, respectively). 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.

6.4.3. Reduced glutathione concentration and glutathione peroxidase activity

Comparing to control, heat exposure for both time durations caused decrease in GSH concentrations in the liver of chickens (**Figure 22/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 22/B, 22/C**). There was a significant, more than 2-fold increase of GPx observed in the liver (P = 0.002) after 8 h heat exposure (**Figure 23/A**). Meanwhile, after the same incubation time, decreased enzyme activity was found in the kidney (P < 0.001; **Figure 23/C**). Regarding GPx activity in the spleen, no significant differences were observed (**Figure 23/B**).

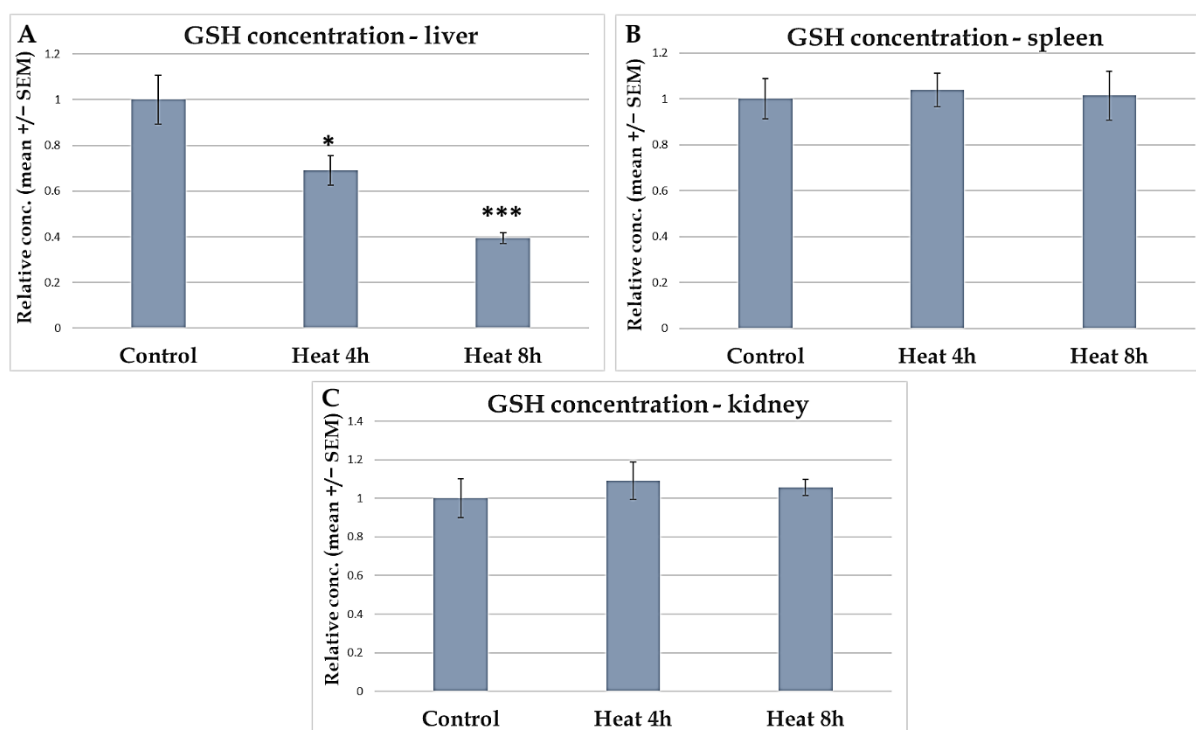

Figure 22. Reduced glutathione (GSH) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C)** assessed by chicken specific colorimetric tests**.** “Control” refers to control group with no heat stress; “Heat 4h” and “Heat 8h” refer to 4- and 8-hour long heat exposure, respectively (applying 37ºC, 50% relative humidity). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis (106.09; 31.8 and 24.54 nmol/mL, respectively). 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.

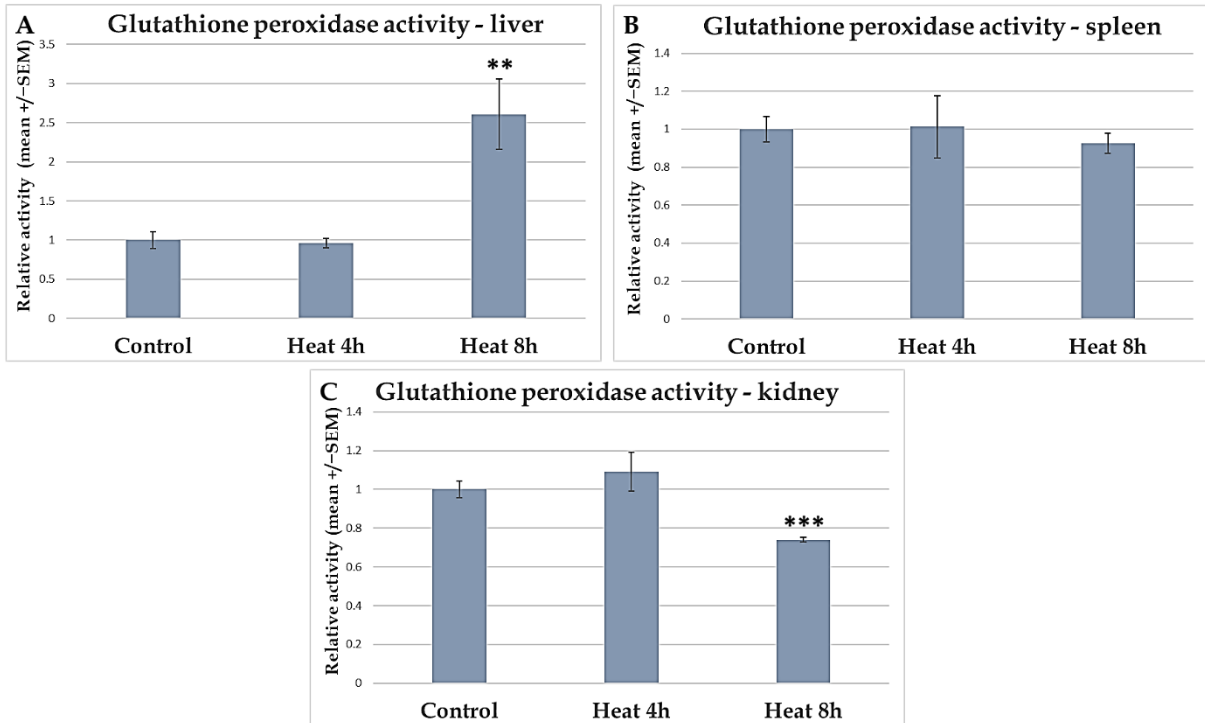

Figure 23. Glutathione peroxidase (GPx) activities of liver **(A)**, spleen **(B)**, and kidney **(C)** assessed by chicken specific colorimetric tests**.** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37ºC, 50% relative humidity). 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.

6.4.4. Protein carbonyl concentration

Hepatic protein carbonyl concentrations were decreased after both treatment periods (**Figure 24/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 24/B, 24/C**).

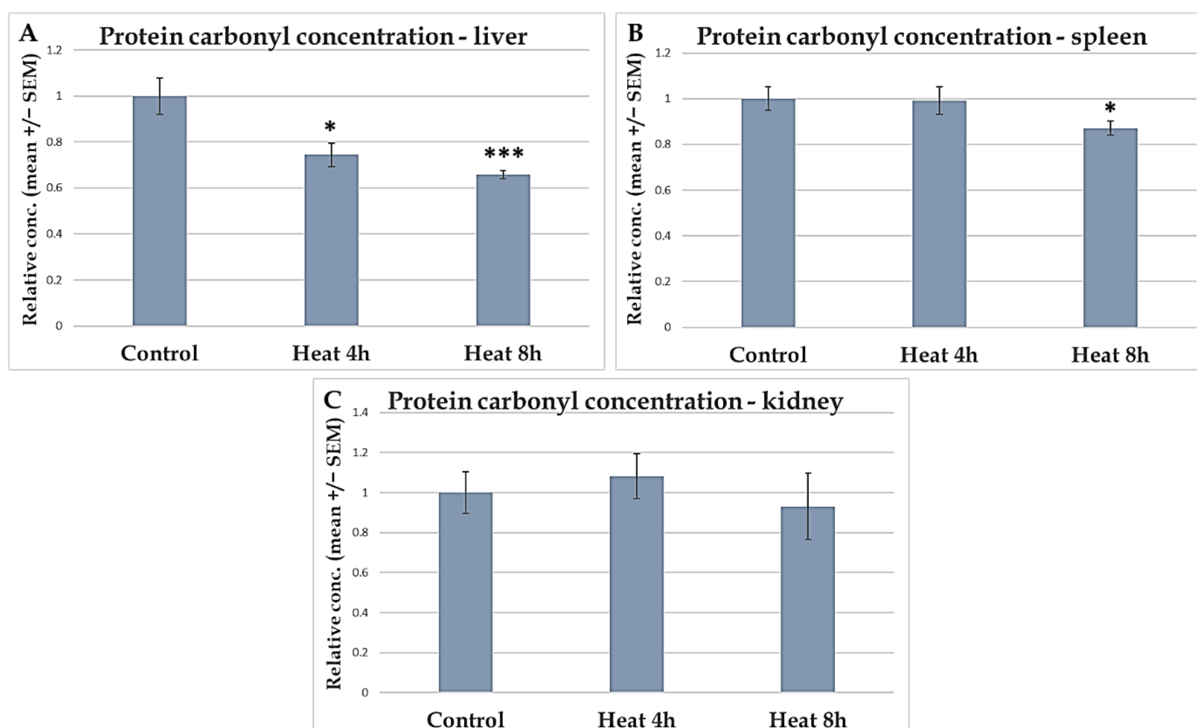

Figure 24. Protein carbonyl concentrations of liver **(A)**, spleen **(B)**, and kidney **(C)** assessed by chicken specific colorimetric tests**.** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis (17.36; 8.07 and 9.53 nmol/mL, respectively). 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. $^{*}P < 0.05$, $^{***}P < 0.001$.

6.4.5. HSP27, αA-crystallin and αB-crystallin concentration

No significant changes were found in the HSP27 concentration after any incubation time in any of the investigated organs (**Figure 25.**). Relative to control, lower αA-crystallin concentrations were observed in the liver after both 4 h ($P = 0.042$) and 8 h ($P = 0.005$) duration of heat stress (**Figure 26/A**), while no changes were found in the other two organs (**Figure 26/B, 26/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 27/A**), while no changes were detected in the other two organs tested (**Figure 27/B, 27/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 6.**).

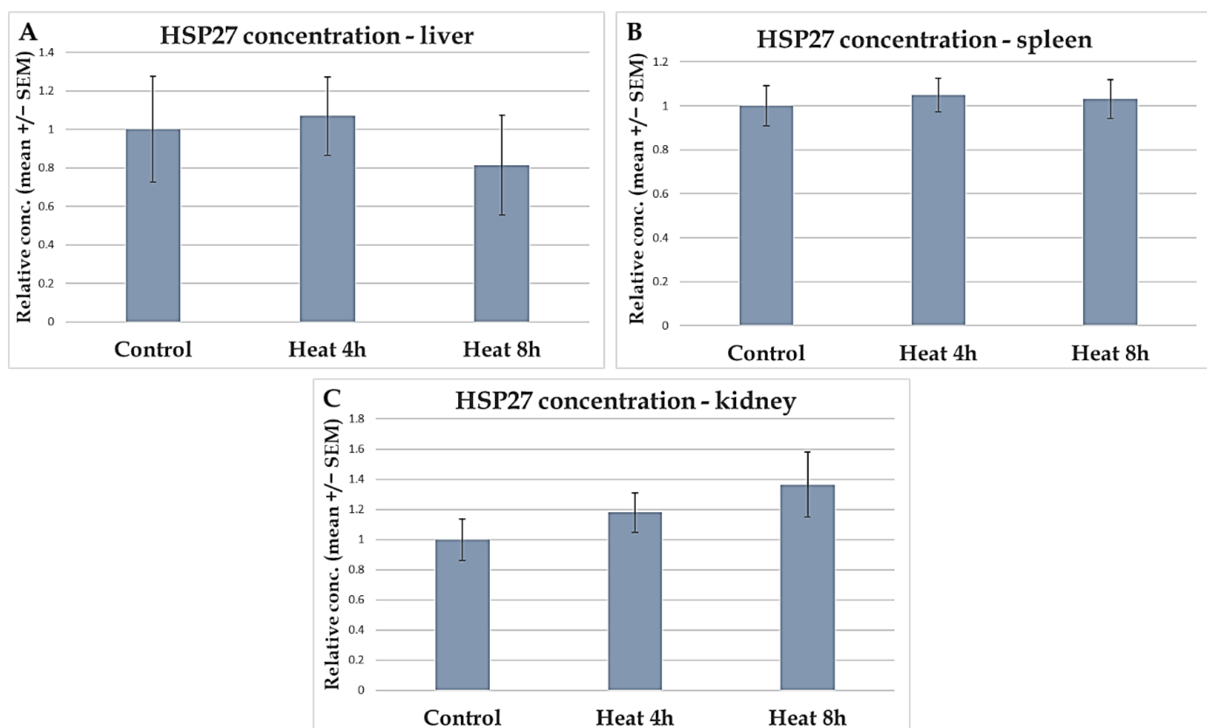

Figure 25. HSP27 (HSPB1) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C)** assessed by chicken specific ELISA tests**.** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis (1.83; 1.52 and 1.05 ng/mL, respectively). Results are expressed as mean ± standard error of the mean (SEM), n = 8/groups.

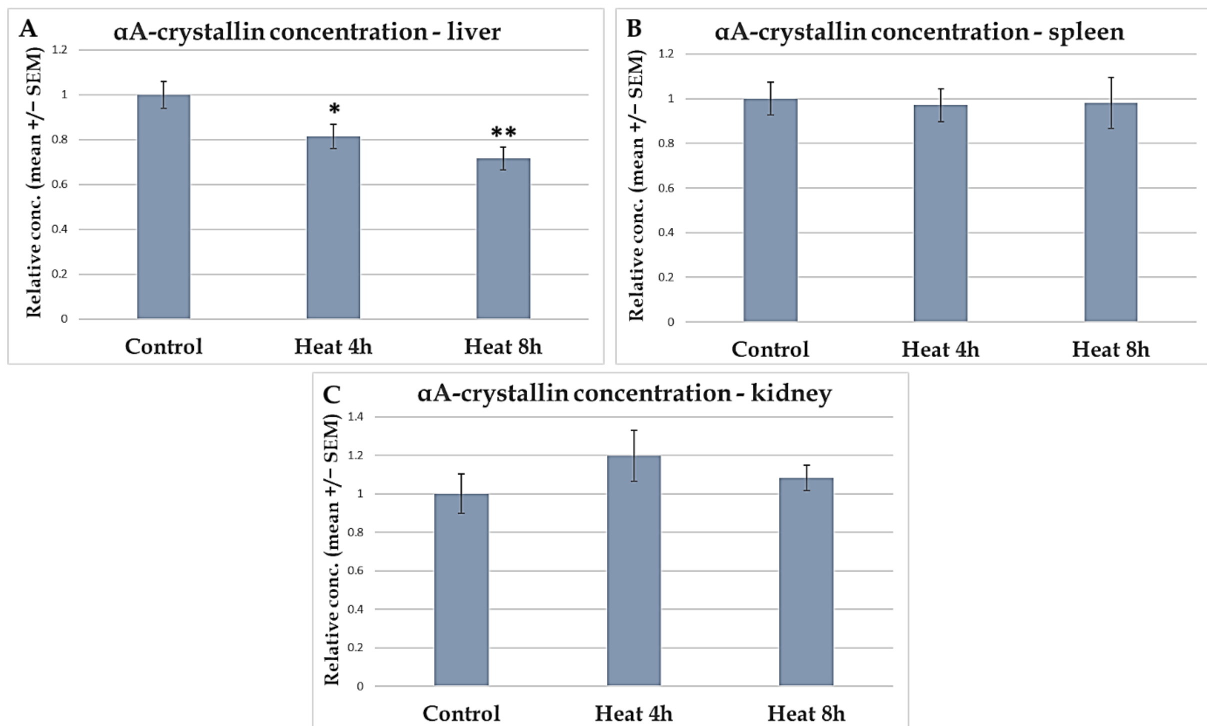

Figure 26. αA-crystallin (CRYAA; HSPB4) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C)** assessed by chicken specific ELISA tests**.** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity). Relative concentrations were calculated by considering the mean value of control group as 1 (40.64; 30.52 and 39.26 pg/mL, respectively). 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.

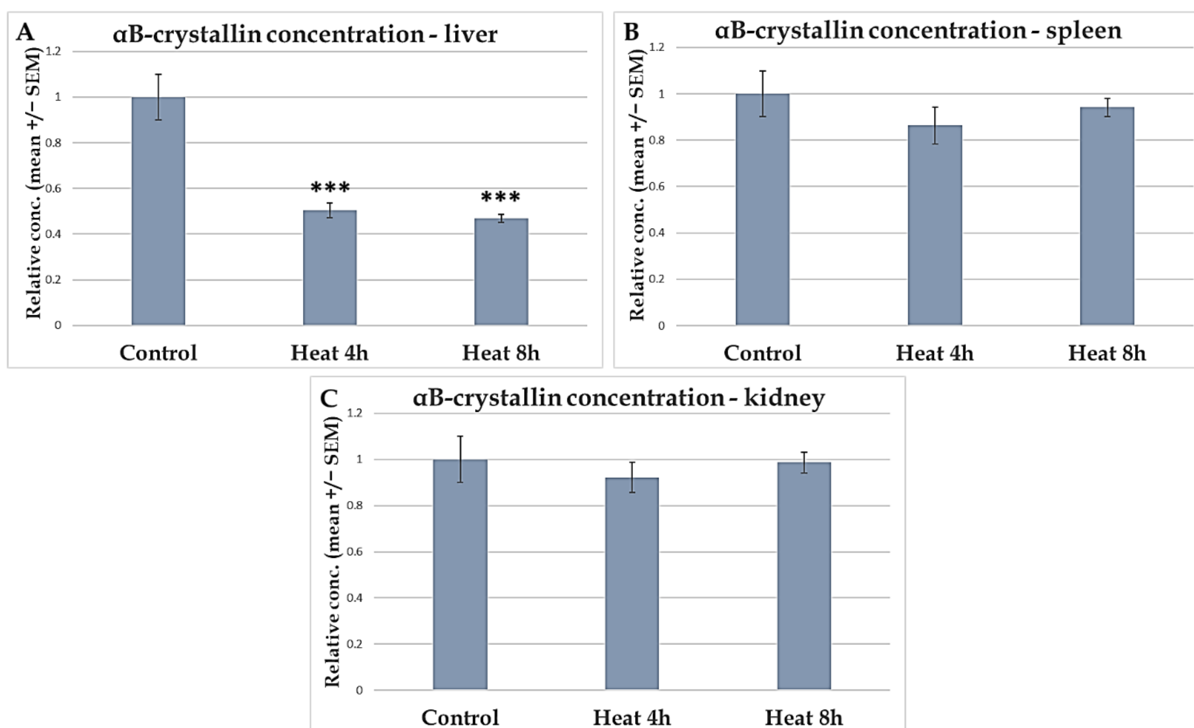

Figure 27. αB-crystallin (CRYAB; HSPB5) concentrations of liver **(A)**, spleen **(B)**, and kidney **(C)** assessed by chicken specific ELISA tests**.** "Control" refers to control group with no heat stress; "Heat 4h" and "Heat 8h" refer to 4- and 8-hour long heat exposure, respectively (applying 37°C, 50% relative humidity). Relative concentrations were calculated by considering the mean value of control group as 1 on the vertical axis (1048; 138.81 and 673.02 ng/mL, respectively). 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.

Table 6. 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.

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

7. Discussion

7.1. Establishment and characterization of novel chicken-derived primary hepatocyte mono-culture and hepatocyte – NP cell co-culture models

In the present study novel primary hepatic cell culture models have been successfully established and applied from chicken origin. 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 (**Study I.**). 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.

A monolayer hepatocyte – Kupffer cell co-culture and a double-layered enterohepatic co-culture have been also established from swine in a former study of our research group (Mátis et al., 2017; Pászti-Gere et al., 2014), but to the best of our knowledge, no similar avian cell cultures were available until this time. Considering similar cell culture models in chickens, mainly tumorigenic cell lines, such as the Chicken Hepatocellular Carcinoma Cell Line (indicated as LMH cells) or embryonic liver cell cultures were available so far (Amin et al., 2012; Kawaguchi et al., 1987; Kumar et al., 2003). The newly prepared chicken cell models enable 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 (Mátis et al., 2017). 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. The main advantage of these models is that they are non-tumorigenic primary cell cultures, hence results gained in these studies can be better extrapolated to the *in vivo* conditions of the healthy or inflamed chicken liver.

7.2. Cellular effects of heat stress on hepatic cell cultures of chicken origin *in vitro*

The other major goal of the present PhD work was to investigate the cellular effects of acute heat stress on the newly established cell culture models (**Study II.**). The applied heat exposure and incubation conditions were set based on previously available literature data. In spite of the higher physiological body temperature of birds, avian cells are often cultured at temperatures similarly to cultured mammalian cells. For instance, the aforementioned hepatic LMH cells, chicken embryonic fibroblast (CEF) cells or chicken primary myocardial cells were cultured at 37°C in various studies (Ibtisham et al., 2018; Sun et al., 2015; Xu et al., 2017), while a chicken macrophage-like cell line was maintained at 41.5°C (Slawinska et al., 2016). Similarly, different protocols do exist for the *in vitro* modelling of heat stress. An elevation of the ambient temperature from 37°C to 43°C have triggered maximal heat stress response in LMH cells (Gabis et al., 1996; Sun et al., 2015), while CEF cells were heat stressed by increasing temperature from 37°C to 40-44°C (Ibtisham et al., 2018). Further, heat stress response was evoked in myocardial cells by elevating temperature from 37°C to 42°C (Xu et al., 2017), and heat stress was modelled in chicken macrophage-like cells by incubation at 45°C in contrast to control cells, cultured at 41.5 °C (Slawinska et al., 2016). The time course of heat exposure was also different in various studies; the incubation time mostly ranged between 1 and 5 h to provoke short or medium-term heat stress (Slawinska et al., 2016; Sun et al., 2015; Xu et al., 2017). Based on the parameters of all the described avian *in vitro* experiments, and considering the characteristics of primary hepatocytes, control cells were incubated at 38.5°C while heat exposed cell cultures at 43°C for 1 and 2 h to mimic the cellular effects of intense acute heat stress.

According to our results, excessively high incubation temperatures had a strong impact on the metabolic activity, redox state, HSP70 and pro-inflammatory cytokine production of cultured chicken liver-derived cells. Shorter-term, 1 h heat exposure remarkably increased the catabolic activity of both hepatocyte mono-cultures and hepatocyte – NP cell co-cultures, with a higher extent in the latter case. Heat-associated enhanced metabolic rate may contribute to a better adaptation of cells to the increased temperature, which may be reflected in the alleviation of metabolic activity following 2 h of heat stress. On hepatocyte mono-cultures, cellular metabolic activity was still increased after the longer heat exposure compared to controls, but with a lower extent; however, on co-cultures it was already slightly lower after 2 h of heat treatment than in control cells. These results suggest a time-dependent metabolic adaptation to heat stress, hence cultured liver cells tended to recover after the longer heat exposure, which pattern can be also detected observing further parameters, such as HSP70, IL-6 and IL-8 concentrations of cell supernatants. The heat-triggered changes in the metabolic

activity of mono-cultured hepatocytes and co-cultured parenchymal and NP cells showed similar results, but co-cultures seemed to accommodate faster to the altered temperature conditions than hepatocyte mono-cultures. Based on our findings, extracellular LDH activity was not affected by heat stress indicating that the applied heat exposures were not cytotoxic and did not induce death of the cultured cells. Compared to hepatocyte mono-cultures, the lower LDH activity of co-cultures may relate to the increased metabolic activity of these cultures.

Numerous studies reported that heat stress is commonly associated with increased oxidative stress caused by elevated ROS production or inadequate amounts of antioxidants (Surai and Kochish, 2017). In comparison with other organs, liver was found the most sensitive to heat-triggered oxidative stress response in chickens (Lin et al., 2006). In the present study, 1 h long heat stress increased the H_2O_2 concentration of cell supernatants on both cell culture models, indicating an elevation in hepatocellular ROS release and presumably contributing to increased oxidative stress in the liver. Similarly to the metabolic activity, extracellular ROS levels tended to be normalized after 2 h of heat exposure. Comparing hepatocyte mono-cultures with hepatocyte – NP cell co-cultures, both models responded in the same manner as no significant differences could be found in the H_2O_2 concentration of culture media gained from different cell cultures.

The concentrations of HSP70, IL-6 and IL-8 in culture media were altered similarly by heat exposure. After the shorter (1 h) heat treatment, the level of extracellular HSP70 and those of the measured pro-inflammatory cytokines were intensively decreased, with an average extent of approx. 90% on both hepatocyte mono-cultures and hepatocyte – NP cell co-cultures. However, all these concentrations tended to be normalized following the longer, 2 h heat exposure. A similar heat stress associated decline in HSP70 level was reported in a previous study with rat myocardial cells, where the intracytoplasmic HSP70 concentration was reduced by short term, 1 h long heat exposure, whereas it was restored after 2 h heat incubation (Chen et al., 2015). It can be suggested that the utilization of heat-shock proteins in an intense short term heat stress exceeded their synthesis, resulting in decreased HSP70 levels, but after 2 h, cells could produce sufficient amount of HSP70 to fulfill the increased requirements and contribute to the restoration of physiological cell function, reflected by normalized metabolic activity and ROS levels. Intracellular HSP70 protein abundance was gradually increased by heat stress of 1 to 5 h in another *in vitro* study carried out using chicken myocardial cells (Xu et al., 2017). In addition, a 2 h long heat stress induced the expression of the HSPA2 gene encoding HSP70 on a chicken macrophage-like cell line (Slawinska et al., 2016).

The effects of heat stress on the immune response have been described in certain studies, but limited data is available concerning heat-triggered changes in hepatic immune function. Heat stress could provide controversial immunomodulatory action on the pro-inflammatory

cytokine production as it stimulated splenic IL-4 and IL-12 (Ohtsu et al., 2015), but in another study, decreased splenic IL-6, IL-12 gene expression, and also that of IL-1β and IL-10 in caecal tonsils of chickens (Quinteiro-Filho et al., 2017). However, it should be underlined that these data were gained following chronic *in vivo* heat stress. The latter immunosuppressive actions were observed only in *Salmonella* Enteritidis infected chickens, reflecting to a possible interaction of infection with inflammatory and stress response (Quinteiro-Filho et al., 2017). Heat stress can also suppress cellular immunity by reducing total white blood cell count and macrophage activity (Mashaly et al., 2004; Bartlett and Smith, 2003). In the present study, 1 h of heat stress down-regulated the hepatic production of both measured pro-inflammatory cytokines, IL-6 and IL-8, but their levels returned to the baseline after 2 h of heat exposure, indicating fast cellular adaptation.

The connection of HSPs and inflammatory mediators has been already studied by analyzing the liver transcriptome of chickens. Most HSPs, such as HSP70, were up-regulated by a 3 h long heat stress, while the expression of pro-inflammatory cytokines was mostly low or not affected by heat exposure (Lan et al., 2016). However, it can be suggested that HSPs possess key regulatory role in mitigating immune and inflammatory response under heat stress (Pockley, 2003). In the present cell culture study, HSP70 and pro-inflammatory cytokines responded to heat stress in a similar manner as they were down-regulated after 1 h, but normalized within 2 h of heat exposure.

Based on the results explained, and in line with previous studies (Lan et al., 2016), it can be suggested that the potential impact of a shorter heat stress on liver cell function may be in some aspects much higher than that of a longer heat exposure. Both 1 and 2 h incubation at 43°C can be considered as acute heat stress, but there were significant differences between the shorter and longer heat exposures. The 1 h lasting intense heat stress could provoke dramatic changes, such as stimulating catabolic metabolism and ROS release and strongly decreasing HSP70 as well as pro-inflammatory cytokine production, but the majority of these alterations have been mitigated within 2 h exposure by successful cellular adaptation. Hence, the critical role of short term heat stress in broiler farming has to be emphasized as it can impair liver function and the health of chickens.

When comparing the applied different cell culture models, mostly similar results were received on hepatocyte mono-cultures and hepatocyte – NP cell co-cultures. In case of metabolic activity, the co-culture, serving also as an inflammatory model, showed higher baseline level, which was altered by heat stress to the same extent as on mono-cultured hepatocytes. However, it can be suggested that the metabolic rate of co-cultures was normalized faster as already a moderate decrease was observable after 2 h of heat stress compared to controls. These results indicate an increasing action of inflammation (mimicked by including mostly macrophages as NP cells in co-cultures) on cell metabolic rate and suggest

a better capability of the models in presence of Kupffer cells for the adaptation to short term acute heat stress. This is consistent with the observed lower baseline level of extracellular H_2O_2 concentration in the hepatocyte – NP cell co-cultures compared to the hepatocyte monocultures.

In conclusion, the established novel primary cell culture models provide useful tools for studying the inflammatory and stress response in the liver of chickens. The successful separation of hepatocytes and NP cells and the preparation of a hepatocyte – NP cell co-culture from chickens enable investigations on the role of different cell types and on the interaction of stress and hepatic immune response. Based on our results, short term (1 h) intense heat stress can greatly influence 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.

7.3. Effects of T-2 toxin on hepatic cell cultures of chicken origin *in vitro*

In **Study III.**, cellular effects of T-2 toxin have been investigated in the abovementioned mono-culture and co-culture models. Concerning the major effects of T-2 toxin, numerous studies can be found in the literature, the results are; however, conflicting. This inconsistency may originate from the dissimilar study designs and the various applied T-2 toxin concentrations, or it could be explained by the species-specific differences alike. Therefore, in order to clarify the questions related to this scientific field, the aim in this study was to investigate the effects of T-2 toxin on the cellular metabolic activity and furthermore, on the oxidative and inflammatory status of the abovementioned hepatic cell culture models. The incubation conditions and the applied T-2 toxin concentrations were determined based on our pilot studies and on literature data. According to this, T-2 toxin was applied in 10, 100, 1000 nmol/L concentrations in the culture medium, similarly to other chicken related hepatic *in vitro* experiments (Yang et al., 2016).

In the present study, T-2 toxin treatment decreased cellular metabolic activity in all of the applied concentrations in both of the cell culture models. This result is in correlation with the findings that trichothecene mycotoxins inhibit protein synthesis, and are able to cause severe damage on mitochondrial membranes and on the endoplasmic reticulum resulting in morphological and functional impairments (Yang et al., 2000). Since T-2 toxin can bind to different proteins, it decreases the activity of various enzymes, such as succinate dehydrogenase, which plays crucial role in essential catabolic pathways. This results in cellular energy deficiency by decreasing the rate of the citric acid cycle and by consequently that of the respiratory chain (Adhikari et al., 2017; Wan et al., 2015).

Apart from the DNA- and membrane-related damage, another important harmful effect caused by trichothecene mycotoxins is the generation of reactive oxygen species and consequently appearing oxidative stress. Oxidative stress induced by T-2 toxin was earlier investigated by *in vitro* (Chaudhari et al., 2009) and *in vivo* studies (Mézes et al., 1999). In the literature, reports can be found regarding the *in vitro* oxidative effects of the T-2 toxin in different cell types such as Vero cells (Bouaziz et al., 2013), porcine ovarian granulosa cells (Maruniakova et al., 2014) and human monocytes (Huang et al., 2007). Studies were also carried out on human hepatoma cells (Bouaziz et al., 2008) and chicken primary hepatocyte cell cultures (Yang et al., 2016); however, regarding the exact hepatic effects caused by T-2 toxin – especially in avian species – and the role of hepatocytes or liver-derived macrophages concerning the mechanism of action is still unknown.

In the actual study no differences were found regarding the effects of T-2 toxin on extracellular H_2O_2 concentration either in the hepatocyte mono-culture or in the hepatocyte –

NP cell co-culture models. Besides, differences were observed in the cellular metabolic activity, interleukin production and HSP70 levels at the same time. It is also worth mentioning, that in several trichothecene toxin related studies carried out in broiler chicken DNA damage and apoptosis were described, whereas oxidative stress was not observed measuring ROS levels and the concentration of different lipid peroxidation markers in human colon carcinoma cells *in vitro* (Bensassi et al., 2009) and in broiler chickens *in vivo* (Awad et al., 2012; Rezar et al., 2007). Therefore, it may be suggested, that oxidative damage is probably not the main contributive factor to trichothecene mycotoxin (T-2 and DON) toxicity in chickens; however, further investigations are necessary to clear this question. Comparing the two applied cell culture models, lower H_2O_2 concentration was found in the co-cultures independently of the T-2 toxin treatment. Bozem et al. (2018) showed, that real time extracellular H_2O_2 concentration was affected by several extra- and intracellular mechanisms, such as the production and degradation intensity of the molecule (Bozem et al., 2018). In the study of Spolarics et al. (1996), increased H_2O_2 breakdown was reported in endotoxin challenged Kupffer cells as a protective mechanism against phagocytosis related oxidative stress which may explain the reason of the measured lower H_2O_2 concentration in our Kupffer cell containing co-cultures (Spolarics et al., 1996).

Significant increase was found in the HSP70 concentrations of culture media after 24 h of 100 nmol/L and 1000 nmol/L T-2 toxin incubation in hepatocyte mono-cultures. This finding is in correspondence with other *in vitro* studies carried out on Vero cells (El Golli et al., 2006). In spite of these findings, Bouaziz et al. (2013) observed increased HSP70 protein level in case of Vero cells treated with T-2 toxin, only in combination with zearalenol, but not alone (Bouaziz et al., 2013). Similarly, the trichothecene mycotoxin DON did not influence HSP70 *in vitro*, despite its general harmful effects (Bensassi et al., 2009). The effects of mycotoxins on HSP70 production can be refined by species-specific differences as well. Up to date, there is no data in the literature regarding the effects of T-2 toxin on HSP70 production in chicken.

Concentration of pro-inflammatory cytokine IL-6 was elevated as a result of 100 nmol/L and 1000 nmol/L T-2 toxin treatment in both of the applied models after 8 h incubation time as a consequence of the rapid toxin attack. However, in the cell culture models treated with T-2 toxin for 24 h – corresponding of a longer toxin exposure accompanied with an adaptation response – no significant differences were found. These results are similar to those described by Wang et al. (2012) and Wu et al. (2014). These latter studies have shown a significant up-regulation in the mRNA expression of different inflammatory factors such as IL-1β, IL-6 and TNF-α in RAW264.7 murine macrophages in dose-dependent manner (Wang et al., 2012; Wu et al., 2014a). Similarly, significant increase in IL-6 mRNA expression and serum IL-6 cytokine levels were reported in rats *in vivo*, treated by T-2 toxin (Zhou et al., 2014). Correspondingly to the abovementioned results, Fu et al. (2001) observed a similarly higher IL-6 level after T-2

treatment measured from the cell culture medium of *in vitro* cultured human foetal chondrocytes (Fu et al., 2001). In another study, similarly elevated IL-6 and IL-8 concentrations were observed in IPEC-J2 cell cultures as the effect of T-2 toxin (Pomothy et al., 2020).

Significant differences were found after 8 h of incubation time in the co-culture models respecting IL-8 concentrations, while in the case of hepatocyte mono-cultures, higher IL-8 concentration was only found after 24 h of incubation time. These differences were shown in both cases in the 1000 nmol/L T-2 toxin treated groups. Along with our findings, investigations carried out on Caco-2 cell lines also demonstrated concentration dependent significant increase of IL-8 (Kruber et al., 2011). Further, trichothecene mediated MAPK pathway activation has been also described, resulting in the up-regulation of pro-inflammatory cytokines, contributing to functional disorders and apoptosis of the cells (Pestka, 2010).

Comparing the applied cell culture models, significant differences were found in extracellular H_2O_2, HSP70 and IL-8 levels, but the cellular response of various culture types to T-2 toxin did not significantly differ from each other. The concentration of these molecules in cell culture supernatant is determined by their cellular synthesis, release, utilization and breakdown, highly depending on the comprising cell types and the influencing factors, such as the concentration and the duration of T-2 toxin exposure. Considering only our presented results, the observed differences between mono- and co-cultures cannot be completely explained, but the critical role of the comprising cell types should be strongly emphasized.

General harmful effects of T-2 toxin were already widely investigated, although there are numerous unanswered questions regarding certain cellular consequences in chickens. 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.

7.4. Consequences of acute heat stress in parenchymal organs of chickens *in vivo*

In the last phase of the project (**Study IV**.), our main aim was to investigate the *in vivo* effects of acute short term heat stress on parenchymal organs such as the liver, the kidneys and the spleen of chickens. According to our results, out of all tissues, the liver was found to be the most affected by heat-triggered molecular damage. This highlights the special susceptibility of the organ to the harmful effects of heat stress, suggested by previous studies as well (Cheshire, 2016). Alterations in the cardiorespiratory system lead to hepato-splanchnic vasoconstriction together with reduced splanchnic blood flow (Epstein and Roberts, 2011). These factors combined with increased hepatocellular metabolic demands can rapidly result in severe hypoxia, heavily exposing the liver to the negative effects triggered by heat shock response (Hall et al., 2001). Further, heat-associated impairment of the intestinal wall integrity increases its permeability, allowing for a severe leak of endotoxins into the circulation even in healthy animals (Le et al., 2020). In case of heat stroke, decreasing portal blood flow in combination with the decline of hepatic functions intensely reduces the detoxifying capacity of the liver, resulting in diminished biotransformation of endotoxins and xenobiotics (Epstein and Roberts, 2011). The special hepatic sensitivity to heat stress may also correlate with the high concentration of PUFA in the liver, especially compared to the proportion of potential antioxidants (Lin et al., 2006). These PUFA molecules are particularly prone to oxidation and consequently susceptible to oxidative damage generated by heat stress, increasing the exposure of this organ to the harmful effects of elevated environmental temperature (Nunzio et al., 2016).

Aligning with the abovementioned factors, **Study IV**. showed elevated MDA concentration in the liver of the chickens after 8 h heat exposure, while no alteration was found in the other investigated organs. The significant increase in hepatic MDA level may be presumably caused by the already mentioned relatively high PUFA content and their intense peroxidation (Lin et al., 2006). Similarly, in another study involving different broiler chicken strains, elevated MDA concentration was observed in red blood cells after acute heat exposure. Remarkable strain-specific differences were also found suggesting that Ross chickens exhibit much higher vulnerability to heat stress than other breeds, resulting in more severe damage of cell membrane lipids (Altan et al., 2003). In a further study, a similar pattern was observed in the MDA concentration of the blood plasma and the liver in chickens after heat exposure (Tsikas, 2017; Yang et al., 2010).

In the hepatic GPx enzyme activity assay, a remarkable 2.5-fold increase was observed after the 8 h long heat exposure, demonstrating the intense heat-provoked antioxidant defense response of the liver. This finding is in line with the results of studies carried out in other

species, such as Sprague Dawley rats, pikeperch (*Sander lucioperca*), and pigs, on the level of both mRNA and enzyme activity (Yun et al., 2012; Wang et al., 2019; Cui et al., 2016). Further, Habashy *et al.* (2019) described a similar increase in GPx activity only after longer heat exposure (12 days, 35°C, 40-50% relative humidity) in Cobb 500 broiler chickens, but no difference was found following short-term heat stress (24 h) in the liver (Habashy et al., 2019). Comparing the mentioned study with our recent results, the importance of age-dependency (15-day-old vs. 32-day-old chickens) as well as the possible role of the already mentioned strain-specific differences (Ross 308 vs. Cobb 500) along with the dissimilarity in the experimental set up (37°C vs. 35°C) can be strongly suggested.

Interestingly, despite highly increased hepatic GPx activity, the same parameter significantly decreased in the kidney after 8 h treatment, highlighting also possible organ-dependent variations in acute heat shock response. It is important to mention that similar effects of short-term heat stress in the kidney were described *in vitro* in renal LLC-PK1 cells of pig origin (Zhang et al., 2014) and *in vivo* in Antarctic *Notothenia* fish species (Forgati et al., 2019).

In accordance with the elevated GPx activity of the liver, hepatic GSH concentration was subsequently diminished after heat exposure since the enzyme is responsible for the GSH – GSSG conversion, providing effective cellular defense against ROS (Flohé, 2013). This decrease in hepatic GSH levels caused by several stressors was also found in numerous species including shelducks (Ma et al., 2014), rats (Tirkey et al., 2005), mice (Wu et al., 2010), and humans (Kaffe et al., 2015), but it was first described in the liver of broiler chickens under short-term acute heat stress in the present study.

According to our findings, acute heat stress significantly diminished protein carbonyl concentration – a marker of oxidative protein damage – in the liver after both incubation times and in the spleen after 8 h of heat exposure. Similar changes were observed by Oksala *et al.* (2014) in *Garra rufa* fish living in hot springs, exposed to moderately elevated water temperatures. A strong connection between the function of HSPs and protein carbonyl content was also suggested in the adaptation mechanisms to heat stress (Oksala et al., 2014). The central role of sHSP oligomers (particularly that of HSP27) in the cellular defense pathways against severe, acute oxidative stress was also confirmed in a different study with transgenic mice (Hollander et al., 2004).

In the present research, investigations of possible correlation between acute heat stress and changes in sHSP levels in the investigated organs were also aimed. Although, αA- and αB-crystallins are widely known as prominent components of the eye lens, they can both be found in different concentrations in the liver, kidney, spleen, adrenal gland, and cerebellum, depending on the animal species (Fan et al., 2014; Kato et al., 1991; Lee et al., 2005; Srinivasan et al., 1992). It is also important to mention, that their presence or absence in

various organs of chickens has not yet been well investigated. According to our results – beyond the important observation that both αA- and αB-crystallin proteins were detectable in the liver, kidney, and spleen of chickens – a remarkable, strongly significant hepatic decline was observed in αA- and αB-crystallin levels of the heat stressed groups. It is important to mention that in **Study II.** applying primary chicken-derived hepatic cell culture models, a similar heat stress-associated decrease in the HSP70 level was described after short-term treatment, whereas it tended to be restored after longer heat exposure. Changes in HSP levels of the same manner following acute heat stress were also observed in other models, such as in rat myocardial cells, where intracellular HSP70 concentration was decreased after short-term heat exposure and normalized following longer incubation times (Pockley, 2003). Regarding the HSP abundance in *m. longissimus dorsi* following transportation-driven stress in pigs, a similar decreasing tendency was observed in αB-crystallin concentration (Yu et al., 2009).

Based on the results of the present study, it can be suggested that the utilization of αA- and αB-crystallin proteins exceeded their synthesis during intense acute heat stress, resulting in decreased cellular sHSP levels. Hence, αA- and αB-crystallins are suggested to play a key role in the hepatic adaptation process triggered by acute heat stress in chickens. Based on our results, the rapid utilization of these sHSPs is in correlation with the decreased rate of protein carbonylation, possibly as a result of an overcompensation mechanism to restore cell function. A similar correlation was found between crystallin and GSH levels, strengthening the hypothesis, that there may be also a relationship between the activity of sHSPs and the function of the glutathione system (Christians et al., 2012). Further, HSP27 and αB-crystallin overexpression has been found to affect glutathione levels by enhancing the activity of glucose-6-phosphate dehydrogenase, glutathione reductase, and glutathione-S-transferase (Christians et al., 2012). A strong correlation between the protective function of HSPs and protein carbonyl content has also been suggested (Beltran et al., 2015). Thus, all these molecules and enzymes represent a complex protective system that acts to eliminate free radicals; even modest differences in physiological concentrations and enzyme activities may have remarkable effects on the resistance to cellular oxidative damage (Das, 2011). In correspondence with the finding, that acute heat stress did not result in alterations of the mentioned factors in the spleen and liver, no correlation was found between sHSPs and the mentioned parameters in these organs.

Despite the previously mentioned results concerning αA- and αB-crystallins, no alteration was found in HSP27 concentration after acute heat stress in any of the investigated organs. This suggests the possibility that HSP27 does not play a crucial role in the protective mechanisms against severe, short-term heat stress in the investigated parenchymal organs of broiler chickens. Supporting our findings, in another study, HSP27 protein levels were unchanged in the Malpighian tubules of the insect *Lucilia cuprina* after 1 h long thermal stress,

while on the other hand, up-regulation of the HSP27 was found on the transcriptional level. These results suggest that some proteins involved in the heat shock response require longer time to be expressed after exposure to the stressor (Singh and Tiwari., 2016). In case of vertebrates, such as in *Xenopus*, a similar pattern has been reported in early-stage embryos, where short-term heat shock did not cause any considerable change in the expression of HSP27 *in situ*, even on the mRNA level (Singh et al., 2017). Further, after 2 h exposure to heat stress in chicken myocardial cells *in vitro*, a significant increase of several HSPs was observed (including HSP60, HSP70 and HSP90), while no alteration of HSP27 was detected until after a longer 5 h incubation time (Wu et al., 2016). Finally, beyond the fact that sHSPs can be induced by elevated temperatures in general, different sHSPs are activated in different environmental and body temperature ranges. This dissimirality in expression of sHSPs also depends on species-specific characteristics (Janowska et al., 2019).

Taking every detail into consideration, despite the fact that sHSPs are highly conserved proteins, represented in some form in all living creatures including animals, plants, fungi, and prokaryotes (Zininga et al., 2018), all our results strengthen the hypothesis about their sometimes dissimilar roles in various organisms. In addition to this, reviewing the results of other research groups, our present study highlights even species- or strain-specific as well as age-dependent differences in the involvement of sHSPs in different metabolic processes.

In **Study IV.**, 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 GPx 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 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 only 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.

7.5. Conclusion

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 our planet. Extreme weather conditions seriously affect the agriculture as well, including the repeated exposure of livestock to heat stress and the more commonly occurring contamination of food and feed by mycotoxin producing molds. Poultry species with special emphasis on modern broiler strains are particularly exposed to heat stress conditions due to several reasons including among many things their relatively high physiological body temperature, intense metabolism and fattening, the presence of feathering and the absence of sweat glands. Further, according to recent findings, the prevalence of trichothecene mycotoxins (including T-2 toxin) can be in some regions, including Europe, more than 50% considering all feed samples, serving with serious hazards for broiler industry, too.

Among various harmful effects and animal welfare issues, the mentioned factors often have direct and indirect impact on the immune system, leading to further severe health problems together with higher susceptibility to complex multifactorial diseases. This situation results not just in massive economic loss, but also in serious danger considering the whole food-chain along with the One Health concept, because of the more frequent occurrence of poultry-related facultative pathogens infecting possibly also further animal species and even humans, or due to the presence of mycotoxins in the animal tissues.

By this reason, one of the main achievements of our study was the newly developed and successfully established liver-derived primary cell culture systems from chicken origin, described previously. Other important accomplishments are the resultful investigations of the molecular effects of heat stress and T-2 toxin exposure in broiler chickens applying the mentioned *in vitro* and *in vivo* models with special emphasis on the liver. 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. 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.

8. 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.

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Intest-Plus® matrix encapsulated butyrates stimulate endogenous VFA production in the caecum of broilers
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Az illó- és hosszú szénláncú zsírsavak összehasonlító vizsgálata vaddisznóban és házi sertésben
MTA Akadémiai Beszámolók, Budapest, Hungary, 2021.

Sebők Csilla, Vörösházi Júlia, Mackei Máté, Tráj Patrik, Szentgyörgyi Ákos, Neogrády Zsuzsanna, Mátis Gábor
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Varga Bence Tibor: **Modulation of the cytotoxic effects of T-2 toxin by epoxide hydrolase on chicken hepatic cell culture mode**. DVM thesis. Supervisors: Máté Mackei and Réka Fanni Barna, Budapest, 2019

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