

The role of glutathione in idiosyncratic and non-idiosyncratic drug induced liver injury

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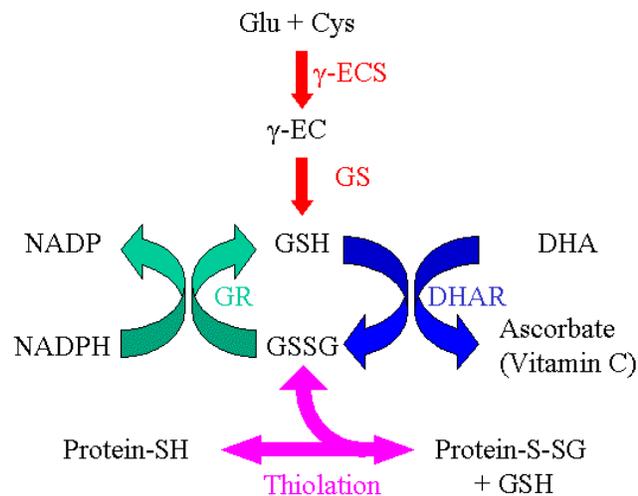
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Abstract

Introduction: The probability of idiosyncratic drug-induced liver injury (IDILI) occurrence is defined as a rare combination of several events at the same time, such as chemical properties of the drug, toxicant exposure, environmental and genetic factors. In the present study, we addressed the interaction of drugs and an environmental factor. It is known that different environmental factors can enhance the probability of IDILI: tobacco, alcohol consumption, diet, pre-existing diseases, viral or bacterial infections, etc. Accordingly, the aim of this study was to investigate the effect of an inflammatory agent, lipopolysaccharide (LPS), on the redox cellular homeostasis and to discover the role of glutathione in IDILI manifestation.

Material and Methods: Mouse and human precision-cut liver slices (PCLS) were incubated for 24 h with acetaminophen (APAP) and its isomer 3'-hydroxyacetanilide (AMAP), different IDILI-related drugs: diclofenac (DF), ketoconazole (KT), clozapine (CZ), carbamazepine (CBZ), troglitazone (TGZ); and two non-toxic comparator drugs of KT and CZ, voriconazole (VC) and olanzapine (OZ) respectively, in the absence or presence of LPS. Furthermore, drugs, that did not reveal synergistic toxicity with LPS following 24 h incubation of mouse PCLS, were tested for 48 h. Viability of PCLS was assessed by means of total and reduced (GSH) glutathione levels, GSH/GSSG ratio, ATP content and LDH leakage from PCLS to incubation media.

Results: AMAP was shown to be less toxic in mouse PCLS than APAP, while in human PCLS, AMAP revealed higher toxicity in comparison with APAP. It was shown that LPS by itself noticeably but not significantly increased total glutathione and GSH levels in mouse PCLS after 24 h incubation, which was not found after 48 h. Statistically significant synergistic toxicity was observed when mouse PCLS were incubated for 24 h with KT+LPS and CZ+LPS with a decline in glutathione level, and for 48 h with TGZ+LPS without changes in glutathione level. Similar results were demonstrated with human PCLS after 24 h incubation: supra-additive toxicity with a decline in glutathione level was shown in KT+LPS-/CZ+LPS-treated groups, though it was not found to be statistically significant.

Discussion: The maintenance of a glutathione level under conditions of mild liver injury after LPS exposure is possibly caused by the up-regulated production of reducing power, NADPH, and/or induction of glutathione synthesis *de novo*. Different molecular mechanisms seem to be responsible for IDILI development. Accordingly, glutathione was shown to play a role in KT and CZ synergistic hepatotoxicity, while TGZ-induced toxicity appears to be independent of the antioxidant status and glutathione level. The present study demonstrated once more, that inflammation can play a vital role in IDILI development. Additionally, it was shown, that glutathione content may be regarded as a potential novel biomarker for certain IDILI prediction and prevention.

Keywords: idiosyncratic drug-induced liver injury, precision-cut liver slices, glutathione

List of abbreviations

γ GCS	γ -glutamylcysteine synthetase
AMAP	3'-hydroxyacetanilide
APAP	acetaminophen
ATP	adenosine triphosphate
CBZ	carbamazepine
CZ	clozapine
DF	diclofenac
IADRs	idiosyncratic adverse drug reactions
IDILI	idiosyncratic drug-induced liver injury
GR	glutathione reductase
GSH	glutathione
KT	ketoconazole
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
OZ	olanzapine
PCLS	precision-cut liver slices
ROS	reactive oxygen species
SEM	standard error of the mean
TGZ	troglitazone
VC	voriconazole
UW solution	University of Wisconsin solution

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I. Introduction

The term “idiosyncratic adverse drug reactions” usually refers to rare and sporadic drug-induced toxic reactions (0.01-0.1%) (1), which occur only in the minority of people taking the drug and are characterized with high morbidity and mortality, and have a delayed onset after drug exposure. (2-4) These reactions usually manifest within clinical concentrations and are not connected to the known pharmacological activity of drugs. (2,5) Because of the rare incidence of idiosyncratic adverse drug reactions (IADRs) and lack of understanding of the mechanisms involved, they usually cannot be predicted by preclinical and clinical studies, or in clinical practice. (6) That brings uncertainty into the drug development process and it has become a cause of drug black-box warnings or withdrawals from the market. (3, 6-7)

Drug-induced liver injury is a main reason of liver failure in USA: more than 50% of liver failure cases are due to drug exposure. (3,7) IADRs account for 10-20% of acute liver failure cases that lead to death or require liver transplantation. (1,3,7) Understanding idiosyncratic drug-induced liver injury (IDILI) mechanisms will greatly contribute to both drug development process and clinical practice by providing reliable predictive tests or biomarkers. (8)

It is hypothesized that IDILI is immune-mediated. (6, 8) Though there is compelling evidence that the immune system is involved in some IADRs, its role in IDILI is still disputed. (2,7,9)

There are two different hypothesis considering the question of what is toxic in the organism: the drug itself or its active metabolites? Some drugs form active metabolites, followed by adducts formation, without any manifestation of toxicity. (2,9) Additionally, covalent binding to macromolecules only or exposure to a drug itself seems to be insufficient to trigger an immune response or overcome previously established drug tolerance. (3-4,7) On the other hand, the rapid onset of toxicity in some cases suggests that a parent drug is responsible for IDILI rather than its metabolites. Additionally, IADRs are considered to be a result of a unique combination of genetics, environmental and stress factors, as well as concurrent diseases, including infection and/or inflammation. (6-7) Thus, under certain circumstances, such as viral or bacterial infection, drug toxicity threshold can be lowered or additional “danger signals” can be formed, that results in IDILI manifestation. (2,5-6,8) These additional stress factors can significantly potentiate the immune response. (3,7) It is suggested that inflammagens can play a role of such risk factors, and simultaneous exposure to them together with a drug may lead to IDILI development. (5) The abovementioned hypothesis, called ‘inflammatory stress hypothesis’, receives support from many scientists, and we have investigated this hypothesis in our studies. (3,5,56)

Currently, no animal model exists to study or predict IDILI (7, 10). *In vivo* methods mean the use of a large numbers of animals. In turn, the use of *in vitro* methods, such as cell cultures, means IDILI is studied in a non-physiological way. (7) Therefore, other *in vitro* or *ex vivo* models should be used for toxicity studies. The precision-cut liver slices (PCLS) model, which was used in our study, already proved to be efficient and functional in various pharmacological and toxicological studies. (11-14) We addressed the inflammatory stress model using lipopolysaccharide (LPS): a component of gram-negative bacterial cell walls (3,11), as an inflammatory agent. It has been demonstrated *in vivo* that modest inflammation can underlie susceptibility of individuals to some drugs leading to IADRs development. (3,5,9,15)

It has been shown that LPS can influence the pharmacological and toxicological properties of drugs, for example, by altering their metabolism or increasing the sensitivity of cellular components under their exposure. (16) LPS inhibits P450 activity, in particular CYP3A, which can lead to disruption of the supposed way of drug biotransformation. (17-18) Also, LPS can alter expression of drug transporters, including the ATP-binding-cassette transporters in hepatocytes. (18) On the other hand, LPS is considered to be a main inducer of the inflammatory response in the host. (19) It is known that LPS causes the release of various cytokines, such as TNF α , IL-1 β , IL-10, reactive oxygen species (ROS) generation and up-regulation of cell surface molecules in mammals (11,17,20-21) An excess amount of oxidants can lead to cell damage and death by oxidizing proteins, membrane lipids, nucleic acid or carbohydrates. (22)

Moreover, it has been shown that LPS-induced ROS production is associated with decline in cellular glutathione (GSH) level. (20) Glutathione is a major intracellular non-protein thiol (23), which has several vital functions, such as antioxidant defense by detoxifying electrophiles and free radicals, maintaining the essential thiol status of proteins and modulating synthesis of DNA and various biological mediators. (23-25) Additionally, glutathione plays an important role in drug and/or their reactive metabolite detoxification (26-27), which has been hypothesized to be the cause of IADRs development. (27) In cells glutathione exists in reduced (GSH) and oxidized (GSSG) forms. GSH/GSSG ratio serves as an indicator of antioxidant and redox status. (24, 28) Under normal conditions, glutathione reductase (GR) keeps cellular glutathione in its reduced state. (25)

Besides, it has been reported that rat primary cultured hepatocytes with cellular GSH depletion can be used for a better evaluation of potential DILI and IDILI risk in early drug development due to higher cytotoxic sensitivity of certain drugs in this model. (27) Additionally, biomarkers that reflect cell damage, may predict the risk of IDILI. (9) For example, a decrease in GSH and increase in GSSG level is known as an oxidative stress biomarker (29). Based on this,

we hypothesized that a so-called 'danger signal', such as a decrease in cellular glutathione level, may play a role of a prospective biomarker for IDILI risk prediction.

In our study we investigated toxicity of several drugs: acetaminophen (APAP) and its isomer 3'-hydroxyacetanilide (AMAP), which is considered to be non-hepatotoxic, diclofenac (DF), ketoconazole (KT), clozapine (CZ), carbamazepine (CBZ), troglitazone (TGZ) which associated with IDILI development, and two non-toxic comparator drugs of KT and CZ, voriconazole (VC) and olanzapine (OZ) respectively, in the absence or presence of LPS by using a mouse and human PCLS model. The viability of PCLS was evaluated by measuring GSH, GSH+GSSG, GSH/GSSG ratio, adenosine triphosphate (ATP) content and lactate dehydrogenase (LDH) leakage.

The aim of this study was to investigate the intervention of moderate inflammation with glutathione levels and the role of glutathione in IDILI development, in order to test it as a biomarker for IDILI prediction and prevention.

II. Materials and Methods

General

2.1 Chemicals

Acetaminophen (APAP), 3'-hydroxyacetanilide (AMAP), ketoconazole (KT), clozapine (CZ), diclofenac sodium salt (DF), carbamazepine (CBZ) and LPS from *Escherichia coli* serotype B55:O55 (Lot 050M4014) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LPS possessed an endotoxin level of 600,000 EU/mg, as stated by the manufacturer. Olanzapine (OZ) and voriconazole (VC) were kindly provided by Dr. Willem Schoonen (MSD, Oss, the Netherlands) and Jan-Willem Alffenaar (UMCG, Groningen, the Netherlands) respectively. Troglitazone (TGZ) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Stock solutions of each drug were made in the solvent DMSO (VWR, Briare, France). All other drugs and chemicals used in this study were procured from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2 Human liver tissue

Human liver tissue was obtained from donors as surgical waste after split liver transplantation (TX) or from patients after partial hepatectomy (PH) due to removal of carcinoma,

as described previously. (30) The study protocols were approved by the Medical Ethical Committee of the University Medical Center Groningen.

2.3 Animals

For preparation of PCLS, female C57BL/6 mice, weighing 18-22 g, were purchased from Harlan (Horst, the Netherlands) and housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle with access to food (Harlan chow no 2018, Horst, the Netherlands) and tap water *ad libitum*. The mice were allowed to acclimatize for at least one week before experiments started. The research protocols were approved by the Animal Ethical Committee of the University of Groningen.

2.4 Excision of mouse liver

Mouse surgical procedures were carried out under isoflurane/O₂ anesthesia. The liver was excised and placed in ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical Care, Waukegab, IL, USA).

2.5 Preparation of PCLS

PCLS (5 mm diameter, 200-300 µm thick and 5 mg wet weight) were prepared according to the protocol described earlier. (31) Briefly, with a 5mm biopsy punch (Kai Industries, Seki, Japan) 5mm cylindrical liver cores were made and put into an ice-cold UW solution. These cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer saturated with carbogen (95%O₂/5%CO₂). After slicing, PCLS were kept again in an ice-cold UW solution until incubation.

2.6 Pre-incubation and incubation of PCLS

PCLS were incubated according to the protocol described previously (31) for 24 or 48 h in pre-warmed 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria). Accordingly, PCLS were transferred individually to each well containing 1.3 ml Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25 mM D-glucose and 50 µg/ml gentamicin (Gibco, Paisley, UK) (WEGG medium) and saturated with 95%O₂/5%CO₂, and pre-incubated at 37°C for 1 h while shaking 90 times/min. Pre-incubation and subsequent refreshing the medium allow PCLS to restore their ATP level and to remove cell debris. After pre-incubation, PCLS were transferred to fresh WEGG medium, with or without LPS (30 µg/ml for mouse and 40 µg/ml for human), in combination with DF [200 µM], KT [15 µM], VC [1500 µM], CZ [45 µM], OZ [45 µM], CBZ [400 µM], TGZ [30 µM] or the solvent DMSO (concentration during incubation ≤0.5%) and

incubated for 24h. Also, incubation with APAP and AMAP [1mM, 5mM for mouse and 5mM for human] for 24h was performed. For 48 h incubation, a lower concentration of 25 µg/ml LPS was used, considering the longer incubation time. PCLS were transferred to fresh WEGG medium, with or without LPS (25 µg/ml) and in combination with OZ [40 µM], CBZ [400 or 550 µM], TGZ [30 or 42 µM] or the solvent DMSO (concentration during incubation ≤0.5%) and incubated for 48h without changing the medium. The decision regarding which drugs should be tested for 48h incubation was made based on results obtained from 24h-incubation studies.

Sample analysis

2.7 Glutathione determination

Glutathione exists in the cells in reduced (GSH) and oxidized (GSSG) states. The GSH and total glutathione (GSH+GSSG) levels were measured in mouse PCLS following 24h or 48h incubation according to the modified Fishers method. (32) Three replicate PCLS were washed briefly in 0.9% NaCl solution, collected and snap-frozen in liquid nitrogen for further storage at -80°C until analysis. Each slice was homogenized for 45 sec with a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) in 400 µL ice-cold 50 mM Tris-HCl buffer, which contains 1mM EDTA (pH 7.4) and centrifuged for 3 minutes at 13000 rpm and 4°C. The supernatant was used for GSH and total glutathione assays separately, using GSH solution as a standard.

To measure the content of total glutathione, 15 µL of 1mM NADPH solution (Roche Diagnostics GmbH, Mannheim, Germany) and 7.5 µL of 20U/ml glutathione reductase solution (Sigma-Aldrich, St. Louis, MO, USA) were added to 150 µL of the supernatant and incubated for 15 min at 37°C to reduce GSSG into GSH. 15 µL of 50% Trichloroacetic acid solution was used to precipitate the proteins which can disturb GSH measurement due to presence of SH-groups. After 5 min on ice, the samples were centrifuged for 5 min at 4°C at 4600 rpm for total GSH assay or at 13000 rpm for reduced GSH assay. 50 µL of the supernatant was transferred to micro-titer 96-well plate and 200 µL of Ellman's reagent (1 mM solution of 5,5'-dithio-bisnitrobenzoic acid in 0.5 M Tris/10 mM EDTA buffer containing 10% v/v ethanol, pH 8.0) was added to each well, after which the absorbance was read at 405 nm after 5 minutes.

GSSG was calculated as a difference in the amount of GSH measured before and after chemical reduction, considering that GSSG contains two unites of GSH. A shortcoming of this measurement can be found in the possibility of spontaneous autoxidation of GSH that can lead to overestimation of GSSG.

2.8 ATP measurement

ATP was measured using the ATP Bioluminescence Assay Kit CLS II (Roche, Mannheim, Germany, protocol in Appendix II). After incubation, PCLS were placed individually in a safe-lock micro test tube containing 1 ml sonification solution, 70% ethanol (v/v) containing 2 mM EDTA (pH 10.9), and were consequently frozen immediately in liquid nitrogen for further storage at -80°C until the analysis. Each slice was homogenized for 45 sec with a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) and centrifuged afterwards for 5 minutes at 13000 rpm and 4°C. The supernatant was collected in separate tubes and the precipitate was left overnight at 37°C with the lid open to evaporate the ethanol for further protein determination. 5 µL of supernatant was transferred to a black 96-well plate and 45 µL 0.1 M Tris HCl containing 2 mM EDTA (pH 7.8) was added to each well. Afterwards, 50 µL of luciferase was added to each well and luminescence was measured after 5 min with a Lucy1 luminometer (Anthos, Durham, NC, USA) at a room temperature, using a standard ATP calibration curve. All reagents were brought to a room temperature before use. The intensity of the light measured correlates with the amount of ATP in the PCLS.

2.9 Protein measurement

The amount of protein was determined by the Lowry method (33) using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany). In brief, the remaining precipitate was dissolved in 200 µL 5M NaOH by incubation for 30 min at 37°C inside a shaking waterbath. Afterwards, samples were diluted five times in water and homogenized for 45 sec with a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA). 5 µL of sample content was transferred to a micro-titer 96-well plate and 25 µL of reagent A and 200 µL of reagent B were added. The absorbance was read at 650 nm after keeping a plate in the dark for 15 minutes, using bovine serum albumin (BSA) as a standard.

2.10 LDH leakage determination

Aside glutathione and ATP measurement, the viability of PCLS was also assessed by measuring the lactate dehydrogenase (LDH) activity in the slice and incubation media. The results were presented as percentage of the enzyme leakage from the slice into the medium. For the total LDH content in fresh PCLS determination, three PCLS were collected after 1h pre-incubation and homogenized in 1.3 ml of fresh medium (as incubation volume) using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA). Afterwards, samples were centrifuged for 3 minutes at 13000 rpm, 4°C, and 50 µl of supernatant from each tube was collected to one sample of the total volume 150 µl. To determine LDH activity in the incubation media, 50 µl medium was

collected from each of the three replicate PCLS after incubation to one sample of the total volume 150 μ l for each experimental group. The amount of LDH in the medium sample was analyzed using the Roche/Hitachi Modular system (Roche, Mannheim, Germany) according to a routine laboratory procedure.

Statistics

2.11 Statistical analysis

Each experiment was performed with a minimum of 3 mouse or human livers using PCLS in triplicates from each liver. To investigate whether the obtained results are statistically significant, a two-tailed paired Student's t-test for comparison of two groups or a two-way ANOVA for comparison of more than two groups were used. The level of significance was defined at P-value <0.05. In all graphs the mean values and standard error of the mean (SEM) are shown.

III. Results

24 h incubation animal studies

3.1 Glutathione content in samples

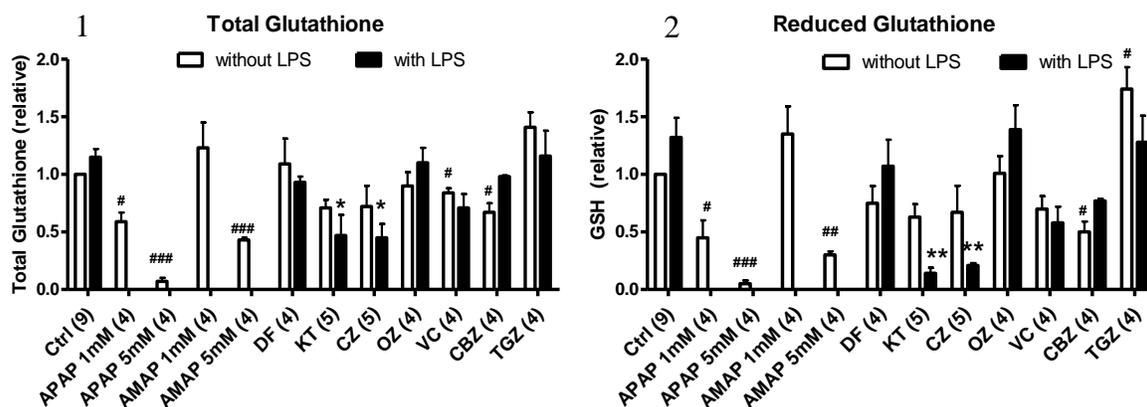


Figure 1, 2. Total and reduced glutathione content of mouse PCLS after 24 h incubation with different drugs in absence (open bars) or presence (black bars) of LPS. The total and reduced glutathione content has been expressed as relative values to the control without LPS treatment. Graphs represent the mean values \pm SEM of 4-9 experiments, using 3 PCLS for each group in every experiment. #P<0.05, ##P<0.01 and ###P<0.001 by the two-tailed paired Student's t-test for comparison of two groups: the control and drug-treated only. *P < 0.05 and **P < 0.01 by the two-way ANOVA for comparison of four groups together to test the drug+LPS interaction: the control, LPS-only, drug-only and LPS+drug groups.

After 24 h incubation PCLS retain the ability to synthesize glutathione and maintain the reduction of GSSG to its reduced state: GSH. The mean absolute values of total and reduced glutathione in control group samples was 38 and 20 μM respectively. According to the results (Fig.1,2), APAP in both concentrations of 1mM or 5mM, AMAP 5mM and CBZ itself significantly decreased total glutathione and GSH levels in liver tissue. Additionally, VC caused significant decrease in total glutathione level in mouse PCLS. In contrast TGZ increased total glutathione and GSH levels in PCLS, however, only the increase in GSH level was found to be significant ($P < 0.05$). Other drugs that were tested did not cause any considerable changes in cellular glutathione level.

Following 24 h incubation, LPS has been shown to consistently but not significantly increase total glutathione and GSH levels. Furthermore, LPS treatment caused a significant decrease in total glutathione ($P < 0.05$) and GSH ($P < 0.01$) levels after 24 h incubation of mouse PCLS treated with KT and CZ. Remarkably, LPS co-treatment noticeably, though not significantly, increased GSH level in DF-treated PCLS, and increased total glutathione and GSH levels in OZ- and CBZ-treated PCLS as well.

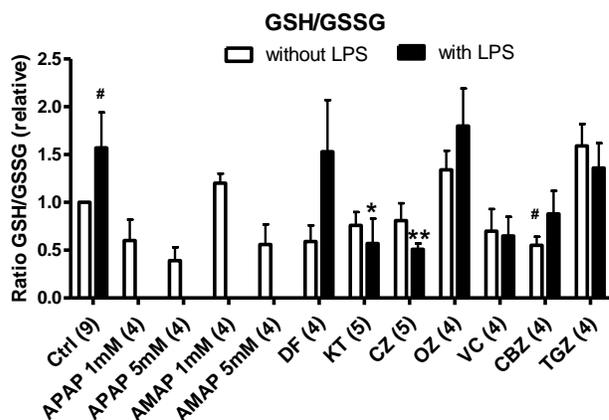


Figure 3. GSH/GSSG ratio in mouse PCLS after 24 h incubation with different drugs in absence (open bars) or presence (black bars) of LPS. GSH/GSSG ratio has been expressed as relative values to control without LPS treatment. The graph represents the mean values \pm SEM of 4-9 experiments, using 3 PCLS for each group in every experiment. [#] $P < 0.05$ by two-tailed paired Student's t-test for comparison of two groups: the control and drug-treated only. ^{*} $P < 0.05$ and ^{**} $P < 0.01$ by two-way ANOVA for comparison of four groups together to test the drug+LPS interaction: control, LPS-only, drug-only and LPS+drug groups.

The GSH/GSSG ratio is believed to be an indicator of cellular antioxidant and redox state. Decline in the ratio implies a failure in ROS detoxification and thus increased ROS accumulation. The mean absolute value of GSH/GSSG ratio in control group was 2.7. In the present study, it has been shown that certain drugs slightly increased (AMAP 1mM, OZ, TGZ), whereas others

decreased the GSH/GSSG ratio: significant decline has been demonstrated only with CBZ ($P<0.05$). Remarkably, LPS by itself significantly increased the GSH/GSSG ratio ($P<0.05$). In addition, a similar increase has been observed in LPS+DF/ LPS+OZ/ LPS+CBZ/ LPS+TGZ-treated groups in comparison with control and/or drug-only treated groups. However a significant LPS-enhanced decline of GSH/GSSG ratio has been shown in LPS+KT ($P<0.05$) and LPS+CZ ($P<0.01$) groups,

3.2 Viability of mouse PCLS: LDH leakage

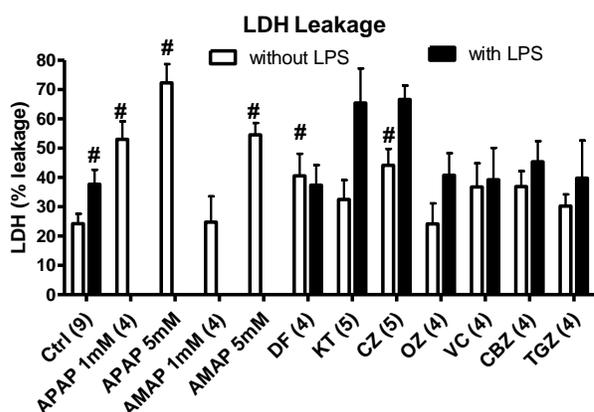


Figure 4. LDH leakage from PCLS into incubation media following 24 h incubation with different drugs in absence (open bars) or presence (black bars) of LPS. LDH leakage has been expressed as the percentage of the total LDH content in a slice. The graph represents the mean values \pm SEM of 4-9 experiments, using 3 PCLS for each group in every experiment. # $P<0.05$ by two-tailed paired Student's t-test for comparison of two groups: control and drug treated only.

The viability of mouse PCLS was assessed by LDH leakage measurement into an incubation medium from a slice after 24 h incubation. (Fig.4) The mean value of LDH leakage in control group was 24.2%. All tested drugs showed toxicity in varying extent, with the exception of AMAP 1mM and OZ where LDH leakage after treatment of these drugs was equal to that of control group. APAP in both concentrations 1mM and 5mM, AMAP in a high concentration 5mM, DF and CZ showed significant toxicity ($P<0.05$) after 24 h incubation. Furthermore, it has been shown that LPS by itself caused slight, but significant toxicity on mouse PCLS. No synergistic toxicity was found after PCLS incubation with DF, OZ, VC, CBZ or TGZ in the presence of LPS. Even though LPS enhanced the toxicity of KT and CZ up to 66%, it was not statistically significant.

LDH leakage was elevated in all experimental groups, including the control group, while in previous experiments mean values were at a lower range: LDH leakage in the control group was 11%. Therefore, to assess the reliability of our LDH data, measurement of alanine transaminase

(ALT) level in all control group samples was performed: ALT level was within normal range (data not shown).

24 h incubation human studies

3.3 Glutathione content in samples

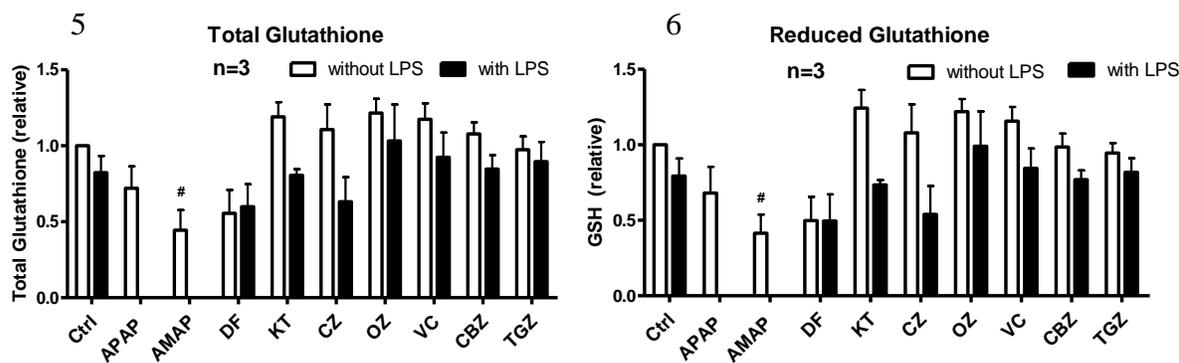


Figure 5,6. Total and reduced glutathione content of human PCLS after 24 h incubation with different drugs in absence (open bars) or presence (black bars) of LPS. The total and reduced glutathione content has been expressed as relative values to control without LPS treatment. Graphs represent mean values \pm SEM of 3 experiments, using 3 PCLS for each group in every experiment. # $P < 0.05$ by two-tailed paired Student's t-test for comparison of two groups: control and drug-treated only.

It has been demonstrated that after 24 h incubation human PCLS, like mouse PCLS, retain the ability to synthesize glutathione. The mean absolute values of total and reduced glutathione in control group samples was 53 and 51 μ M respectively. Due to the negligible difference in total glutathione and GSH level in human PCLS, it was not accurate to calculate the GSH/GSSG ratio: in some samples GSH accounted for more than 99% of total glutathione. Moreover, insufficient sensitivity of the glutathione tests prevents accurate determination of the GSSG content due to the small difference between total and reduced glutathione values.

The present study has shown that following 24 h incubation, in human PCLS AMAP caused a significant decline in both total and reduced glutathione levels in contrast to the findings in mouse PCLS. Though DF by itself and DF+LPS noticeably decreased the total glutathione and GSH level, it was not found to be statistically significant because of high variation due to the differences in results obtained from "responder" and "non-responder" livers. "Responders" and "non-responders" refers to individuals who show or don't show a measurable synergistic effect upon drug + LPS exposure, respectively. Incubation of human PCLS with the other drugs was shown to have no significant influence on PCLS glutathione content in comparison with control. In contrast to mouse data, LPS by itself caused a slight but not significant decline in total and reduced glutathione levels in human PCLS. Moreover, LPS further decreased total glutathione

and GSH content in drug+LPS co-treated groups. Though, this further decrease in glutathione levels was not found to be significant in any drug+LPS group, a noticeable decrease in LPS+KT and LPS+CZ treated groups in comparison with drug-only treated groups was demonstrated (Fig.5,6).

3.4 Viability of human PCLS: LDH-leakage

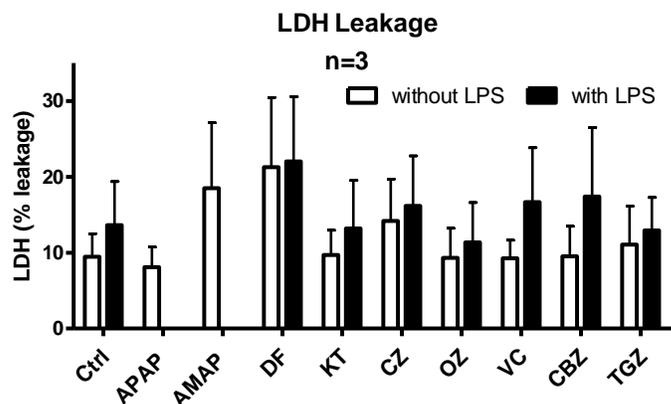


Figure 7. LDH-leakage from human PCLS to the incubation media following 24 h incubation with different drugs in absence (open bars) or presence (black bars) of LPS. LDH leakage has been expressed as the percentage of the total LDH content in a slice. The graph represents the mean values \pm SEM of 3 experiments, using 3 PCLS for each group in every experiment.

The viability of human PCLS was assessed by LDH leakage measurement to an incubation medium from a slice after 24 h incubation. (Fig.7) The mean value of LDH leakage in the control group was 9.5%. Almost all tested drugs did not enhance LDH leakage to medium following 24 h incubation, whereas certain drugs, such as AMAP and DF, caused noticeable, though not significant, increase in LDH leakage. Furthermore, it was found that LPS by itself caused slight toxicity on human PCLS. Nevertheless, there was no synergistic toxicity revealed after human PCLS incubation with any of the drugs in the presence of LPS.

However, despite the LDH data demonstrated in Fig.7, our previous human studies showed that LDH measurement is not informative for toxicity assessment in human PCLS.

3.5 Viability of mouse PCLS: ATP-content, LDH-leakage

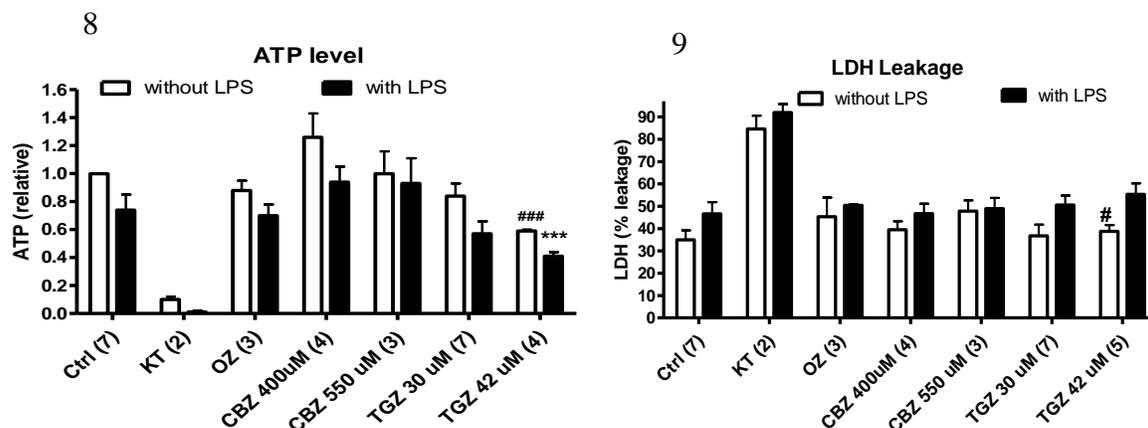


Figure 8,9. ATP content of mouse PCLS and LDH leakage from PCLS to incubation medium after 48 h incubation with different drugs, in absence (open bars) or presence (black bars) of LPS. ATP content has been expressed as relative values to control without LPS treatment. LDH leakage has been expressed as the percentage of the total LDH content in a slice. Graphs represent mean values \pm SEM of 3-7 experiments, using 3 PCLS for each group in every experiment. # $P < 0.05$ and ### $P < 0.001$ by two-tailed paired Student's t-test for comparison of two groups: control and drug-treated only. *** $P < 0.001$ by two-way ANOVA for comparison of four groups together to test the drug+LPS interaction: control, LPS-only, drug-only and LPS+drug groups.

Viability of mouse PCLS following 48 h incubation was assessed by ATP content and LDH-leakage measurement. (Fig.8,9) According to the obtained results, mouse PCLS retained the ability to synthesize ATP following 48 h incubation without changing the medium: the mean value of ATP in control group without LPS was 8.26 pmol/ μ g protein. The mean value of LDH leakage in control group was approximately 35%.

Based on ATP content and LDH-leakage data, TGZ 42 μ M caused significant toxicity in mouse PCLS, whereas other tested drugs revealed no or slight toxicity. LPS by itself was shown to be slightly, though not significantly, toxic after 48 h incubation: ATP value dropped to 5.87 pmol/ μ g protein and LDH-leakage reached 46.6 %, which is higher than in a control group. Though LPS further decreased the ATP level in LPS+TGZ 30 μ M- and LPS+TGZ 42 μ M- treated groups, only in the latter group synergistic supra-additive toxicity was found to be statistically significant ($P < 0.001$). In other LPS-co-treated groups this phenomenon was not observed. Furthermore, LPS noticeably, though not significantly, increased LDH leakage in LPS+TGZ 30/40 μ M-treated groups compared to TGZ alone. Due to high toxicity of KT by itself following 48 h incubation, estimation of synergistic toxicity with LPS was not possible: ATP content was lower than 1 pmol/ μ g protein and LDH leakage was approximately 85 % in KT- only treated group.

3.6 Glutathione content in samples

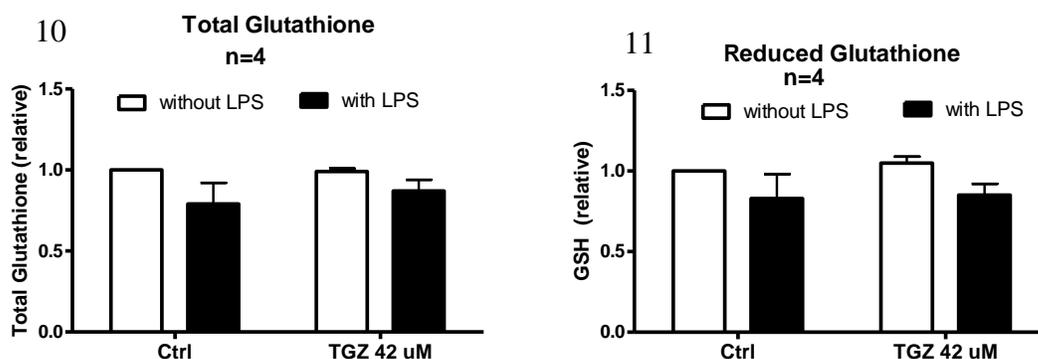


Figure 10, 11. Total and reduced glutathione content of mouse PCLS after 48 h incubation with TGZ 42 μ M in absence (open bars) or presence (black bars) of LPS. Total and reduced glutathione content has been expressed as relative values to control without LPS treatment. Graphs represent mean values \pm SEM of 3 experiments, using 3 PCLS for each group in every experiment.

Our results have shown that after 48 h incubation without changing the medium mouse PCLS retain the ability to synthesize glutathione and maintain the reduction of GSSG to its reduced state: GSH. The mean absolute values of total and reduced glutathione in control group was 36 and 30 μ M respectively. Remarkably, in contrast to the results after 24 h where around 53 % of total glutathione is in the reduced form, after 48 h most of the glutathione is in the reduced form (83%). Therefore due to the negligible difference in total glutathione and GSH levels, just as in human PCLS, it was not possible to calculate the GSH/GSSG ratio.

In contrast to the mouse glutathione data obtained after 24 h incubation, LPS was shown to cause slight decrease in total glutathione and GSH levels following 48 h incubation. However, the high variation in LPS-treated PCLS is due to one mouse experiment that exhibited higher sensitivity to LPS: the relative values of total and reduced glutathione were around 0.45 in LPS only treated group. Moreover, upregulation of glutathione synthesis after 48 h incubation of PCLS with TGZ 42 μ M did not occur: glutathione level in this group was comparable with control. Additionally, just as with 24 h incubation, no enhanced reduction in glutathione level in TGZ 42 μ M group co-treated with LPS was revealed.

IV. Discussion

As it has been mentioned earlier, there is evidence based on results from experimental *in vitro* and *in vivo* models, confirming that inflammatory stress could be a susceptibility factor for IDILI manifestation. It is noteworthy, that microorganisms from the gastrointestinal tract can periodically induce mild inflammation in their host. Thus, LPS, reaching the blood circulation, can play the role of inflammatory mediator. Different factors, such as alcohol, diet, concurrent diseases, and antibiotic treatment, can contribute to the elevation of LPS level in blood. (18)

In several studies, it was suggested that LPS induces a decline in the GSH/GSSG ratio. LPS decreased GSH/GSSG ratio in human monocyte-derived dendritic cells (DC) (20), and also in phagocytes and lymphocytes in mouse (22) and rat (34) septic shock model caused by intraperitoneal injection of endotoxin. However, conflicting results have been reported, making this suggestion still controversial (35). Accordingly, we hypothesized that a decline in GSH level induced by LPS may contribute to IADRs development.

Many mammals protect their tissues by strict regulation of defense mechanisms. Glutathione is included in the first line of defense against oxidative processes and is a substrate in the GSH redox cycle (22). GSH detoxifies reactive intermediates and electrophilic metabolites, which has been thought to be one of the causes of IDRs, and which produced as a result of exposure to various toxicants or stress factors, (27, 36-37). Moreover, glutathione plays an important role in the regulation of apoptosis. Thus, GSH depletion is a trigger factor of apoptotic pathways. (38)

Cellular glutathione level represents the ability of cells to handle oxidative stress and is an important parameter in the tissue viability evaluation. (32) Decrease in the total glutathione level can characterize conjugation processes or can be a result of direct cell toxicity, leading to a drop in cellular GSH synthesis or its extracellular degradation. Decline in GSH level and GSH/GSSG ratio, on the other hand, are markers of oxidative stress. Thus, the observed drop in total glutathione, GSH levels and GSH/GSSG ratio in mouse PCLS after APAP 1mM, KT, CZ, CBZ-treatments, which we found in our study, can be explained by GSH-conjugates formation with changes in oxidant pressure and/or cellular ability of GSH synthesis. (39-43) Our results were in line with a previous study where a decline of the GSH/GSSG ratio in rat liver was demonstrated after CBZ administration. (43) Additionally, GSH depletion following APAP-treatment of hepatocyte cell culture resulted in a rapid loss of cell viability, which led to the conclusion that APAP-hepatotoxicity appears to be critically dependent on cellular glutathione level. (26) The significant decrease in all glutathione parameters with congruous increase in LDH-leakage following mouse PCLS-treatment with high concentrations of APAP and AMAP (5mM) may be due to the GSH-conjugation of the reactive metabolite which is accompanied by a considerable

drop in cells viability. On the other hand, the observed decrease in the GSH level and GSH/GSSG ratio without any changes in the total glutathione level with DF treatment may indicate glutathione consumption due to ROS generation and oxidative stress that is in line with literature data regarding its mechanisms of toxicity. (18, 44-46)

All drugs, except AMAP 1mM and OZ, increased in varying extent the LDH-leakage from mouse PCLS following 24 h incubation: for APAP 1mM- and 5mM-, AMAP 5mM-, DF- and CZ-treated groups the increase was found to be significant ($P < 0.05$). These results correlate with our shown glutathione data and are in agreement with previous reports, that AMAP in low concentrations is not toxic in mice. (47)

In the present study, we have demonstrated that a low dose of LPS increased total glutathione, GSH levels and GSH/GSSG ratio in mouse PCLS after 24 h incubation in comparison with control. Our findings are in line with previous studies, which demonstrated the increase in baseline GSH content in hepatic macrophages on exposure to a low dose of endotoxin *in vivo* (35), but in contrast to several others above mentioned studies, that showed a decrease in GSH/GSSG ratio after LPS exposure (20,22,34).

There are several possible hypotheses to explain this phenomenon. First of all, the augmented supply of NADPH through the hexose monophosphate shunt in LPS-stimulated cells can contribute to the fast recovery of cellular GSH level after exposure to toxins, including drugs, in comparison with control. (35) Moreover, it has been suggested that LPS stimulates glutathione synthesis in Kupffer cells *de novo*, as a higher total glutathione level was detected. (35)

Additionally, it has been demonstrated that LPS produces an increase in nuclear accumulation of NF- κ B. (19) NF- κ B is a central mediator of the immune response by enhancing transcription of genes responsible for the expression of cytokines, chemokines and adhesion molecules production, involved in ROS generation. (19,48) Previously it has been reported that GSH depletion blocks the phosphorylation of the inhibitory I κ B complex and therefore reduces its degradation, resulting in abolishment of LPS-induced NF- κ B nuclear localization. (49) These results contradicted with the subsequently published data showing that decrease in GSH and increase in GSSG levels leads to rapid ubiquitination, phosphorylation of the I κ B complex resulting in its degradation, leading to the NF- κ B nuclear localization. (22) On the other hand, it has been reported that NF- κ B, aside its pro-inflammatory properties, is implicated in the up-regulation of the γ -glutamylcysteine synthetase (γ GCS), enzyme for GSH synthesis: up-regulation of γ GCS leads to an increase in cellular glutathione level. (19) To sum up, a moderate concentration of LPS seems to stimulate up-regulation of protective cellular mechanisms.

Therefore, these findings can explain our results regarding the trend of elevation (though not significant) in total glutathione and GSH levels, as well as GSH/GSSG ratio due to LPS.

Furthermore, similar results were reported following chronic administration of low doses of APAP to rats and mice: mild liver injury caused by low doses of APAP leads to upregulation of hepatic glucose-6-phosphate dehydrogenase and glutathione reductase, resulting in an increase in glutathione content, contributing to the protection against the lethal dose of APAP. (16)

Interestingly, the drop in total glutathione, GSH levels and/or GSH/GSSG ratio were more pronounced in drug-only-treated PCLS, than in LPS co-treated PCLS with several tested drugs, such as DF, OZ and CBZ. This can be explained by LPS-stimulated alternative protective mechanisms mentioned above. Nevertheless, LPS co-treatment had no influence on VC-treated PCLS. A significant decrease in total glutathione, GSH levels and in GSH/GSSG ratio has been demonstrated with interaction of LPS+KT or -CZ in comparison with other experimental groups: control group, LPS- or drug only-treated groups. Furthermore, this study found that hepatic damage resembled oxidative stress: LDH leakage considerably increased, though not significantly, following LPS+KT or -CZ treatment. The synergistic hepatotoxicity of the same degree was not demonstrated with other drugs. Additionally, our data correlates with previous research from Hadi *et al.* In their study it was shown, that LPS not only triggers LDH leakage in mouse PCLS following 24 h incubation, but also significantly decreases ATP level in slices treated with KT and CZ (data not shown).

The variance of the inflammatory co-stimulation impact on drug-treated PCLS showed once more that mechanisms of IDILI differ between drugs, and include diverse downstream toxicological molecular pathways. Metabolic pathways of drugs can serve as a probable explanation of the inflammatory-induced synergistic toxicity with preceding glutathione depletion in LPS+KT or -CZ treated PCLS. Recently, several new reactive and toxic metabolites of KT were found in mouse liver that are N-oxides. The concentration of these metabolites was much smaller than those of the main metabolites: N-deacetyl ketoconazole, dialdehydes and oximes. The natural way of detoxification of the formed N-oxides is reduction. (40) As this process is NADPH dependent, a rapid and extensive detoxification process can lead to the decrease in tissue NADPH concentration, eventually leading to depletion. As a result, a lot of NADPH-dependent processes in cells, including the reduction of GSSG to GSH, are affected, leading to failure in compensation mechanisms and, as a result, toxicity manifestation. (40), One of the main reactive CZ metabolites is a Nitrenium ion, which aside conjugation with GSH, can also be directly reduced by NADPH, which may result in failure of cellular protective mechanisms. (42) However,

the data cannot exclude that other previously not described pathways can be responsible for observed toxicity.

It has been hypothesized that hepatotoxicity of TGZ is mediated through intra-mitochondrial oxidative stress. TGZ-induced dissipation of the mitochondrial inner transmembrane potential can lead to a rise in permeability of mitochondrial membranes, which causes progressive deterioration, alteration of their integrity and, as a result, cell damage. (50) Also, it has been reported that TGZ forms in total five GSH conjugates (51-52), leading to the assumption that the cellular glutathione level may decrease accordingly. On the contrary, in the present study, increase in total glutathione, GSH levels and GSH/GSSG ratio has been shown following mouse PCLS incubation for 24 h with TGZ with or without LPS. According to the obtained results from several *in vitro* and *in vivo* animal and human studies, TGZ possess antioxidant and anti-inflammatory activities. Using rat lens culture, it has been demonstrated that TGZ significantly prevented lipid peroxidation. Moreover, TGZ increased lens Glutathione Reductase. (53) Additionally, a reduction in ROS generation by leukocytes and an inhibition of lipid peroxidation in patients has been demonstrated. (48) Also, TGZ caused a reduction in intranuclear NF- κ B-binding activity and induced an increase in the expression of κ B (I κ B)-inhibitory complex. (48) Although this data contradicts evidence obtained from other studies mentioned above, it does correlate with our results, supporting the hypothesis that TGZ exerts a potent anti-inflammatory effect in addition to antioxidant activity.

It has been reported that the glutathione cellular level may fall temporarily after exposure to toxins, but usually cells are able to compensate this and rapidly restore the initial glutathione level. (38) Our data showed that after exposure to certain drugs only or in combination with an inflammatory agent (KT+LPS, CZ+LPS), cells failed to tolerate the decrease in glutathione level and failed to compensate it. Therefore, glutathione may play an important role in toxification/detoxification of certain drugs, leading to IDILI development. Moreover, our data supports the concordance between animals and humans in synergistic impairment of defense mechanisms with certain IDILI-associated drugs together with LPS: the decrease in total glutathione and GSH levels was found to be more pronounced in LPS+KT, LPS+CZ-treated human PCLS when compared to other drug-treated human PCLS following 24 h incubation. On the other hand AMAP in human PCLS was shown to be more toxic than APAP in respect of LDH-leakage and glutathione depletion, that correlates well with earlier obtained results by Hadi *et al* (data not shown). This phenomenon possibly may be related to different metabolic pathways and downstream molecular toxicological mechanisms in mouse and human.

Additionally, the differences in hepatotoxicity among PCLS from various individuals after certain drug treatment, such as DF, suggest that the individual variability in isoenzyme

expression, differences in genetic, environmental factors and/or defense mechanisms may play a vital role in probably of IDILI development. Especially, inter-individual variability is more pronounced in human, that makes prediction of IDILI in human even more difficult.

It was reported previously that synergistic toxicity was not always observed following 24 h incubation. It was shown that DF exhibits synergistic toxicity with LPS only after 48 h incubation (data not shown). In the present study, to verify this observation, incubation of mouse PCLS for 48 h with drugs was performed only if no synergistic toxicity after 24 h incubation in presence or absence of LPS was found. LPS only, OZ, chosen as a non-toxic drug, CBZ 400-/ 550 μ M- only or in combination with LPS rendered no or insignificant influence on mouse PCLS viability. KT, chosen as a positive control, was demonstrated to be highly toxic with and without LPS following 48 h incubation, rendering evaluation of any additive-toxicity impossible. Based on ATP and LDH data, we have demonstrated synergistic toxicity when TGZ 42 μ M was incubated together with LPS (ATP: $P < 0.001$) for 48 h. Glutathione content was not altered, neither by TGZ 42 μ M, nor by its combination with LPS, which is probably due to the antioxidant activity of TGZ mentioned above. Also, this observation indicates that toxicity of TGZ is unrelated to glutathione cellular level and antioxidant protection. One interesting point is that the GSH content in the control group was higher following 48 h incubation in comparison with 24 h, while total glutathione remained the same. This might imply that cellular molecular processes in PCLS recover even more after 48 h of incubation: glutathione reductase is able to reduce GSSG to GSH at a higher rate. Elevated total glutathione and GSH levels as well as the capacity of cells to maintain glutathione in its reduced state, play an important role in protection against oxidative stress and detoxification of different electrophiles and ROS species.

It is known that patients with impaired defense mechanisms, often due to age, genetic or environmental factors, can develop severe adverse reactions. (39,46) For example, protein malnutrition, that affects mostly the hospitalized and elderly, may lead to a decrease in tissue antioxidant level and, as a result, to an increase in sensitivity to infections and ROS-induced tissue damage. (28) Decreasing GSH and NADPH level with age also could have a marked effect on the detoxification capacity. (54) Additionally, women have much lower levels of plasma GSH than men (55), which may partly explain the higher rate of IDILI in females. Therefore, many different factors should be taken into account in evaluation of drug safety, such as age, sex, genetic polymorphism of drug metabolizing enzymes and genetic or acquired mitochondrial abnormalities. (43) Thus, individuals who might have a genetic or acquired deficiency in oxidant defense system or other mitochondrial abnormalities might be more susceptible for toxicity manifestation. (50) For example, the IDILI induced by CBZ has been associated in humans with disturbance in glutathione metabolism (43), while it was suggested that GSH depletion may play

a role in DF-induced hepatotoxicity. (46) Furthermore, patients with GSSG reductase or glutathione synthetase (GSS) deficiency or genetic defects resulting in synthesis of an unstable GSS molecule, exhibit increased sensitivity to chemical toxicants and oxidative stress. (36) One interesting point to mention is that studies on mutant microorganisms showed that a lack of GSS does not always lead to an increase in sensitivity to radiation and to certain chemical toxicities (36). This brings us to the hypothesis that disturbance in antioxidant cellular status can be a predisposing but not exclusive factor in DILI development. And, as mechanisms of IDILI are even more complicated, it is likely that the combination of several stress factors is needed to trigger a pathological cascade leading towards IDILI.

In summary, the drug itself or its reactive metabolites can lead to glutathione depletion by forming GSH-conjugates or stimulating ROS generation, as was shown by the present and several previous studies. (16) Also, we demonstrated that LPS by itself did not cause any significant changes in cellular glutathione level, neither in mouse nor human PCLS, contrary to what was hypothesized. Furthermore, our study revealed synergistic hepatotoxicity of certain IDR- related drugs, such as KT and CZ, with the inflammatory agent, LPS, following 24 h incubation and TGZ together with LPS following 48 h incubation. These results add evidence to the hypothesis that interaction of LPS with potentially hepatotoxic drugs may lead to IDILI development. Moreover, based on these results, the glutathione level can play a role as a novel biomarker for certain IDILI prediction, as statistically significant selective downregulation of total glutathione, GSH levels and GSH/GSSG ratio after PCLS treatment with KT or CZ in combination with LPS was demonstrated. It is unlikely that one mechanism is responsible for all IDILI development and different factors can have an impact on the outcome of the drug therapy. Although there are still many unresolved questions and areas of controversy, a rather large number of experimental observations point to inflammation as an important participant in IDILI.

In conclusion, our study provided additional evidence to support the inflammation-stress hypothesis in IDILI development in precision-cut liver slices and revealed that glutathione levels can be regarded as potential biomarkers that could be valuable in prediction, prevention and monitoring of toxicity development.

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Appendix 1

Glutathione assay protocol (modified)

Materials:

- DTNB
 - 19.8 mg DTNB in 5 ml ethanol, then 1:10 dilute in 0.5 M Tris/10mM EDTA buffer, pH 8 (45ml)
- 0.5 M Tris-HCl 10 mM EDTA buffer, pH 8
MW Tris 121.14 so 6.057 g/100ml mQ).
(to 6.057 g Tris add 70 ml mQ, then add 10 ml 100 mM EDTA, bring to pH 8 and volume 100 ml)
- 50 mM Tris-HCl buffer + mM EDTA, pH 7.4. Tris (Mw 121.14 weighting room. 605.7 mg Tris/100 ml mQ).
- 50% TCA: weight 5 g TCA and fill till 10 ml.
- 100 mM EDTA (to 1.4612 g EDTA add 50 ml mQ).
- Stock solution 1mM reduced GSH (0.307 mg/ml 50 mM Tris-HCl + 1mM EDTA).
(7.675 mg GSH in 250 μ l buffer, from here 50 μ l + 4,95 ml buffer).
- Micro-titer plate, 96 wells flat bottom with lid. Nontreated 20/pack. 100/case Costar 3370.
- NADPH (1mM)
NADPH, fridge human lab (47) MW=833.4
8.33 mg/10 ml mQ= 1mM
or 10 μ L of 100 mM NADPH in 990 μ L mQ
- Glutathione reductase (20U/ml), keep cold. GR, fridge, human lab (63).
22 μ L of GR (available: 2.2 mg protein/ml, 205 unites/mg protein) + 474 μ L of buffer (50 mM Tris-HCl + 1mM EDTA).
Calculations: $2.2 \times 205 = 451$ unites per ml, so 9.922 unites in 22 μ L. We need 20 U/ml, so we should dilute 22 μ L of initial GR in 474 μ L of buffer ($9.922 \times 1\text{ml}/20$ unites and minus 22 μ L).

GSH

Methods:

1. Use slices which are washed in NaCl and/or medium samples from -80.
2. Homogenise slices using MiniBeater (45 sec) in 400 µl ice cold 50 mM Tris-HCl buffer which contains 1 mM EDTA pH 7.4.
3. Centrifuge all samples 3 min at 13000 rpm, 4°C.
4. Prepare standard series samples according to the table.

Stock solution of 1mM reduced GSH (0.307 mg/ml)

	Concentration		50 mM Tris-HCl + 1mM EDTA buffer 7.4
A	0.200 mM	100 µL stock solution	400 µL
B	0.150 mM	100 µL stock solution	565 µL
C	0.100 mM	100 µL stock solution	900 µL
D	0.050 mM	250 µL C	250 µL
E	0.025 mM	200 µL C	600 µL
F	0.000 mM	0	500 µL

5. Take 150 µl supernatant to microcentrifuge tube and standard series.
6. Add 15 µl 50% TCA to samples and standard series and keep on ice.
7. Centrifuge all samples 5 min at 13000 rpm, 4°C.
8. Carefully transfer 50 µL of supernatant to a micro-titer plate in duplo.
9. Transfer 50 µl standard series to a micro-titer plate in duplo (A, H)
10. Add 200 µl of DTNB to each well. Do it fast (within 10 min).
11. Measure the absorbance at 405 nm (plate reader, 3 floor) after 5 min.

GSH+GSSG

1. Take 150 µl supernatant to microcentrifuge tube.
2. Add 15 µl NADPH to each tube (1mM)
3. Add 7,5 µl glutathione reductase to each tube (20U/ml)
4. Incubate tubes at 37°C 15 min.
5. Add as quickly as possible 15 µL 50% TCA to the samples and standard series and put on ice.
6. Centrifuge all samples 5 min 2000 g (4600 rpm) at 4°C.
7. Transfer 50 µl supernatant to a micro-titer plate in duplo.
8. Transfer 50 µl standard series to a micro-titer plate in duplo (A, H)
9. Add 200 µl of DTNB to each well. Do it fast (within 10 min).
10. Measure the absorbance at 405 nm (plate reader, 3 floor) after 5 min.