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Maintenance of high quality rat precision cut liver slices during culture to study hepatotoxic responses: Acetaminophen as a model compound

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ABSTRACT

Precision cut liver slices (PCLiS) represent a promising tool in reflecting hepatotoxic responses. However, the culture of PCLiS varies considerably between laboratories, which can affect the performance of the liver slices and thus the experimental outcome. In this study, we describe an easily accessible culture method, which ensures optimal slice viability and functionality, in order to set the basis for reproducible and comparable PCLiS studies. The quality of the incubated rat PCLiS was assessed during a 24 h culture period using ten readouts, which covered viability (lactate dehydrogenase-, aspartate transaminase- and glutamate dehydrogenase-leakage, ATP content) and functionality parameters (urea, albumin production) as well as histomorphology and other descriptive characteristics (protein content, wet weight, slice thickness). The present culture method resulted in high quality liver slices for 24 h. Finally, PCLiS were exposed to increasing concentrations of acetaminophen to assess the suitability of the model for the detection of hepatotoxic responses. Six out of ten readouts revealed a toxic effect and showed an excellent mutual correlation. ATP, albumin and histomorphology measurements were identified as the most sensitive readouts. In conclusion, our results indicate that rat PCLiS are a valuable liver model for hepatotoxicity studies, particularly if they are cultured under optimal standardized conditions.

1. Introduction

Drug-induced liver injury (DILI), reflecting both, the intrinsic as well as the severe idiosyncratic form, is a multifaceted form of organ damage, which still represents a major pitfall in the drug development process and for the safety of newly marketed drugs (Fung et al., 2001; Lee, 2003; Uetrecht, 2008; Roth & Ganey, 2010; Watkins, 2011; Goldring et al., 2015). The intercellular communication of parenchymal hepatocytes with non-parenchymal cells such as liver resident macrophages (Roberts et al., 2007; Adams et al., 2010) as well as zone-specific damage (Kleiner et al., 2014) play an important role in DILI. Liver models with the relevant cell types and structural features of the in vivo tissue would thus improve the ability to predict hepatotoxicity in the early pre-clinical screening process of drug candidates and thereby enhance their safety. Currently, widespread used in vitro models for the prediction of DILI often lack the three-dimensional architecture and multicellular complexity of the target organ and therefore cannot mimic crucial tissue dynamics in vitro (Brandon et al., 2003). Precision cut liver slices (PCLiS), a liver model that is used by a growing number of researchers to study a number of open questions in the areas of toxicology, pharmacology and the metabolism of xenobiotics (Lerche-Langrand & Toutain, 2000; Lake & Price, 2013; Olinga & Schuppan, 2013), retain the original liver tissue architecture with all naturally occurring cell types and offer a great variety of possible readouts (Lerche-Langrand & Toutain, 2000; Vickers & Fisher, 2005; de Graaf et al., 2010). Importantly, in addition to the frequently used biochemical methods in in vitro/ex vivo models, clinically relevant biomarkers and histomorphological techniques can be applied to the PCLiS model. This test system, which mirrors to a great extent the in vivo situation in the liver, might help in finding a more reliable liver

Abbreviations: AST, aspartate transaminase; BSA, bovine serum albumin; C_{max}, maximal plasma concentration; DILI, drug-induced liver injury; EC50, half maximal effective concentration; GLDH, glutamate dehydrogenase; KH, Krebs-Henseleit; LDH, lactate dehydrogenase; NL, normal liters; PCLiS, precision cut liver slices; SD, standard deviation; SER, smooth endoplasmic reticulum

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model to predict human DILI. However, this promising liver model can only be as good as its performance and stability and the reproducibility of the results obtained with it. These fundamental quality aspects of the system strongly depend on the use of high quality tissue and, just as important, on a culture setup, system and protocol that together ensure optimal culture conditions in order to maintain the slice viability and functionality and to accurately reproduce an in vivo outcome (Vickers & Fisher, 2005; de Graaf et al., 2010). Although a great effort has been made to optimize the PCLiS technique, there is no consensus among the laboratories regarding the culture setup, system and protocol as well as the longest possible incubation period that will support an optimal tissue performance. While the preparation of the slices is very similar between laboratories and everyone agrees that PCLiS can be better maintained in dynamic cultures (Fisher et al., 1995; Olinga et al., 1997a, 1997b; de Graaf et al., 2007, 2010) and in an atmosphere with an enhanced oxygen concentration (Drobner et al., 2000; Evdokimova et al., 2002; Szalowska et al., 2013), there are great differences in the used culture setup (incubator, gas supply) and system (culture container), the applied oxygen concentration in the culture atmosphere, the culture medium including the added supplements, the pre-incubation period, the total culture period and whether a perfusion of the liver is performed before its removal or not (compare Supplementary Table 1). Based on this high number of possible variations, it becomes obvious that the extremely different management of PCLiS cultures in each particular laboratory can affect the performance of the liver slices in a different way and to a different extent, thereby strongly influencing the experimental outcome and preventing the routine use of this valuable liver model. Both may lead to results that are neither representative nor comparable. Many working groups are aware of this fact and make great effort to prove the functionality and viability of their cultured PCLiS before they start their actual study (Karim et al., 2013; Koch et al., 2014; Westra et al., 2016). To prevent the need of this additional expense and to increase the reproducibility of results in future PCLiS studies, our aim was to demonstrate that as long as the PCLiS culture is performed under optimal conditions representative and comparable results can be obtained. A standardized culture method ensuring optimal culture conditions would furthermore allow similar liver slice quality and outcomes regarding model hepatotoxicants at different laboratories enhancing the comparability of PCLiS studies. To this end we established a simple and easily accessible culture setup, system and protocol for the optimal maintenance of PCLiS during culture and characterized extensively the incubated liver slices regarding their quality. For a better comparability, our study was performed with liver tissue from the most studied animal species, the rat, and the most accepted and used culture parameters based on a comprehensive literature search. The characterization was based on a number of different readouts including viability (lactate dehydrogenase [LDH]-, aspartate transaminase [AST]- and glutamate dehydrogenase [GLDH]-leakage, ATP content) and functionality parameters (urea and albumin secretion) as well as histomorphology and other descriptive characteristics (protein content, wet weight and thickness of the slices). After verifying the performance of the rat PCLiS during a 24 h culture period, we exposed the PCLiS to increasing concentrations of the model compound acetaminophen (APAP) to assess the significance of the ten readouts in the evaluation of hepatotoxic responses. In this context, we furthermore aimed to identify whether our cultured PCLiS provide a robust basis for the generation of reproducible and comparable responses. By presenting a well-described and -characterized culture setup, system and protocol, which ensure an optimal tissue quality during a 24 h culture, as well as meaningful readouts, we contribute to push forward the standardization of the PCLiS technique and to improve the reproducibility of PCLiS studies.

2. Materials & methods

2.1. Animals

Male Wistar rats (Crl:WI(Han); 220–240 g; 7 weeks old) were obtained from Charles River (Sulzfeld, Germany). Three animals per Makrolon Type III cage were housed for one week under standard environmental conditions (20 ± 2 °C, 55 ± 15% relative humidity and 12 h dark/light cycles) for acclimatization before sacrification. They were fed with the standard diet ssniff V 1534 (ssniff-Spezialdiäten GmbH, Soest, Germany) and had access to water ad libitum. All experiments were conducted in accordance with the German Animal Protection Act.

2.2. Excision of rat liver

The non-fasted rats were sacrificed under Narcoren®-induced deep anesthesia (160 mg pentobarbital/kg BW; Merial GmbH, Hallbergmoos, Germany) by exsanguination via an incision of the vena cava caudalis. Thereafter, the liver was removed and immediately placed in ice-cold, sterile and oxygenated Krebs–Henseleit (KH-) buffer (pH 7.4, with 11 mM glucose) (Sigma-Aldrich GmbH, Taufkirchen, Germany) supplemented with 25 mM $NaHCO_3$ (Sigma-Aldrich) and 2.5 mM $CaCl_2 \cdot H_2O$ (Sigma-Aldrich), to prevent warm ischemia. Oxygenation of the buffer was achieved by saturation with carbogen (Carbogen®LAB (95% O_2, 5% CO_2); Linde Gas GmbH, Stadl-Paura, Germany). For this purpose, the ice-cold buffer was gassed with 3 normal liters (NL) carbogen/min for 30 min. The slice preparation started about 10 min after liver removal.

2.3. Preparation of PCLiS

With the help of a biopsy punch (pfm medical AG, Cologne, Germany), tissue cylinders (Ø 8 mm) were punched out of the whole liver without distinction of the lobes and collected in ice-cold oxygenated KH-buffer on melting ice until slicing. Cylindrical liver cores were placed in a Krumdieck tissue slicer MD 6000 (Alabama Research and Development, AL, USA) filled with ice-cold oxygenated KH-buffer and pushed down with a weight (5.68 g). Tissue slices of 249 ± 33 µm thickness and a wet weight per three pooled slices of 58 ± 4 mg (approx. 19 ± 1 mg per slice) were cut. The slicing buffer and blade were replaced at least three times during the slicing process. The freshly cut slices were collected in ice-cold oxygenated KH-buffer on melting ice until culture (about 45 min cold ischemia time). Slices were inspected and those with processing-related defects were rejected. The selected slices for the culture were randomly assigned to 25 mL flasks.

2.4. Culture setup and incubation protocol for PCLiS

The culture setup was composed of four culture boxes (dimensions: approx. 11 × 11 × 21 cm, width × high × depth), which were located in an incubation shaking cabinet (Certomat® CT Plus; Sartorius Stedim Systems GmbH, Göttingen, Germany) in an atmosphere of 37 °C and 90% humidity (Fig. 1A). The boxes were gassed with humidified carbogen, which was enabled by intercalating a gas washing bottle (filled with ~125 mL sterile aqua dest.) in the incubator and connected in series with plug-in couplings. Thereby, it was possible to separate the boxes from one another without losing the accumulated gas within the boxes. Eight 25 mL flasks containing the culture medium and the slices were placed into each box and shaken gently (80 times per minute) for an optimal medium rotation and to limit the mechanical stress for the slices. The gassing regime included a flooding of the boxes with 4 NL carbogen/min for 10 min; thereafter, the gas flow was reduced to 0.8 NL/min during the rest of the culture period in order to compensate for any potential gas loss. Each time that the boxes were opened to handle the slices, for example for a medium exchange, the flooding

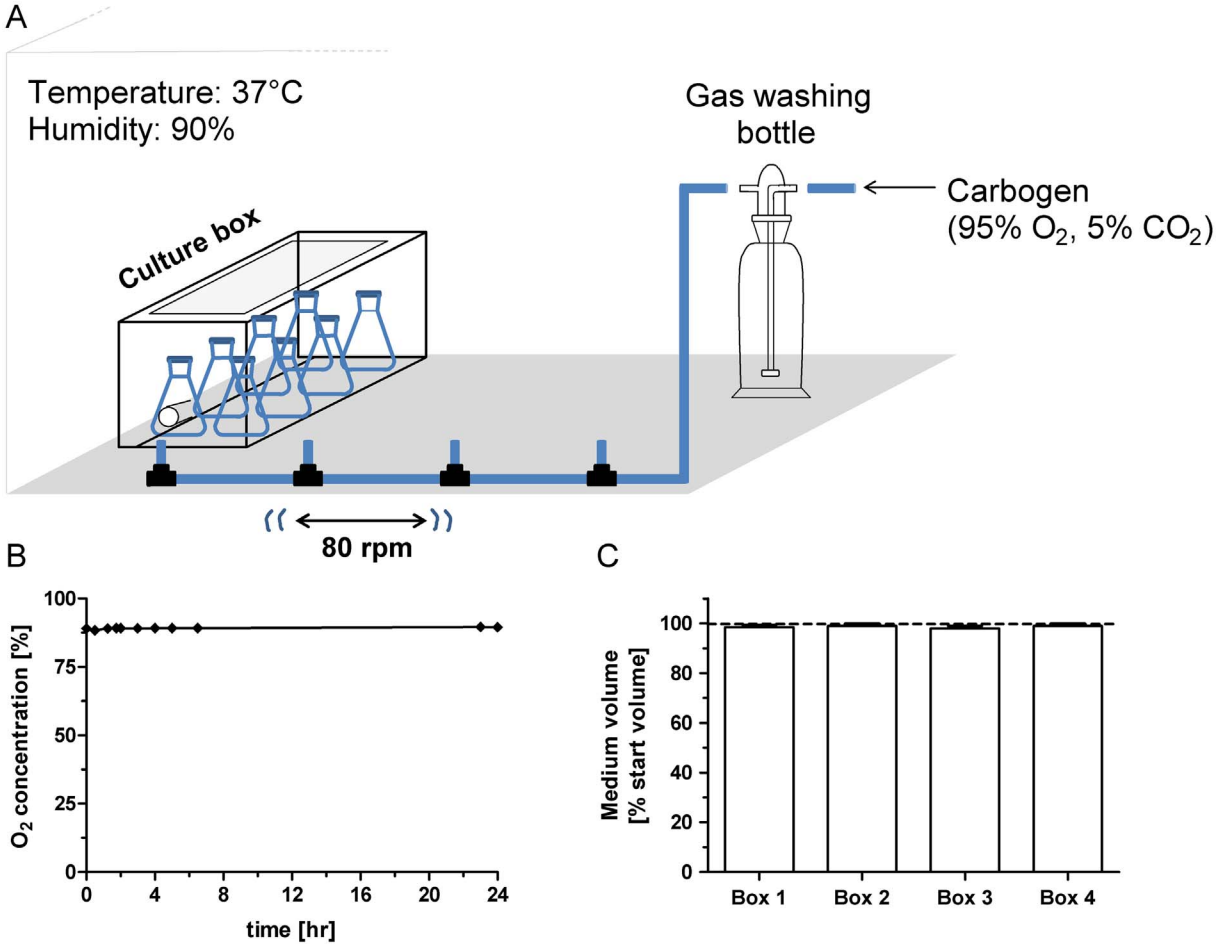

Fig. 1. Schematic illustration and characterization of the culture setup for PCLiS. (A) Culture boxes are located in an incubation shaking cabinet with an atmosphere of 37 °C and 90% humidity and are connected to the carbogen supply, including an intercalated gas washing bottle for gas humidification. (B) The setup was evaluated by the determination of the oxygen concentration that was reached in the culture boxes (88%) by applying the described gassing regime and by determining whether the oxygen supply was stable during the 24 h culture period. (C) Average medium volume in the eight Erlenmeyer flasks located in Box 1–4 after a 24 h culture period. All data are expressed as mean ± SD. Statistical analyses were performed using a one-way ANOVA with Tukey post hoc test.

process was repeated. The oxygen concentration in the boxes was determined with a GOX 100 oxygen analyzer (Greisinger Electronic GmbH, Regenstauf, Germany).

Immediately after completing the slicing process, three slices per 25 mL flask were co-cultured in 6 mL pre-warmed and oxygenated William's medium E (with L-glutamine; Life Technologies, CA, USA) supplemented with 25 mM glucose (final concentration of glucose in the medium: 36 mM) and 50 µg/mL gentamicin (Life Technologies). The tissue slices were allowed to recover from the slicing process and to replenish tissue ATP levels for 1 h (Toutain et al., 1998; Lerche-Langrand & Toutain, 2000; de Graaf et al., 2010). After this pre-incubation period (PI), the slices were transferred into new flasks containing fresh medium to remove any surface debris and cytoplasmic factors such as proteases that may be released from the damaged outer cell layers. Depending on the experiment, the fresh medium contained no additional compounds or different concentrations of acetaminophen or vehicle. A schematic overview of the time schedule of the PCLiS preparation, culture and gassing regime can be found in the supplementary material section (Supplementary Fig. 1).

2.5. Treatment protocols

For the characterization of PCLiS under control conditions, untreated slices were cultured for up to 24 h post PI. The slices were examined immediately after the 1 h PI period as well as 2 h, 6 h and 24 h post PI. Slices treated with acetaminophen (APAP) (Sigma-Aldrich) or vehicle (dimethyl sulfoxide (DMSO); Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were incubated for 24 h (post PI). Slices were exposed to increasing concentrations of APAP (2.55–15 mM), the maximal final concentration of DMSO being 0.47% v/v. The selected concentrations were in the range of 100-fold C_{max}, which Xu et al. (2008) previously considered to be clinically relevant. Samples for the characterization of the slices, which was based on ten readouts including parameters to evaluate slice viability (LDH-, AST- and GLDH-leakage, ATP content) and functionality (urea and albumin secretion) as well as histomorphology and other descriptive characteristics (protein content, wet weight and thickness of slices), were taken at each above-mentioned point in time and treatment condition.

2.6. Wet weight, ATP content and protein content of PCLiS

The three co-cultured slices were simultaneously removed from the medium and the adhering fluid was removed by laying them on a filter paper (Machery-Nagel GmbH & Co. KG, Düren, Germany). Then, the three slices were transferred into a tared Eppendorf tube, the wet weight was recorded and the pooled tissue slices were snap-frozen in liquid nitrogen and stored at − 80 °C until analysis. Freshly cut slices, which were not cultured, were used as controls. The determination of the ATP content was based on the previously described method of Szalowska et al. (2013). In brief, the frozen tissue was transferred into Lysing Matrix D tubes (MP Biomedicals, LLC, CA, USA) containing

ceramic spheres and 20 µL ice-cold CelLytic™ MT buffer (a cell lysis reagent for mammalian tissue; Sigma-Aldrich) per mg wet weight of the pooled slices. The tissue was immediately homogenized (without thawing) for 40 s at a speed of 6.0 m/s in a FastPrep-24 cell and tissue homogenizer (MP Biomedicals). The homogenates were placed on ice and subsequently centrifuged at 14000 rpm and 4 °C for 10 min to remove cellular debris. Two hundred microliters of the supernatant were mixed with 100 µL of the ATP cell lysis solution from the ATPlite Luminescence Assay Kit (PerkinElmer, MA, USA) at RT. For the following protein determination, the remaining supernatant was kept on ice. After a second centrifugation at 14000 rpm and RT for 3 min, 50 µL of the ATP-samples were transferred into a white 96-well plate that contained 100 µL PBS per well. The ATP content in the samples was measured according to the manufacturer's protocol and calculated from a standard ATP calibration curve (0.1–1000 µM). Luminescence was measured with a Wallac 1240 Victor^3 Multilabel counter (PerkinElmer). The ATP determination was performed in technical duplicates and results were expressed as nmol ATP per mg wet weight.

The protein content of the three pooled slices was determined according to the method of Smith et al. (1985) with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. To this end, 20 µL of the supernatant were diluted 15-fold in PBS and the color change of the samples was measured in technical triplicates with a SpectraMax® 340 PC Microplate Reader (Molecular Devices, LLC, CA, USA) at 562 nm. The concentrations were determined by using a bovine serum albumin (BSA) standard curve (7.8–500 µg/mL) and the protein content was expressed as mg protein per three pooled slices.

2.7. LDH, AST and GLDH measurements

The leakage of LDH, AST and GLDH, into the culture medium was determined as a marker for plasma membrane damage/lysis, whereby AST is located primarily in the mitochondria and to a smaller extent in the cytoplasm and GLDH is located solely in the mitochondria of liver parenchymal cells (Zimmerman, 1974; Schmidt & Schmidt, 1988). Alanine transaminase (ALT) is mostly present in the liver, while AST is found in various different tissues besides the liver. However, ALT was neglected as a possible hepatotoxicity marker because the high pyruvate content in the culture medium would falsify the results.

For the analyses, the culture medium was collected by placing the culture containers on ice to stop the reaction and to counteract enzyme degradation. The medium was centrifuged at 2000 rpm and 4 °C for 5 min to remove cellular debris. Aliquots of the supernatants were sampled separately for each enzyme. Samples for LDH measurement were stored at 4 °C for a maximum of one day, while samples for the AST and GLDH measurements were stored at − 20 °C until analysis. Cellular lysates for the determination of the total LDH activity in the three pooled slices were prepared by adding 0.1% Triton® X-100 (Sigma-Aldrich) to 6 mL medium and the slices after the respective culture period and for each treatment condition. The slices were homogenized for 20 s in the detergent-containing medium by using an Ultra-Turrax® T8 (IKA®-Werke, Staufen, Germany). Lysates were centrifuged at 2000 rpm and 4 °C for 5 min and the debris-free supernatant was aliquoted and stored at 4 °C until analysis. LDH activity was determined using the Cytotoxicity Detection Kit (LDH) (Roche Diagnostics GmbH, Penzberg, Germany). The total LDH samples were diluted 1:10 in pure cell culture medium. Fifty microliters of the samples and 50 µL of the reaction mixture from the kit were incubated in a clear 96-well plate for 11 min at RT. The absorbance of technical triplicates per sample was measured with a SpectraMax® 340 PC Microplate Reader (Molecular Devices) at 490 nm. The LDH leakage into the medium was expressed as % total LDH of three pooled slices for each point in time and treatment condition.

AST and GLDH leakage into the culture medium was determined by using two standard clinical chemistry kits, namely the AST reagent (Randox Laboratories, Crumlin, UK) in the presence of pyridoxal-5-phosphate FS* (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) and the GLDH FS* kit (DiaSys Diagnostic Systems), both performed with the Cobas Fara II analyzer (Roche Diagnostic Systems, NJ, USA). Positive controls for both enzymes were prepared by homogenizing three freshly cut slices in 6 mL culture medium with the Ultra-Turrax® T8 without the detergent, which would disturb the above-mentioned methods. Aliquots of the supernatant of the samples centrifuged at 2000 rpm and 4 °C for 5 min were stored at − 20 °C until analysis. Concentrations were determined by the use of the clinical calibrator CAL 2 (Randox Laboratories). For each series of measurement a quality control (assayed chemistry premium plus level 3 control, Randox Laboratories) was tested in parallel. One technical replicate per biological replicate was measured. Pure medium was used as negative/background control and these values were subtracted from the sample values. Data were expressed as U/L.

2.8. Urea and albumin measurements

The secretion of the two liver metabolism products urea and albumin from the liver slices into the culture medium was used to assess the functionality of the cultured tissues. Medium was collected from the slice incubations that were used for the determination of the wet weight and the ATP/protein content and centrifuged at 2000 rpm and 4 °C for 5 min. Aliquots for the measurement of urea were stored at − 20 °C and for albumin at − 80 °C until analysis. Urea was measured according to a colorimetric endpoint method (Urea CT* FS**, DiaSys Diagnostic Systems) and the concentration was determined by using the standard of the test kit, which was diluted 3-fold in 0.9% NaCl. The quality control CAL 2 was diluted 7-fold in 0.9% NaCl. Dilutions were necessary to ensure an optimal concentration range for the evaluation of the data. Measurements were performed using the Cobas Fara II analyzer. Albumin was measured with a competitive Enzyme Linked Immunosorbent Assay (ELISA) (Nephrat II; Exocell, PA, USA) according to the manufacturer's instructions. Samples were diluted up to 5-fold when necessary and measured with the Epoch Microplate Spectrophotometer (BioTek, VT, USA) at 450 nm. The concentrations were determined by using a rat serum albumin standard curve (1.56–100 µg/mL). For both functionality parameters, pure medium was used as negative/background control and these values were subtracted from the sample values. Data were expressed as nmol urea or µg albumin per mg wet weight.

2.9. Histology and slice thickness determination of PCLiS

Immediately after the respective treatment or cultivation period, one slice out of three co-cultured slices per biological replicate was transferred into a 4% buffered formalin solution. Accordingly, four slices per treatment as well as four freshly cut control slices were fixed for at least 24 h and vertically embedded in paraffin. Transversal sections located in the middle of the slices were prepared after dehydration in xylol and ethanol. In addition, the caudate lobe of the liver was fixed in formalin immediately after dissection of the liver lobes and was used as a tissue control that was not stressed by the slicing procedure and therefore mirrored the liver tissue status at the beginning of an experiment (fixing period ≥ 24 h). The morphological assessment of the PCLiS and the caudate lobe was performed by analyzing paraffin sections (3 µm thick) stained with the hematoxylin solution according to Mayer (Merck KGaA, Darmstadt, Germany) and eosin/phloxin (Merck KGaA/Waldeck GmbH & Co. KG, Münster, Germany) (H & E) using a standard procedure. The slice thickness was determined by means of scans of the H & E stained paraffin sections with the Pannoramic Viewer 1.15.4 program (3DHISTECH, Budapest, Hungary), by measuring the distance between the intact upper and lower cell surface layers of the corresponding slices. Dead, not attached outer cell layers were not included in the thickness measurement. Three

out of four fixed slices per treatment, which did not show embedding artifacts, were analyzed. The measurement took place at five different homogeneously distributed points of each slice and the average of these five values was calculated for each slice. The thickness of the three measured slices per treatment condition and experiment was again averaged.

2.10. Statistics

All experiments were repeated three times, each of them conducted with liver tissue from a different rat, and all data were recorded as mean ± SD. For each experiment, four biological replicates, each one consisting of the three co-cultured and afterwards pooled slices or the culture medium from three co-cultured slices, were investigated per point in time, treatment condition and readout, except for LDH measurements; in the latter case only two biological replicates were used. Four replicates were also measured in the case of positive and negative controls. The data was first checked for normal distribution according to David et al. (1954) and for outliers according to the Grubbs' test (Grubbs, 1969). The identified outliers (a maximum of one out of the four values) were excluded from further analyses. Thereafter, a one-way ANOVA with Tukey's post hoc test was performed to compare different pairs of group means within an experiment. The statistical analyses as well as the calculation of the half maximal effective concentration (EC50) of APAP were carried out with Prism 4 software version 4.03 (GraphPad Software, CA, USA). A p-value of < 0.05 was considered significant.

3. Results

3.1. Characterization of the culture setup and system

In a first but crucial step, we characterized our culture setup and system (Fig. 1A) to ensure optimal culture conditions for the liver tissue and to create the basis for the optimal maintenance of the tissue functionality and viability during the whole culture period. The oxygen-enriched atmosphere, in which the PCLiS were cultured, was achieved by gassing the culture boxes with pure carbogen (95% O_2/5% CO_2). The effective oxygen (O_2) concentration that was reached in the boxes by flooding them with the gas (4 NL/min) for 10 min was 88%. By compensating the gas leakage from the boxes with a reduced supply of carbogen (0.8 NL/min), it was possible to maintain the maximum O_2 concentration of 88% without fluctuations during the whole culture period of 24 h (Fig. 1B). The humidification of the carbogen, which was achieved by the intercalated gas washing bottle, prevented a significant loss of the culture medium due to evaporation after 24 h culture (Fig. 1C). In addition, we demonstrated by means of a simple indicator reaction with carbon dioxide that extensive gas transport into the culture medium was achieved (Supplementary results 1).

3.2. Characterization of rat PCLiS based on viability, functionality and (histo-) morphological parameters during a 24 h culture period

All parameters used for the characterization of the cultured rat PCLiS were depicted as a function of the incubation time over a period of 24 h. The viability of the tissue slices was assessed by determining their ATP content and LDH leakage (Fig. 2A and B) as well as the release of the two liver enzymes AST and GLDH (Fig. 2C and D). When compared to the ATP content of the freshly prepared (not cultured) control slices (0.194 ± 0.008 nmoles ATP per mg wet weight), only slices cultured for as long as 24 h showed a slight decrease in their ATP levels (0.160 ± 0.014 nmoles ATP per mg wet weight; $p < 0.01$), i.e., 83 ± 10% of the initial average ATP content of fresh slices. The LDH leakage of the slices into the culture medium was found to be relatively high after the 1 h pre-incubation period (PI). After changing the medium following the PI, the LDH activity decreased significantly to below 2% of the total LDH content of the slices at 2 h post PI and after 24 h incubation reached the levels observed after the PI. Generally, the LDH leakage after an incubation of 24 h was found to be low if compared to the total LDH content in the slices (7.3 ± 0.77% of total LDH). The release of AST and GLDH was only moderately enhanced (22 ± 7% and 17 ± 4% of the total enzyme content, respectively) after a 24 h incubation. The liver slices retained the ability to synthesize urea as well as albumin for at least 24 h. The production rate of urea was slightly diminished after 24 h, the albumin production rate increased with time and reached a maximum after 24 h (Fig. 2E and F).

The PCLiS macroscopic appearance was assessed in the formalin-fixed slices collected after the different incubation periods. At each point in time, the tissue slices looked very similar and did not show any obvious structural changes, except for a slight decrease in size (i.e. diameter) (Fig. 3D). The histomorphological evaluation consisted in the comparison of the cultured slices with two controls: (i) the caudate lobe, which reflects the normal rat liver tissue after the organ removal and the status of the liver before any mechanical damage was inflicted to the tissue by the slicing procedure; (ii) freshly prepared (not cultured) slices, which depict the status of the tissue slices after the mechanical insult and before their incubation is started. The general histology of the freshly cut slices revealed a similar overall structure including the typical lobular architecture of the liver, as compared to the caudate lobe control, and parenchymal cell structures were well preserved. Only a few cells at the cutting edge of the slices showed disrupted membranes (Fig. 3E). Hepatocytes were characterized by their typical polygonal shape and large, finely granulated to clear cytoplasmic inclusions. The cellular structure and the tissue architecture were preserved during the whole incubation period (i.e. up to 24 h), while the clear cytoplasmic inclusions disappeared completely after a 24 h culture period. Furthermore, the outer cell layers of the PCLiS started to show a homogeneously light red color 2 h post PI, which is indicative of a band of dying and already dead cells, and started to separate from the remaining viable tissue 6 h post PI. After the 24 h culture period, the tissue slices were surrounded by a band of dead cells, which disintegrated and formed a protein mass in which no cell structures could be distinguished. The remaining liver tissue did not show extensive necrosis, whereby different forms of single-cell necrosis (pyknosis, karyorrhexis) were sporadically and randomly observed throughout the slices.

Furthermore, the wet weight (Fig. 3A) significantly decreased over time from 17.59 ± 1.35 mg per slice to 9.05 ± 0.88 mg (~51% of the fresh slice control) after a 24 h incubation. A decrease over time was also seen in the case of the protein content (Fig. 3B), which was reduced significantly from 1.58 ± 0.23 mg per slice to 1.03 ± 0.13 mg (~66% of the fresh slice control) after a 24 h incubation, and in the case of the slice thickness (Fig. 3C), which was significantly reduced from 218 ± 15 µm (fresh slices) to 184 ± 24 µm after a 24 h incubation (~84% of the fresh slice control).

3.3. Effect of APAP on the rat PCLiS

To assess the validity of and the mutual correlation between the ten selected parameters, we exposed the PCLiS to increasing concentrations (2.55–15 mM) of acetaminophen (APAP) for 24 h. The measurement of the ATP content revealed an initial significant and dose-related increase in ATP synthesis, which reached a maximum in slices treated with 5.5 mM APAP. Higher concentrations (10–15 mM) led to an extensive loss in slice viability (Fig. 4A). The half maximal effective concentration (EC50) of APAP in rat PCLiS after a 24 h exposure was found to be 8.8 mM. A slight but significant increase in LDH leakage was only observed at the highest tested concentration (Fig. 4B). The release of AST and GLDH in the culture medium was decreased if compared to the vehicle control. GLDH showed a dose-dependent and significant decrease over the whole concentration range ($p < 0.01$), while the AST activity varied depending on the added APAP concentrations, but

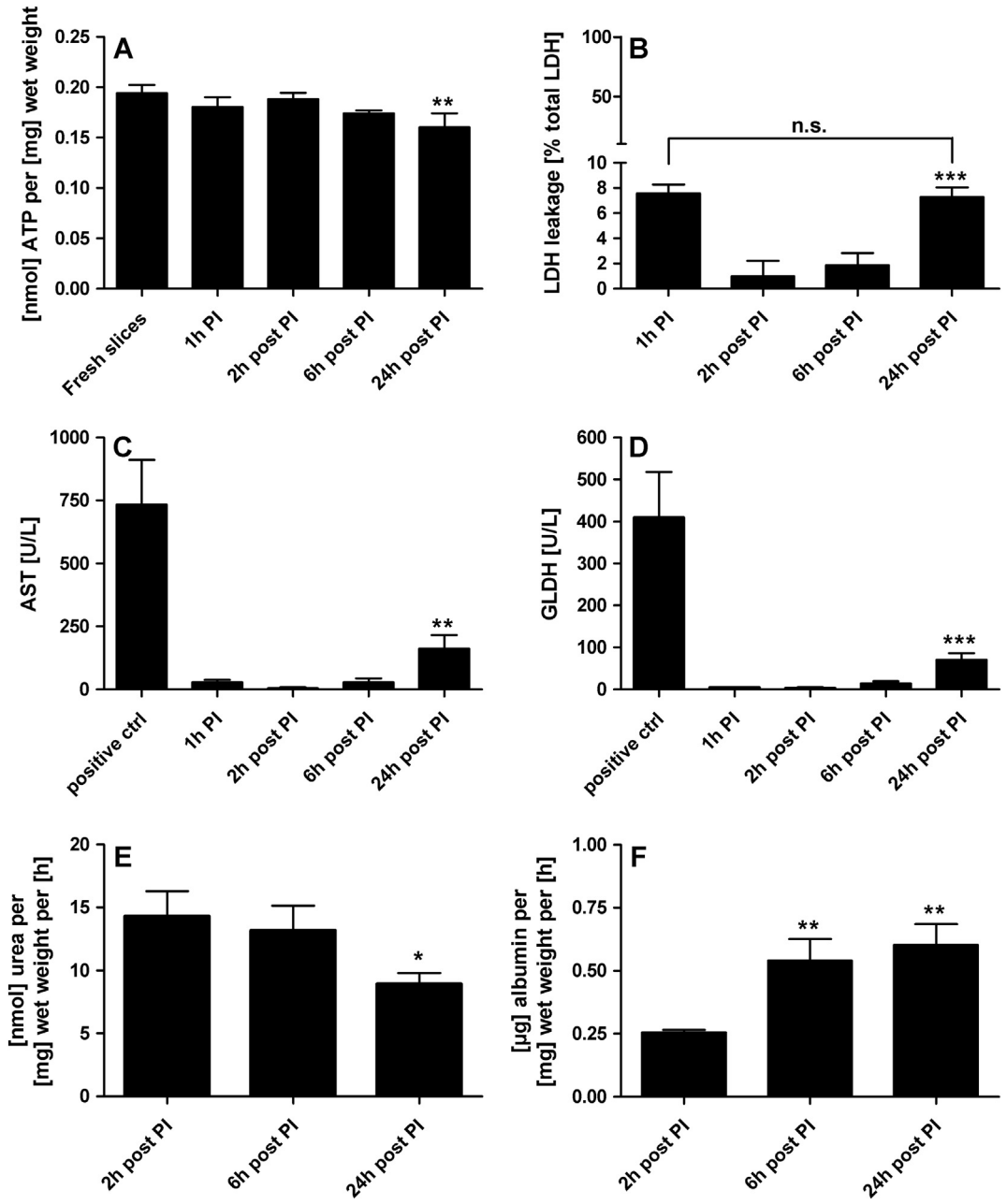

Fig. 2. Characterization of the rat PCLiS over a culture period of 24 h by means of viability and functionality parameters. Samples were taken after the pre-incubation (PI) period of 1 h as well as 2 h, 6 h and 24 h post PI. At the end of the PI a medium change was performed. The viability of the slices was assessed by determining their ATP content (A), the LDH leakage (B) as well as the release of AST (C) and GLDH (D) from the three co-cultured slices. The functionality of the liver tissue was assessed by determining the urea (E) and albumin (F) production rates. Positive controls for AST and GLDH represent the total enzyme content in the three co-cultured slices. All data (n = 3 independent experiments) are expressed as mean ± SD. Statistical analyses were performed using a one-way ANOVA with Tukey post hoc test. Ctrl = control; * The asterisks indicate statistically significant differences when compared to the fresh slice control (ATP content), to 1 h PI (AST and GLDH) or to 2 h post PI (LDH, urea and albumin): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

these variations in no case reached a statistically significant level (Fig. 4C and D). The production of urea and albumin increased dose-dependently up to a concentration of 5.5 mM and this increase was statistically significant when compared to the vehicle control (Fig. 4E and F) and correlates which the increased ATP content and its reached maximum at the same concentration. Also in line with these findings is the observation that a significant loss of functionality was seen at concentrations ≥ 10 mM, the concentration at which the ATP content began to decrease.

The macroscopic appearance of the APAP-treated PCLiS displayed a color change from a light beige in the case of the control slices to a dark brown color of the slices exposed to 10–15 mM APAP (Fig. 5D). Slices exposed to lower concentrations did not show a color change (data not shown). However, the dark colored slices remained intact up to the highest tested concentration of 15 mM. Interestingly, the wet weight and the thickness of APAP-treated PCLiS significantly increased, whereby the increases were first observed at a concentration of 10 mM APAP and reached a maximum at a concentration of 15 mM APAP (Fig. 5A and C). The protein content of the slices remained unchanged (Fig. 5B).

Our biochemical findings are in line with the results from the histomorphological examination (Fig. 6). Thus, the tissue histomorphology also revealed no cell death when the slices were incubated with up to 5.5 mM APAP. Interestingly, a great number of hepatocytes with red-colored areas within the cytoplasm were observed in the slices exposed to 5.5 mM APAP, which also showed an increased ATP content and higher synthesis rates of urea and albumin. In accordance with the

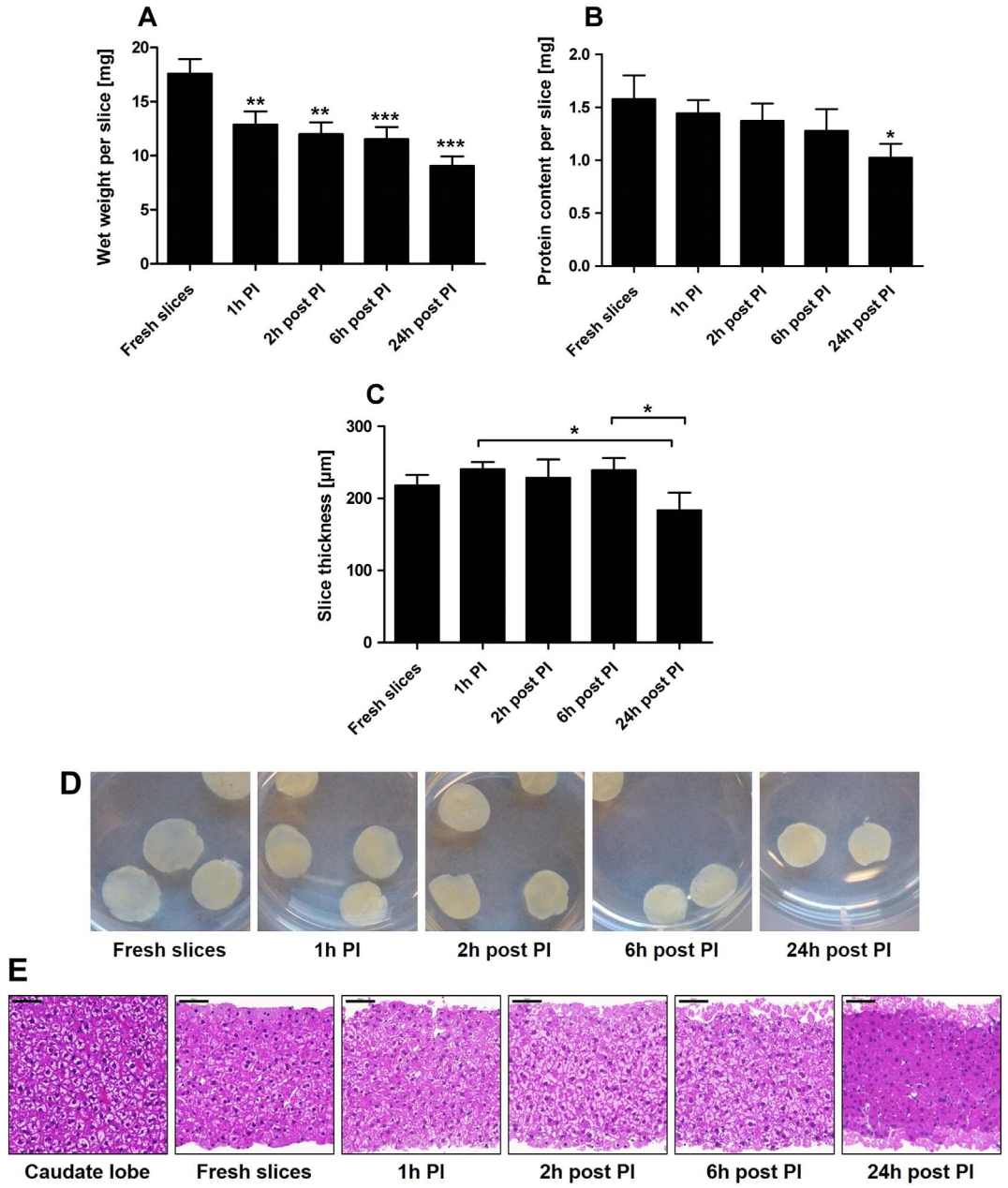

Fig. 3. Characterization of rat PCLiS over a culture period of 24 h by means of slice descriptive and (histo-) morphological parameters. Samples were taken after the pre-incubation (PI) period of 1 h as well as 2 h, 6 h and 24 h post PI. The slices were characterized by their wet weight (A), protein content (B) and thickness (C). Moreover, the liver slices were analyzed macroscopically (D) and histomorphologically (E). Scale bars represent 50 µm. All data (n = 3 independent experiments) are expressed as mean ± SD. One representative picture per point in time is presented. Statistical analyses were performed using a one-way ANOVA with Tukey post hoc test. * The asterisks indicate statistically significant differences when compared to the fresh slice control and when not stated otherwise: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

finding that the viability and the functionality of the slices decreased when treated with 10–15 mM APAP, extensive necrosis was evident in the corresponding slices. Nevertheless, small areas containing viable cells of darker color and preserved polygonal shape were still observed in slices treated with 10 mM APAP. In slices treated with 15 mM hardly any viable cells but mostly rounded or disintegrated cells with a light red homogenous color and a dark nucleus were detected. Totally disintegrated hepatocytes appeared to form a protein mass in the tissue. In addition, the cell structure of slices treated with ≥ 10 mM APAP started to loosen up and to disintegrate. The hepatocytes in these slices showed a reduced connection to other cells and large free spaces were formed between the cells. Importantly, plasma membranes of the APAP-damaged hepatocytes remained predominately intact up to the highest APAP concentration tested.

4. Discussion

Despite the increasing use of the PCLiS technique in experimental toxicology and pharmacology (Lerche-Langrand & Toutain, 2000; Lake & Price, 2013; Vickers & Fisher, 2013), the heterogeneous handling of the slices regarding culture setup, system, protocol, and conditions impacts on the tissue quality of the cultured slices, prevents the standardized use of these cultures and leads to study outcomes that are difficult to compare. Our aim was to make a step forward in standardizing the PCLiS technique and to improve the reproducibility and comparability of PCLiS studies. Therefore, we characterized our culture setup and system and assessed the basic performance of the cultured liver slices on the basis of ten readouts by applying biochemical, clinical and histomorphological methods. All readouts were tested

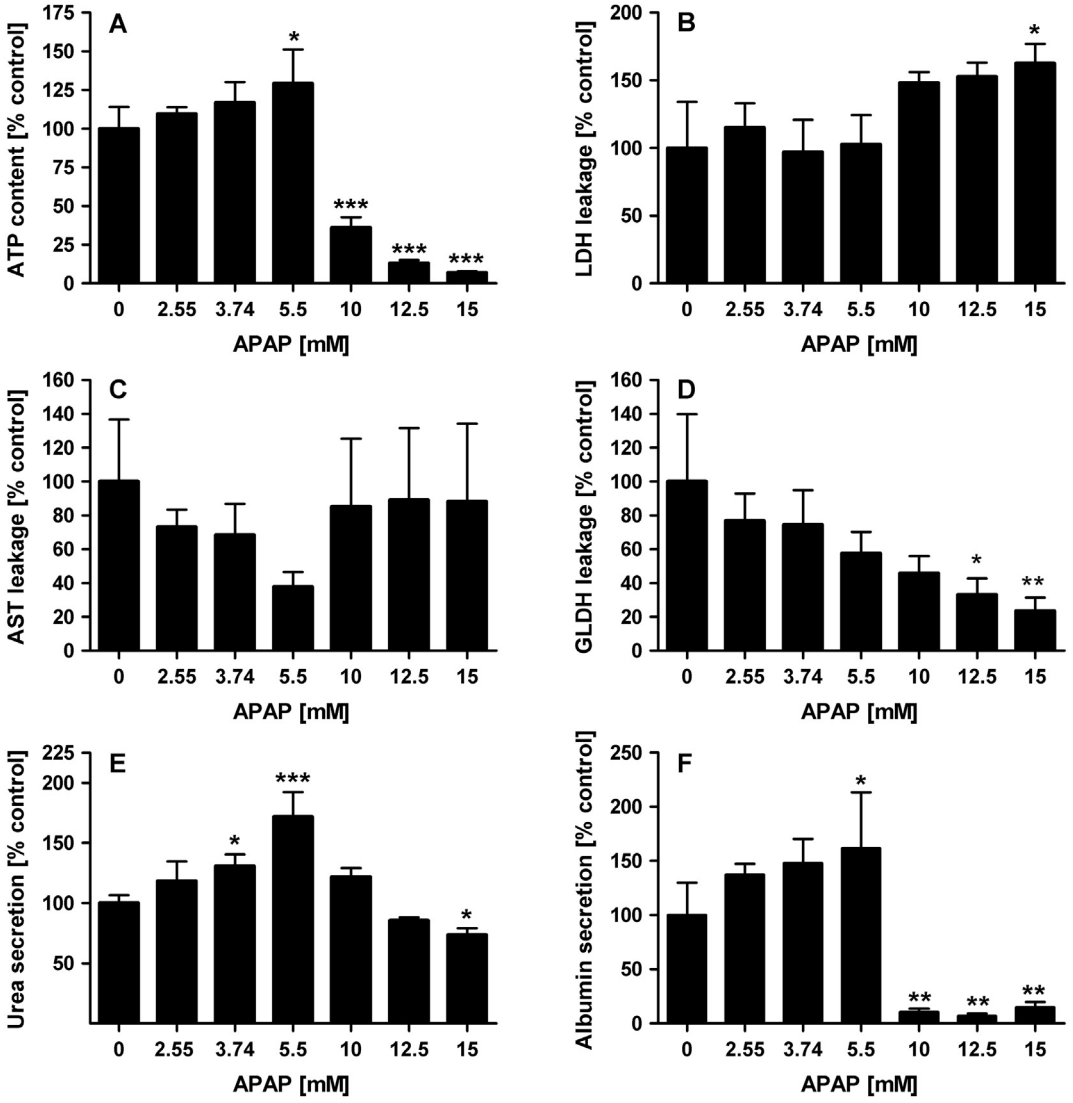

Fig. 4. Characterization of APAP-treated rat PCLiS by means of viability and functionality parameters. Slices were exposed to increasing concentrations of APAP or vehicle (DMSO; final concentration: maximally 0.47%) for 24 h. The viability of the slices was assessed by determining their ATP content (A), the LDH leakage (B) and the release of AST (C) and GLDH (D). The functionality of the liver tissue was assessed by determining the urea (E) and albumin (F) secretion levels. All data (n = 3 independent experiments) are expressed as percent of the vehicle control and mean ± SD. Statistical analyses were performed using a one-way ANOVA with Tukey post hoc test. * The asterisks indicate statistically significant differences when compared to the vehicle control: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

for their suitability to detect hepatotoxic responses using the known hepatotoxicant acetaminophen (APAP) as a model compound.

We based our study on the most widely used and accepted parameters for liver slice cultures derived from many different laboratories applying the PCLiS technique. The selection was made on the basis of a literature search that covered 31 studies and a focus was put on more recent publications and studies that already used optimized parameters (Supplementary Table 1). Table 1 summarizes the selected culture conditions on which the present study is based.

The culture setup represents the basis for optimal conditions for the maintenance of PCLiS. Even though the different culture setups are often described in detail (Olinga et al., 1997a; de Graaf et al., 2010; Hadi et al., 2013a), none of the working groups characterized their setup with respect to the actual oxygen concentration that was present in the atmosphere when gassing with carbogen during the PCLiS culture. Furthermore, it has never been clarified whether the gas in the atmosphere is transported into and dissolved in the culture medium and can thereby reach the tissue to ensure sufficient oxygen supply. We were able to show that the saturation of our culture boxes, did not lead to an oxygen concentration of 95%, which would represent the total oxygen concentration present in carbogen, but slightly less, namely 88%. However, working groups that expose their liver slices to oxygen concentrations as low as 80% did also show well-preserved PCLiS after 24 h (Szalowska et al., 2013; Starokozhko et al., 2015), so that we did not adapt the oxygen concentration to the full 95%. Importantly, the oxygen concentration could be kept constant during the whole culture period of 24 h and an extensive gas transport into the medium was proven by a qualitative indicator reaction. This verification is important, since the hepatocyte functionality and viability is highly dependent on a sufficient oxygen supply (De Bartolo et al., 2006; Kidambi et al., 2009) and several studies confirm that PCLiS can be better maintained when cultured under high oxygen concentrations (Drobner et al., 2000; Evdokimova et al., 2002; Szalowska et al., 2013). The exact quantity of oxygen that diffuses into the medium and the oxygen concentration in the liver slices compared to that in the liver tissue in vivo still remains to be determined. Taken together, the described culture setup and system provides the basic requirements to ensure optimal slice viability and functionality.

The assessment of the viability of the slices according to biochemical techniques, namely the measurement of the ATP content as well as

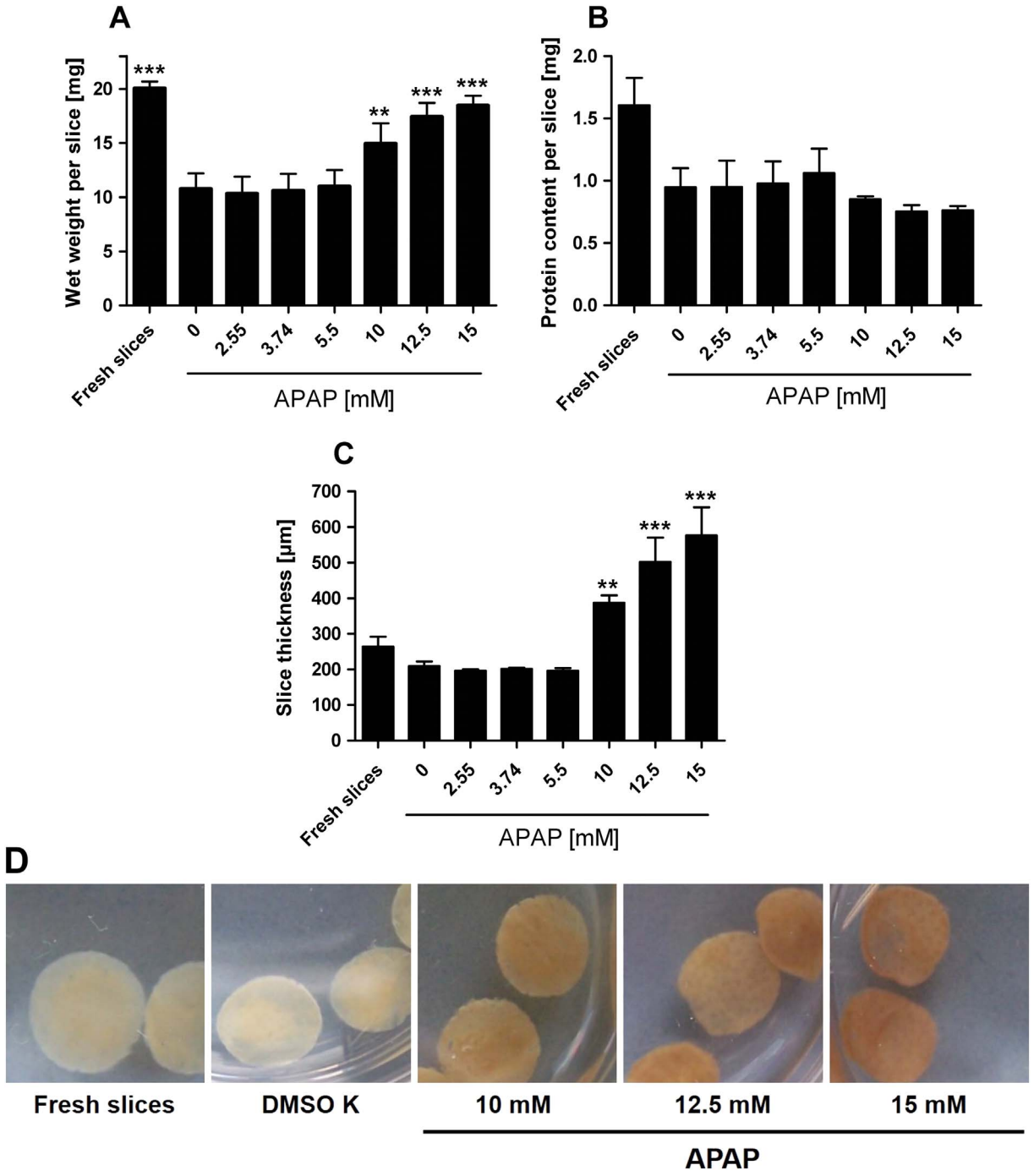

Fig. 5. Characterization of APAP-treated rat PCLiS by means of slice descriptive and morphological parameters. Slices were exposed to increasing concentrations of APAP or vehicle (DMSO; final concentration: maximally 0.47%) for 24 h. The slices were characterized regarding their wet weight (A), protein content (B) and thickness (C). The morphology of the liver tissue was assessed macroscopically (D). All data (n = 3 independent experiments) are expressed as mean ± SD. One representative picture per concentration is presented. Statistical analyses were performed using a one-way ANOVA with Tukey post hoc test. * The asterisks indicate statistically significant differences when compared to fresh slices or the vehicle control: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

the leakage of the three enzymes LDH, AST and GLDH, showed that the tissue remained highly viable during the whole culture period. For example, the ATP content was only slightly diminished after 24 h culture if compared to the fresh control slices. Interestingly, many studies show an increased ATP content in the incubated PCLiS after a 24 h incubation (Vanhulle et al., 2001; Szalowska et al., 2013; Starokozhko et al., 2015), which we did not observe. The reason for this difference remains unexplained at the present time and may be attributed to different culture setups/protocols used. However, in line with our findings, some studies did show a slight decrease in ATP content or MTT reduction after 24 h (Sipes et al., 1987; Obatomi et al., 1998; Kasper et al., 2005).

The leakage of the cytoplasmic enzyme LDH in the culture medium was used as a marker for plasma membrane damage. When compared to previously published studies (Obatomi et al., 1998; Szalowska et al., 2013; Starokozhko et al., 2015), which reported an increased leakage to about 9–13% of the total LDH present in the slices after 24 h, we measured a slightly lower LDH leakage (about 7% of total LDH), thus showing that the tissue slices remained viable during the whole culture period. Our finding that the LDH leakage increased after the PI period

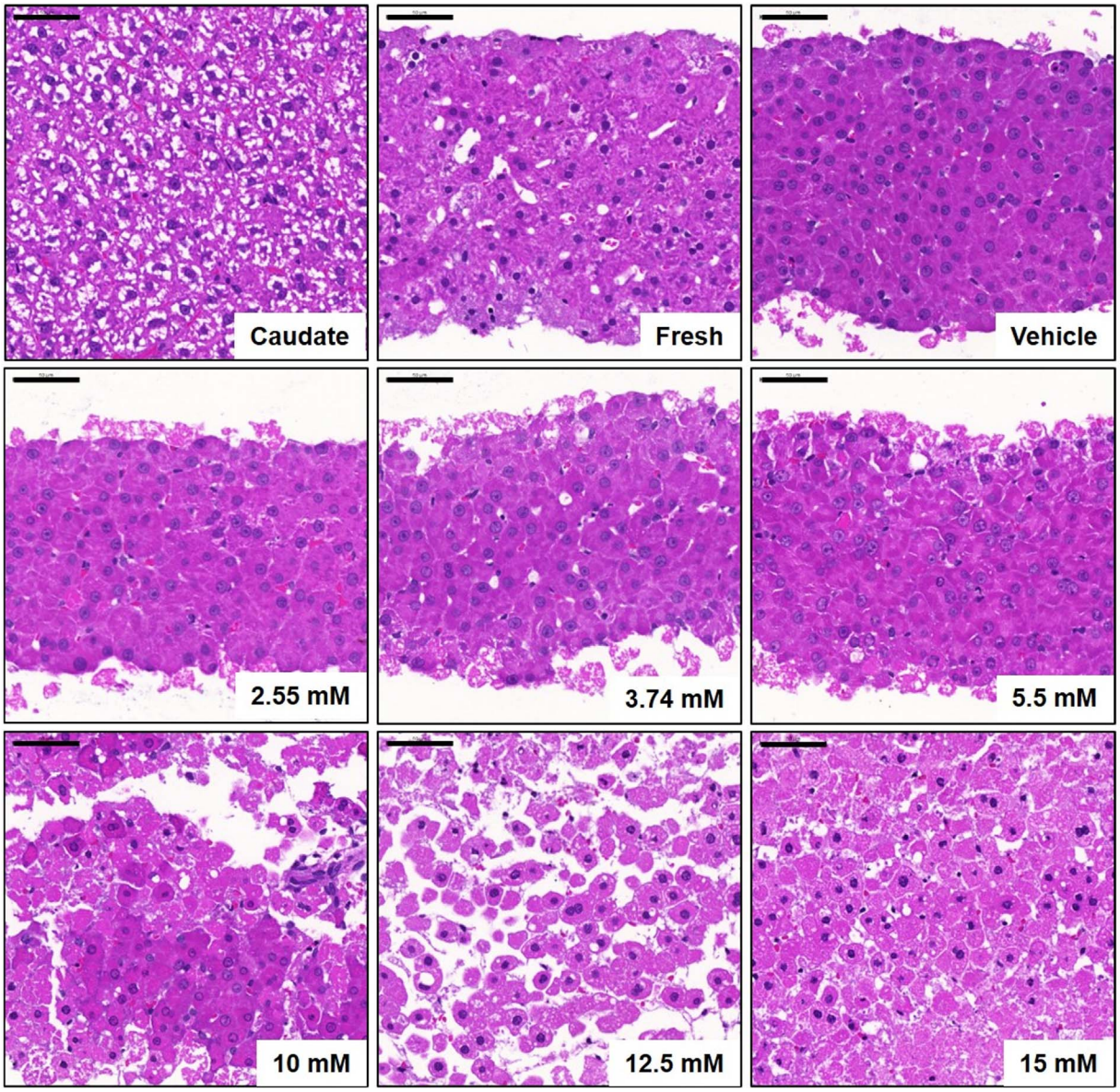

Fig. 6. Histomorphological characterization of APAP-treated rat PCLiS. Slices were exposed to increasing concentrations of APAP or vehicle (DMSO; final concentration: maximally 0.47%) for 24 h. One representative picture per treatment group from three independent experiments is presented. Scale bars represent 50 µm.

and decreased after the medium change and further cultivation is in line with the scientific literature (Miller et al., 1993). This finding can most likely be explained by the membrane damage of the outer cell layers of the tissue, which is an inevitable slicing artifact, and the subsequent removal of the released enzymes by the following medium change.

In accordance with the low LDH leakage, the release of the two liver enzymes AST and GLDH was only moderately increased after a 24 h incubation (17–22% of the total enzyme content). The extent of the AST release is in line with the findings of other working groups, who also reported a 15–20% release of the total AST tissue content into the culture medium after 24 h (Wright & Paine, 1992; Vickers, 2009). Unfortunately, there were no further liver slice data available for the GLDH release. However, our PCLiS culture showed a similar liver enzyme release when compared to that of primary hepatocyte cultures after 24 h (Xie et al., 2014).

The histomorphological assessment revealed that the cell and tissue structures were well preserved during the whole culture period of 24 h. Importantly, research groups culturing PCLiS for longer than 24 h predominantly showed that the slices increasingly developed necrotic areas and that the hepatocytes lost their typical polygonal shape, a first sign of dedifferentiation (Beamand et al., 1993; Toutain et al., 1998; Lupp et al., 2001; Kasper et al., 2005; Starokozhko et al., 2015). Furthermore, Starokozhko et al. (2015), who cultured PCLiS for up to five days, described the formation of an outer cell lining after 72 h culture and extensive tissue remodeling leading to fibrotic structures. Both findings are critical when the slices are planned to be used for the prediction of DILI. On the one hand, viable and well-differentiated cells are needed in a drug screening process, since non-functional, fibrotic or even dead cells respond only in part or not at all. On the other hand, an outer cell lining would be hindering, since the drug could not reach the inner cell layers of the tissue (i.e. the cell lining may prevent the penetration of the drug). Our finding that the outer cell layers die off and detach from the remaining viable tissue after 24 h culture was also previously observed (Neupert et al., 2003; Starokozhko et al., 2015). In addition, several authors describe a decrease in the slice glycogen content with time, as observed by a loss of cytoplasmic glycogen inclusions (Toutain et al., 1998; Lupp et al., 2001; Starokozhko et al., 2015). We observed the same phenomenon in our slices, in which the inclusions were no longer visible after a 24 h culture. Most likely, by

Table 1
Culture conditions underlying the present study.

Parameter	Chosen condition	Justification	References
Species	Rat	Most studied animal	Lerche-Langrand & Toutain (2000); Supplementary Table 1
Liver perfusion	No	Unnecessary for the maintenance of rat tissue slice quality during culture	de Graaf et al. (2007)
PCLiS dimensions	Ø 8 mm, 250 µm thick	Most frequently used dimensions Optimal diffusion of nutrients and oxygen to the inner cell layers Prevention of supply-dependent necrosis	Vickers & Fisher (2005); de Graaf et al. (2010)
Culture system	Submerged, Shaken Erlenmeyer flasks	Frequently used culture system Simplicity and convenience One superior system among five compared systems (shaken flask, stirred well, shaken 6-well, rocker platform, roller system)	Olinga et al. (1997a)
Culture medium	WME + Glucose + Antibiotic	Most frequently used culture medium Most suitable medium to maintain liver tissue quality Serum and/or insulin addition do not affect liver slice quality during 24 h culture	Beamand et al. (1993); Evdokimova et al. (2002); Szalowska et al. (2013); Starokozhko et al. (2015)
Atmosphere	Humidified carbogen (95% O_2, 5% CO_2)	Most frequently used method to obtain a highly oxygenated atmosphere Liver slices can be better maintained in an oxygen-rich atmosphere	Drobner et al. (2000); Evdokimova et al. (2002); Szalowska et al. (2013)
Pre-incubation	1 h	Average of used pre-incubation periods	Supplementary Table 1
Incubation period	24 h	Longer incubation periods can lead to significantly decreased tissue viability and functionality as well as tissue remodeling towards fibrotic structures	Kasper et al. (2005); Elferink et al. (2008); Starokozhko et al. (2015)

this time the stored glycogen was fully consumed by the highly metabolic active hepatocytes. In contrast to the findings of Toutain et al. (1998), who reported a total loss of glycogen after 6 h, our slices kept their glycogen for up to 24 h. This indicates a better supply of our slices with glucose during culture, although we did not supplement our culture medium with insulin. All in all, the biochemical as well as the histomorphological assessment of the rat PCLiS cultured under the conditions described herein revealed viable liver tissue for up to 24 h.

The maintenance of liver-specific functions was assessed by means of the urea and albumin production rates. The ability of the tissue to produce urea was only slightly diminished after 24 h, while the albumin production rate increased with time. This finding proved the functionality of the liver slices for up to 24 h. In contrast, other working groups found a progressive reduction in the albumin production rate when incubated for up to 24 h (Müller et al., 1998; Drobner et al., 2000), which indicates a loss of functionality and differentiation under suboptimal conditions.

The three additional parameters protein content, wet weight and slice thickness showed a significant decrease after a 24 h culture. One explanation could be the loss of the outer cell layers, which was demonstrated by the histomorphological evaluation. The loss of cell material was accompanied by a loss of protein and wet weight as well as a reduction of the slice thickness. In previous studies, the protein content was reported to decrease to about 30–50% of the initial protein content after an incubation of 24 h (Price et al., 1998; Beamand et al., 1993; Szalowska et al., 2013; Starokozhko et al., 2015), which was also seen in this study. An even more pronounced slice protein content decrease was observed after longer incubation periods, e.g. to only 20% of the initial PCLiS protein content after five days (Starokozhko et al., 2015), which is not acceptable in the case of an organ model that claims to reflect the in vivo situation, as proteins build the basis of any functionally correct working tissue. The decreases in wet weight (− 50%) and slice thickness (− 16%) found in this study were also is in line with previously published data (Beamand et al., 1993; Olinga et al., 1997a). All in all, our culture method resulted in high quality liver slices with highly comparable results regarding slice characteristics. As already identified by Godoy et al. (2013), and in line with our findings, the most critical factors in maintaining liver slices were found to be well-prepared and homogenous tissue slices, a slice thickness between 100 and 250 µm as well as a dynamic culture system that is based on optimal penetration of nutrients and oxygen. According to our experience the slice thickness is the most critical factor in regard of the handling and the formation of necrotic tissue cores reducing the tissue viability. Tissue slices of as thin as 100 µm have a very small risk in developing necrotic cores, however, since the liver is a soft tissue, the slices rupture easily and the small amount of tissue impedes some readout measurements, e.g. the ATP content.

To determine whether rat PCLiS cultured under the conditions described herein can be successfully applied to predict and evaluate acute drug-induced hepatotoxicity in a reproducible and representative way, we recorded the response of the rat liver slices to increasing concentrations of the model compound APAP after a 24 h exposure period. The damaging effect of the model compound APAP on rat PCLiS was highly reproducible among the three independent experiments, as shown by the small standard deviations of most of the ten tested readouts. Thus, the application of our culture regime provides a stable basis, which should also facilitate inter-laboratory comparison. In addition, the evaluation of the readouts revealed highly sensitive and less sensitive biochemical viability parameters. The most sensitive parameter to assess the metabolic state of PCLiS and to detect a loss of slice viability was the ATP content. With this parameter it was possible to record a distinct dose-response curve. The derived EC50 of 8.8 mM is in the same range that Hadi et al. (2013b) reported earlier (EC50 = 4.7 mM). However, other working groups using different viability or culture parameters observed no toxic effect of APAP when exposing rat PCLiS to a concentration of 10 mM (Kitamura et al., 1999) or showed a loss of 60–90% viability after exposing the PCLiS to only 2.5 mM APAP for 24 h (Vatakuti et al., 2015). These conflicting results prove the importance of a standardized culture regime for PCLiS in order to enable comparability between different laboratories. Isolated primary hepatocytes show similar EC50 values (Jemnitz et al., 2008), whereas organotypic liver models based on hepatic cell lines needed much higher concentrations to show a toxic effect (Giobbe et al., 2015; Fey & Wrzesinski, 2012). This is most likely due to the higher content/activity of drug metabolizing enzymes in primary liver cells, which can be retained in PCLiS close to the initial value during a 24 h culture and can be better preserved in PCLiS than in isolated hepatocytes (Olinga et al., 1997a; Martin et al., 2003). Although we have used clinically relevant APAP concentrations, it is apparent that a toxic effect in rat PCLiS can only be observed at concentrations higher than those typically causing liver damage in humans (Sevilla-Tirado et al., 2003). A possible explanation may be that rats are able to detoxify the APAP metabolite N-acetyl-p-benzoquinoneimine (NAPQI) more efficient than humans or mice (Miller et al., 1993; Evdokimova et al.,

2002; Hadi et al., 2013b). In terms of extrapolation to human toxicity, PCLiS from human liver tissue would therefore be exceptionally more relevant. The initial increase in the ATP content observed in slices exposed to lower APAP concentrations, might result from an increased metabolic activity of the tissue as a response to a moderate stress, which is also referred to as a hormesis (Calabrese & Blain, 2005; Mattson, 2008). A similar effect was reported for APAP by Fey & Wrzesinski (2012) in a 3D liver cell culture model.

A less sensitive biochemical viability marker was the LDH leakage, which was only slightly increased at the highest tested APAP concentration. Since LDH is only released when the cell membranes are damaged, the membranes seem to remain predominantly intact, even at the highest APAP concentration. This was confirmed by the histomorphological assessment of the slice quality. In line with our finding, Kitamura et al. (1999) did not observe an increase in LDH leakage in rat PCLiS treated with up to 10 mM APAP. However, this phenomenon seems to be an APAP-specific effect, since it is known that other toxins lead to a strong LDH leakage from PCLiS (Onderwater et al., 2004; Guo et al., 2012).

The levels of the liver enzymes AST and GLDH are often increased in the blood of patients suffering from severe liver injury, in which the deterioration of hepatocytes takes place (Giannini et al., 2005). However, by exposing the rat PCLiS to APAP, we observed a decrease of both enzymes in the culture medium, while this effect was clearly dose-dependent in the case of GLDH. Here an artifact in the enzyme measurements can be assumed. However, we were able to exclude an interference of APAP with the assays used by means of confirmatory experiments. In contrast, our results point to a direct interaction between APAP and the enzymes, leading to a dose-dependent decrease in AST and GLDH activities (data not shown). In this context, Halmes et al. (1996) detected in their study that reactive metabolites of APAP can covalently bind to GLDH. Thus, it seems more likely that the observed concentration-dependent reduction in GLDH activity was due to a dose-dependent binding of (reactive) APAP (− metabolites) to the enzyme rather than an APAP-induced cell-related effect. This thesis, however, remains to be elucidated. Nevertheless, the lack in release of AST and GLDH is in line with the observed absence of an enhanced LDH release and the assumption that the cell membranes of the hepatocytes remain mostly intact.

The assessment of the functionality of the APAP-treated rat liver slices by measuring the urea and albumin secretion after a 24 h exposure period revealed an initial dose-dependent increase in urea and albumin production, which reached a maximum with 5.5 mM APAP and a loss of functionality with ≥ 10 mM APAP. This is perfectly in line with our ATP measurements and further reinforces the hypothesis of the increased metabolic activity of the rat PCLiS treated with up to 5.5 mM APAP. Importantly, slices that showed low ATP levels, also showed a reduced to barely available functionality.

A rather unexpected finding was the significant and dose-dependent increase in the wet weight and thickness of rat PCLiS treated with APAP concentrations ≥ 10 mM, which was also the breaking point for the loss of PCLiS viability and functionality, while the protein content remained constant. Both phenomena can be explained by the histomorphological assessment of the slices. As compared to the vehicle control and in contrast to the lower APAP concentrations (2.55–5.5 mM), the liver tissue treated with 10 mM APAP started to loosen up and showed extensive necrotic areas (light red color). Slices treated with higher concentrations of up to 15 mM APAP showed nearly no cell association and mostly cells in the process of dying off or already dead cells. Intercellular swelling of the tissue increased most probably due to an influx of extracellular fluid, e.g. culture medium. It is possible that the cell debris of dead cells and the free cytoplasm, which together formed a mass consisting of proteins and ions, among other components, bound extracellular fluid, thereby leading to an increase of the slice wet weight and thickness. Importantly, at the concentration that led to the color change of the slices, we also observed the onset of the formation of extensive necrotic areas. In accordance with the biochemical evaluation, we were also able to show the hormesis effect at the histomorphological level, predominantly observed in the case of slices treated with 5.5 mM APAP. An increased red staining of certain areas in the cytoplasm of the hepatocytes in the treated slices became visible, which can be attributed to an increased proliferation of the smooth endoplasmic reticulum (SER) (Haschek et al., 2013). The SER contains the majority of cytochrome P450 enzymes involved in xenobiotic metabolism, as well as a number of conjugating enzymes (Cribb et al., 2005). The APAP-stressed hepatocytes seem to up-regulate the proliferation of the SER and thereby strongly increase their drug metabolizing capacity (Remmer & Merker, 1965). This SER up-regulation as an adaptive response to APAP administration was previously observed in isolated rat hepatocytes by Fujimura et al. (1995). This effect was also observed in rats and rabbits after administration of various xenobiotics (Remmer & Merker, 1965) and in humans after acute and chronic alcohol abuse (Lane & Lieber, 1966). Thus, it was possible to correctly reproduce a very specific human in vivo effect in the ex vivo PCLiS model, which moreover underlines the value of this liver model. Therefore, histomorphological evaluations are more than only a sensitive viability parameter as previously concluded by Godoy et al. (2013). Summing up, six out of ten readouts reflected the toxic effect of APAP in the liver tissue, of which the ATP content, the albumin production and the histological findings were found to be the most sensitive to monitor APAP-induced hepatotoxicity.

5. Conclusion

In conclusion, we have presented a culture setup, system and protocol that together ensure an optimal performance of the PCLiS during a 24 h culture, including a high overall slice viability and maintenance of liver-specific functions. The extensive characterization of the 24 h cultured rat liver slices proved that the system is stable under the described conditions and sets the basis for representative and comparable studies. Moreover, 60% of the used readouts proved to be adequate to evaluate hepatotoxic responses and showed a good correlation between each other. Overall, rat PCLiS represent a valuable liver model that can be used under standardized conditions for toxicity studies.

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Transparency document

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tiv.2017.05.001.

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