Use of a Standardised and Validated Long-term Human Hepatocyte Culture System for Repetitive Analyses of Drugs: Repeated Administrations of Acetaminophen reduces Albumin and Urea Secretion

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Summary
Human hepatocytes are the in vitro system of choice to study drug-induced processes in man. Here, we present HEPAC²: a standardised and validated culture system in which human hepatocytes are maintained in HHMM (Human Hepatocyte Maintenance Medium) with HGF (hepatocyte growth factor) and EGF (epidermal growth factor). Cellular viability and hepatocellular functions were monitored daily. Albumin and urea production remained on a relatively constant level for up to 2-3 weeks. Based on this, a standard protocol was established that allows repeated exposure of hepatocytes to study drug metabolism. We used acetaminophen (AAP) to assay the feasibility of this system. Hepatocytes were exposed to AAP (100-2815 mg/l) for 24 h. Subsequently, the culture medium was replaced by medium without AAP and the same exposure scenario was repeated at intervals of 4 days. High doses of AAP (2815 mg/l) diminished urea production by 15-30% and albumin secretion by 70-80%. These effects were reversible. After removal of AAP, secretion of urea and albumin returned to control levels. AAP hepatotoxicity is caused by its biotransformation to the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) mediated by CYP2E1 and CYP1A2. The AAP activating enzymes were active for at least 21 days and their activity was maintained during at least four repeated cycles of exposure to AAP. In conclusion, these data demonstrate the suitability of our long-term culture system to serve as a tool for repetitive screening of drug-mediated changes of hepatocellular functions. This culture technique may help to overcome the sparse availability of human hepatocytes for testing drug-mediated responses in man.

Zusammenfassung: Verwendung eines standardisierten und validierten Langzeit-Kultursystems für humane Hepatozyten zur repetitiven Arzneimittelprüfung: Mehrfachgabe von Azetaminophen reduziert die Albumin- und Harnstoffsekretion
Humane Hepatozyten sind das in vitro System der Wahl, wenn Medikamenten-induzierte Prozesse beim Menschen untersucht werden sollen. Mit HEPAC² stellen wir hier ein standardisiertes und validiertes Zellkultursystem vor, in dem humane Hepatozyten in HHMM (Humanes Hepatozyten Erhaltungsmedium) mit HGF (Hepatozyten Wachstumsfaktor) und EGF (Epidermaler Wachstumsfaktor) kultiviert werden. Täglich wurden zelluläre Vitalität und leberzelltypische Funktionen analysiert. Die Albumin- und Harnstoff-Freisetzung blieb für 2-3 Wochen auf einem relativ konstanten Niveau. Darauf aufbauend wurde ein Standardprotokoll zum Studium von Wirkstoffen etabliert. Dazu werden die Hepatozyten einer Kultur wiederholte Male diesen Wirkstoffen ausgesetzt. Als Modellsubstanz wurde Azetaminophen (AAP) eingesetzt, um die Anwendbarkeit dieses Systems zu untersuchen. Hepatozyten wurden 24 Stunden in Gegenwart von AAP (100-2815 mg/l) inkubiert. Anschließend wurde das Kulturmedium durch AAP-freies Medium ersetzt, und das Applikationsscenario wurde mehrfach in Abständen von 4 Tagen wiederholt. Hohe Konzentrationen von AAP (2815 mg/l) führten zu einer Reduktion der Harnstoff-Freisetzung um 15-30% und zu einer Verminderung der Albumin-Freisetzung um 75-80%. Beide Effekte waren reversibel. Nach Entfernung des AAP erreichten Harnstoff- und Albuminproduktion wieder die Kontrollwerte. Die Hepatotoxizität des AAP wird durch die CYP2E1 und CYP1A2 vermittelte Umwandlung zu N-Acetyl-p-Benzoquinonimin (NAPQI) verursacht. Scheinbar waren die AAP-aktivierenden Enzyme mindestens 21 Tage aktiv, und ihre Aktivität wurde über 4 AAP-Expositionszyklen hinweg aufrechterhalten. Diese Daten belegen die Einsetzbarkeit unserer Langzeitkulturen als Modellsystem zur repetitiven Analyse von Wirkstoff-induzierten Leberfunktionsänderungen beim Menschen. Dieses Kultursystem kann dazu beitragen, die geringe Verfügbarkeit humaner Hepatozyten zu umgehen und die Anzahl von Tierversuchen zu verringern.

Keywords: human hepatocytes, long-term cultures, acetaminophen, repetitive cycles of drug administration

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1 Introduction

The liver is the central organ of energy metabolism, biotransformation and synthesis of plasma proteins under physiological and pathophysiological conditions. Primary hepatocytes are an important model system to analyse and study liver specific processes and functions. So far, most of the studies with hepatocyte cultures have primarily been performed with rat hepatocytes. However, interspecies differences in all aspects of hepatocyte function exist and have been recognised and investigated for more than 30 years (for review see Runge et al., 2000a and references therein). In addition, from data obtained in animal experiments one cannot always predict the effect any given drug might have in man. Therefore, primary human hepatocytes should be the system of choice for the evaluation of liver specific functions in humans, including (i) the biology of human viral liver pathogens or parasites and (ii) phase I and II drug metabolism (Moshage and Yap 1992, Li et al., 1997).

Since human hepatocytes are available only in limited number, the development of culture systems that allow cultivation of differentiated and functional hepatocytes is of great importance. These human hepatocyte cultures should allow the establishment of screening systems for cytochrome P450 inducers and the investigation of drug-drug interactions. Given the fact that the read-out for these screening systems can be non-invasive assays, like for testosterone-6ß-hydroxylase (CYP3A4) or ethoxyresorufin-deethylase (CYP1A), it should be possible for such a culture system to allow repetitive treatment/wash-out cycles to screen for drug activity.

Here we present HEPAC^2, a standardised and validated long-term serum-free human hepatocyte culture system in which hepatocyte morphology and function is maintained for several weeks. Using acetaminophen as a model substance, we are able to demonstrate the robustness of HEPAC^2, showing that repeated exposure of the hepatocytes to the test substance led to a reversible reduction of hepatocellular functions.

2 Characterisation, standardisation and validation of HEPAC^2

Selection of parameters. Which parameters should be used to evaluate the functionality of a given human hepatocyte culture system? Usually, maintenance of liver-specific functions is monitored by measuring the expression of serum proteins and/or drug metabolising enzymes by RT-PCR or Northern blot analysis for RNA detection and by Western blot analysis for protein detection. However, these methods are invasive and require the disruption of the cells. In addition, the quality of these markers as parameters for hepatocyte specific function is arguable for two reasons: First, the detection of a certain mRNA alone does not necessarily indicate the presence of the corresponding protein. Second, even if the protein is detectable by Western blot analysis, one cannot draw any conclusion on its functionality, because cofactors required for enzymatic activities might be missing (Runge et al., 2000b).

Therefore, we decided to monitor hepatocellular function by tracking two products that are synthesised and secreted by hepatocytes. We chose (i) albumin synthesis as a marker for anabolic reactions performed by the hepatocytes, and (ii) the synthesis and excretion of urea. Urea synthesis serves as a parameter for the catabolic process maintained by the hepatocytes. The pathway involves a number of different enzymatic steps, is highly specific for hepatocytes and cannot be performed by any other cells within the liver. An additional advantage is that both products can easily be detected in the cell culture medium without interfering with cellular viability. Furthermore, the release of lactate dehydrogenase activity was assayed as a marker for cellular vitality, and the cell cultures underwent a visual microscopic control.

3 Methods

Isolation and culture of human hepatocytes: Human hepatocytes were isolated from liver resections by three-step collagenase perfusion (Strom et al., 1982; Strom et al., 1987). The viability of cells was determined by trypan blue exclusion test. Only hepatocyte preparations with more than 70% viability were used in the experiments described below. Cells were plated onto collagen-coated 6-well-plates at a density of 10^6 cells per well using MEM (Minimal Essential Medium containing 500 ng/ml insulin and 500 µg/ml gentamycin). Unless otherwise stated, the medium was changed after 3 to 12 h to HHMM (Human Hepatocyte Maintenance Medium; Runge et al., 2000b), containing 10 ng/ml HGF and 20 ng/ml EGF. Medium was changed daily. All studies were performed in accordance with an ethical survey of the ethics commission of the Ärztekammer Mecklenburg-Vorpommern.

Application of Acetaminophen: AAP was dissolved in HHMM containing 40% DMSO to obtain a 0.1 g/ml stock solu-

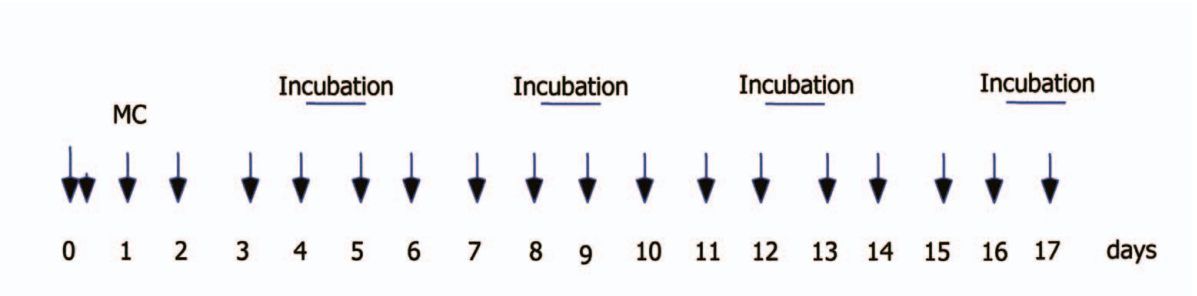

Fig. 1: Experimental design for the implementation of a "recyclable" human hepatocyte culture system.

tion. Human hepatocytes were incubated with AAP, 100-2815 mg/l, or DMSO for control purposes for 24 h. The first incubation cycle was started on day 4 of the culture. After 24 h, medium was replaced by AAP-free culture medium and the hepatocytes were cultured for 72 h in the absence of AAP. This incubation cycle was repeated every 4 days resulting in 24 h incubation with AAP on days 8, 12, 16, and 20. The experimental design is outlined in Figure 1.

Biochemical Assays: The cell culture medium was collected every 24 h for analysis of catabolic (urea release) and anabolic processes (albumin secretion) and for the determination of cellular viability (Lactate-dehydrogenase activity). LDH activity was detected using the Aeroset c8000 system from Abbott (LDH-detection kit, 7D69-20). Urea release was detected by a two step enzymatic conversion (urease / glutamate-dehydrogenase) using the Aeroset c8000 system from Abbott (Urea detection kit, 7D75-20/-30). Albumin release into the culture medium was determined using a sandwich ELISA with antibodies obtained from Bethyl, using goat anti-human albumin (Bethyl, A80-129A) as coating antibody and goat anti-human albumin-HRP conjugate (Bethyl, A80-129P) as detection antibody.

4 Results

Cellular morphology: Cell morphology was assayed by light and electron microscopy. It is evident that although hepatocytes are by far the most prominent cell type, the cultures consist of a mixed population of cells. The cells showed a typical morphology of mature hepatocytes for up to 24 days, mainly mono- and binuclear cells were present (Fig. 2). Electron microscopy revealed round shaped nuclei; mitochondria and Golgi apparatus were well established and maintained from early days in the culture until at least day 24. At all time points, bile canaliculi were readily apparent. Glycogen and desmosomes also developed during the culture, indicating metabolic activity as well as close cell-cell contacts.

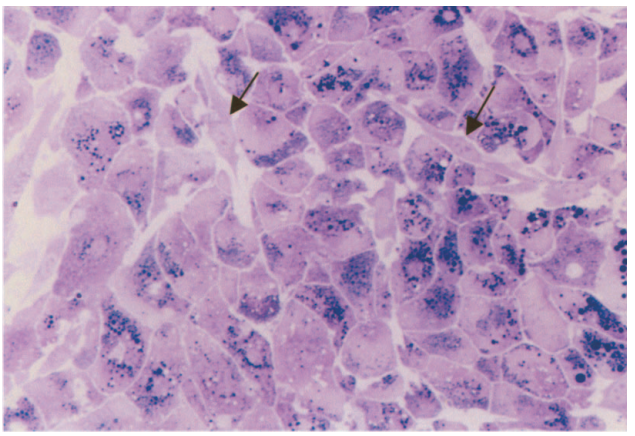

Fig. 2: Toluidine Blue staining of human hepatocytes cultured for 24 days in Human Hepatocyte Maintenance Medium with 40 ng/ml HGF and 20 ng/ml EGF.
A solution of 1% toluidine blue in 1% sodium borate was used to stain epoxide-embedded sections of cell cultures. Hepatocytes were the most prominent cell type. Endothelial- or fibroblast-like cells were detectable in the culture as well (arrows).

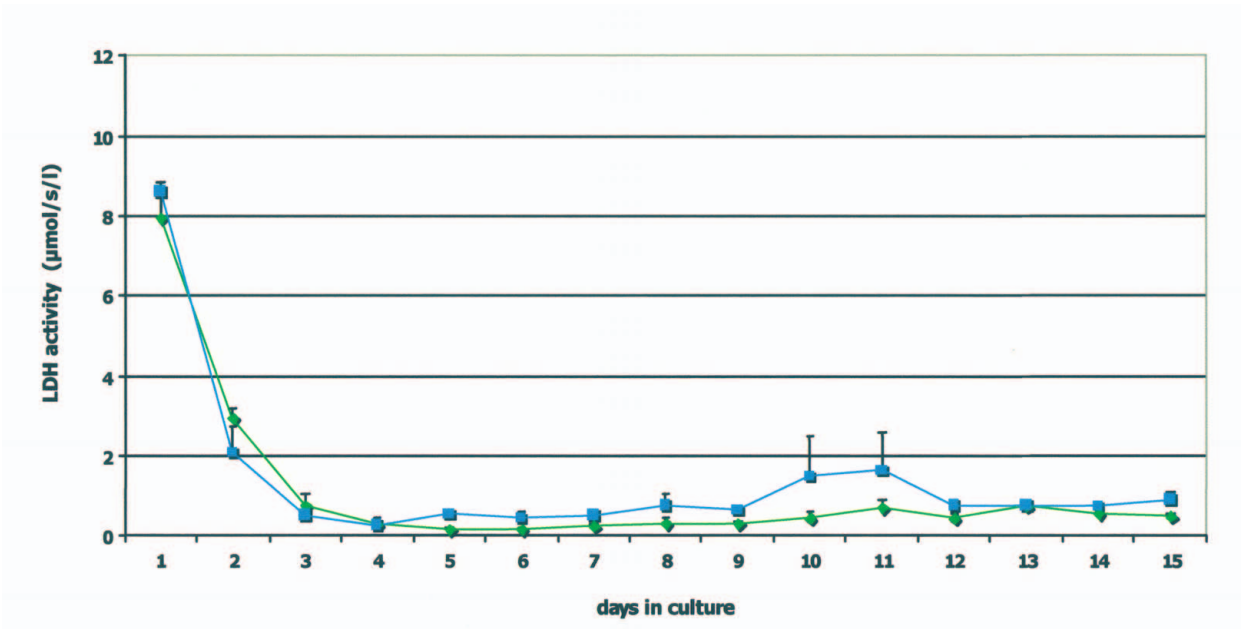

Fig. 3: Release of lactate dehydrogenase in human hepatocyte cultures.
Human hepatocytes were cultured in Human Hepatocyte Maintenance Medium in the presence (blue line) or absence (green line) of HGF and EGF. Cell culture medium was collected every 24 h and assayed for LDH-activity. Data are means ± SEM of 4 to 6 different donors.

Cellular vitality: Cellular integrity was determined by measuring the enzymatic activities of lactate dehydrogenase (LDH) in the culture medium. We monitored the release of LDH in hepatocyte cultures in the presence or absence of growth factors. Initially, within 24 h after plating, LDH activity reached levels of about 8 µmol/s/l. With ongoing culture the daily release of LDH enzymatic activity declined to basal levels of approximately 0.5 µmol/s/l. This basal activity was maintained from day 3 to day 15. The addition of growth factors did not alter the release of LDH into the culture medium (Fig. 3).

Hepatocellular functions: Previously, we showed that expression of phase I drug metabolising enzymes like cytochrome P450 3A4, 1A2, 2E1 and others as well as corresponding testosterone-6β-hydroxylase activity (CYP3A4) were maintained and/or remained inducible for more than 4 weeks within these cultures (Runge et al., 2000b). Expression of liver specific transcription factors C/EBPa, HNF-3 and HNF-4 was maintained for several weeks (Runge et al., 2000c). These factors are held responsible for the maintenance of hepatocyte specific gene expression. Cytochrome P450 3A4 protein expression and corresponding testosterone-6β-hydroxylase activity was maintained and/or remained inducible for up to 30 days (Runge et al., 2000b).

Here, we determined the release of urea as an example for catabolic function of the hepatocytes. Initially, within 24 h after plating, urea release reached levels of about 3-4 mmol/l. Within the next 24 h after plating, urea release dropped to about 1.5 mmol/l. In the absence of HGF and EGF, urea release dropped further to about 1 mmol/l within the next 24 h, reaching a new steady state that was stable at least until day 15. In the presence of growth factors, urea release was maintained from day 2 on at approximately 1.5 mmol/l (Fig. 4). The presence of HGF and EGF seemed to improve urea synthesis and release in human hepatocytes. Statistical analysis using Student's t-test (two sided distribution, two sample equal variance) revealed a significantly higher urea release ($p < 0.05$) in HGF and EGF treated hepatocytes on days 6, 11, and 13, compared to hepatocytes cultured in the absence of these growth factors. In addition, we monitored albumin release as a parameter for hepatocellular anabolic activity. As seen with urea, albumin release reached a steady state between day 3 and 7 during the culture. In the absence of growth factors, albumin release declined from day 7 on. Again, the presence of HGF and EGF seemed to improve hepatocellular functions, since albumin release did not decline but was maintained at the level reached at around day 3 (data not shown).

4.1 Implementation of HEPAC^2 as a recyclable human hepatocyte culture system

After having validated the functional stability of our human hepatocyte culture system, we started to implement a protocol that would take advantage of the robustness of this model system: We culture human hepatocytes in the presence of HGF and EGF until day 4, a time point by which the cells have adopted a new steady state with regard to albumin and urea production. At day 4 the cells are incubated for 24 h with the substance of interest. Then at day 5 the active substance is omitted and the hepatocytes are cultured for 3 days under standard conditions (wash out cycle). At day 8, a second 24 h incubation cycle with the substance of interest is introduced. Again, the next day the foreign substance is omitted, and

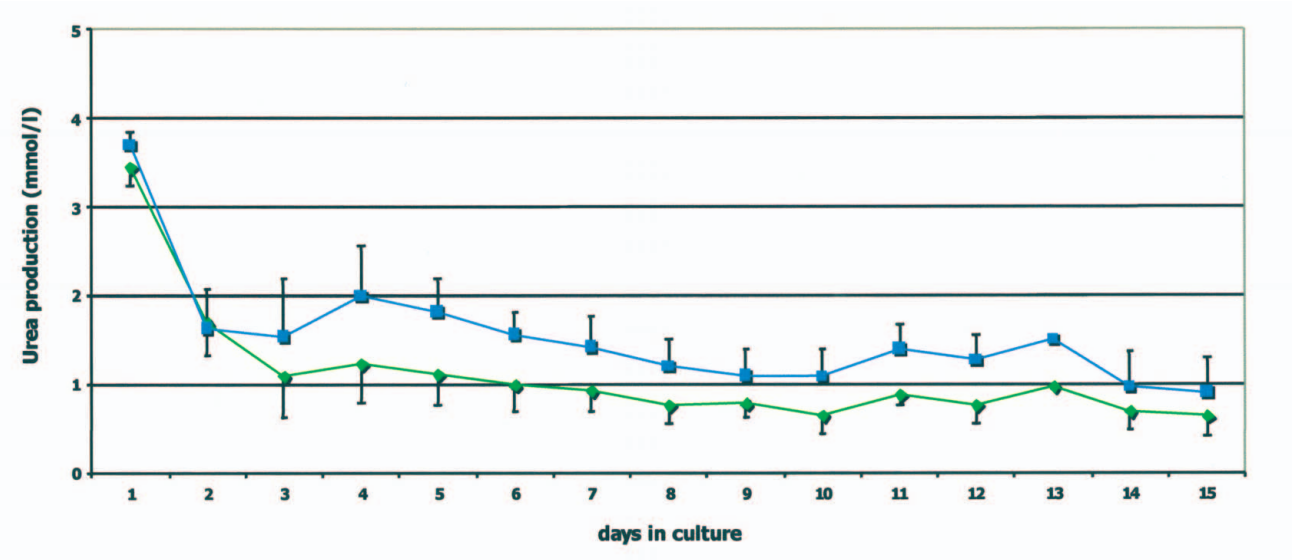

Fig. 4: Release of urea in human hepatocyte cultures.
Human hepatocytes were cultured in Human Hepatocyte Maintenance Medium in the presence (blue line) or absence (green line) of HGF and EGF. Cell culture medium was collected every 24 h and assayed for urea. Data are means ± SEM of 4 to 6 different donors. Statistical analysis using Student's t-test (two sided distribution, two sample equal variance) revealed a significantly higher urea release ($p < 0.05$) in HGF and EGF treated hepatocytes on days 6, 11, and 13, compared to hepatocytes cultured in the absence of these growth factors.

the cells are again cultured under control conditions to wash out any remaining substance or product.

As these assays used to determine (i) metabolism of the active substance and (ii) its influence on hepatocellular metabolism, are non-invasive and, assuming that the active substance is non-toxic, this incubation/wash out cycle may be repeated further until the hepatocytes lose the ability to metabolise the active substance (Fig. 1).

4.2 Acetaminophen as a model substance

AAP, also known as paracetamol, is the first substance we used to evaluate our culture system. In the liver, AAP is detoxified via glucuronidation or sulphation. AAP hepatotoxicity is caused by its biotransformation to the reactive metabolite N-acetyl-p-benzoquinone-imine (NAPQI) mediated by CYP2E1 and CYP1A2. Unless NAPQI is conjugated with glutathione and subsequently excreted, it may bind to proteins and thereby cause hepatotoxicity. As shown in Figure 1, AAP was applied for 24 h on culture days 4, 8, 12, 16, and 20. At day 4 high doses of AAP (2815 mg/l) diminished urea production by approximately 20% and albumin secretion by 70-80%. These effects were reversible. After removal of AAP, secretion of urea returned to control levels within 24 h and albumin secretion returned to normal levels within 72 h (Figs. 5, 6). Three days after removal of AAP, the hepatocytes were again incubated with AAP for 24 h. Again, AAP at a concentration of 2815 mg/l led to a decrease in urea production by 15-30% and albumin secretion by 70-80%. These effects could be repeated several times, no matter whether AAP was added on day 4 or after 2-3 weeks of culture.

5 Summary and outlook

In this report we have presented HEPAC^2: a validated human hepatocyte culture system that allows the serum-free long-term culture of hepatocytes. We have implemented an experimental design that takes advantage of the robustness of our culture system. This protocol allows repetitive analysis of drugs or other substances, as demonstrated here with AAP as a first model substance. Therefore, HEPAC^2 may serve as a suitable tool for repetitive screening of drug-mediated changes. A multi-centre study for external validation of HEPAC^2 has been initiated.

Given the stability of the culture system with regard to the maintenance of hepatocyte specific function and cytochrome P450 expression, HEPAC^2 may also become an alternative to animal experiments for testing long-term effects of drugs and other chemicals. Currently, preclinical analyses of new drugs require oral toxicity studies in rodents and non-rodents to evaluate the toxic characteristics of a chemical. Studies with rodents are designed according to OECD guidelines 407 (Repeated

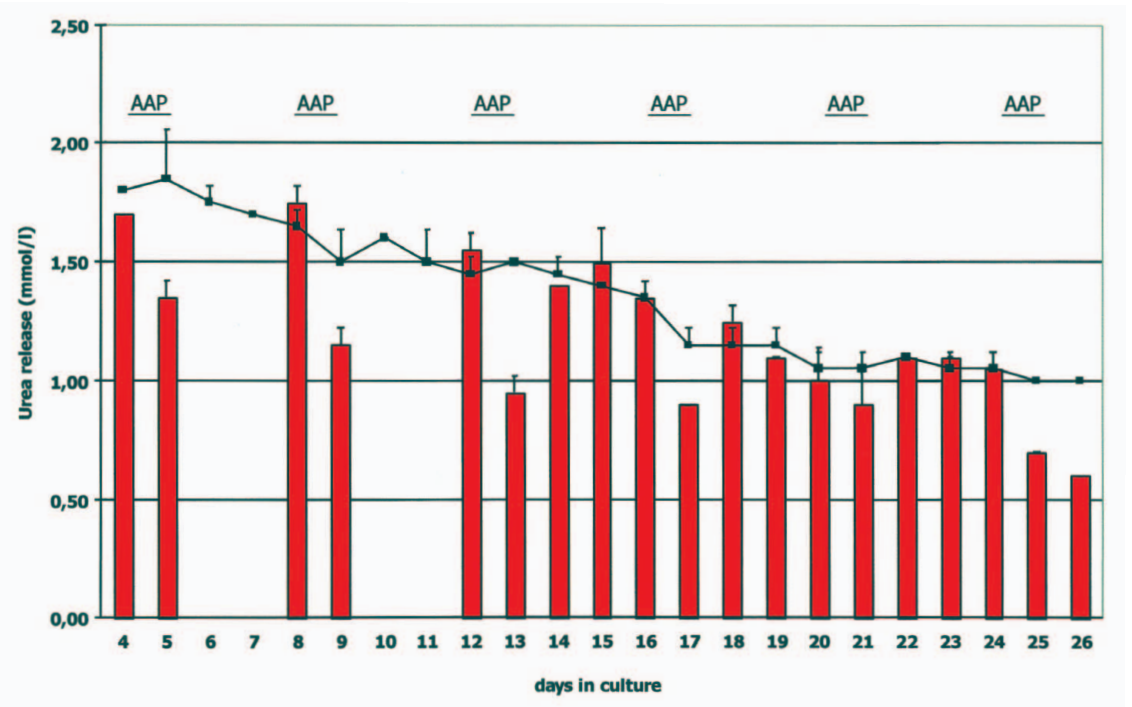

Fig. 5: Acetaminophen reduces urea release in human hepatocytes.
Human hepatocytes were cultured in HHMM with growth factors. Medium was changed every 24 h. Urea was detected in cell culture medium (control, black line). On the days indicated, AAP was added for 24 h. Urea release before, during and after incubation with AAP was determined in the culture medium (red bars). Data represent means ± SD from two determinations of one representative donor out of a total of four different donors.

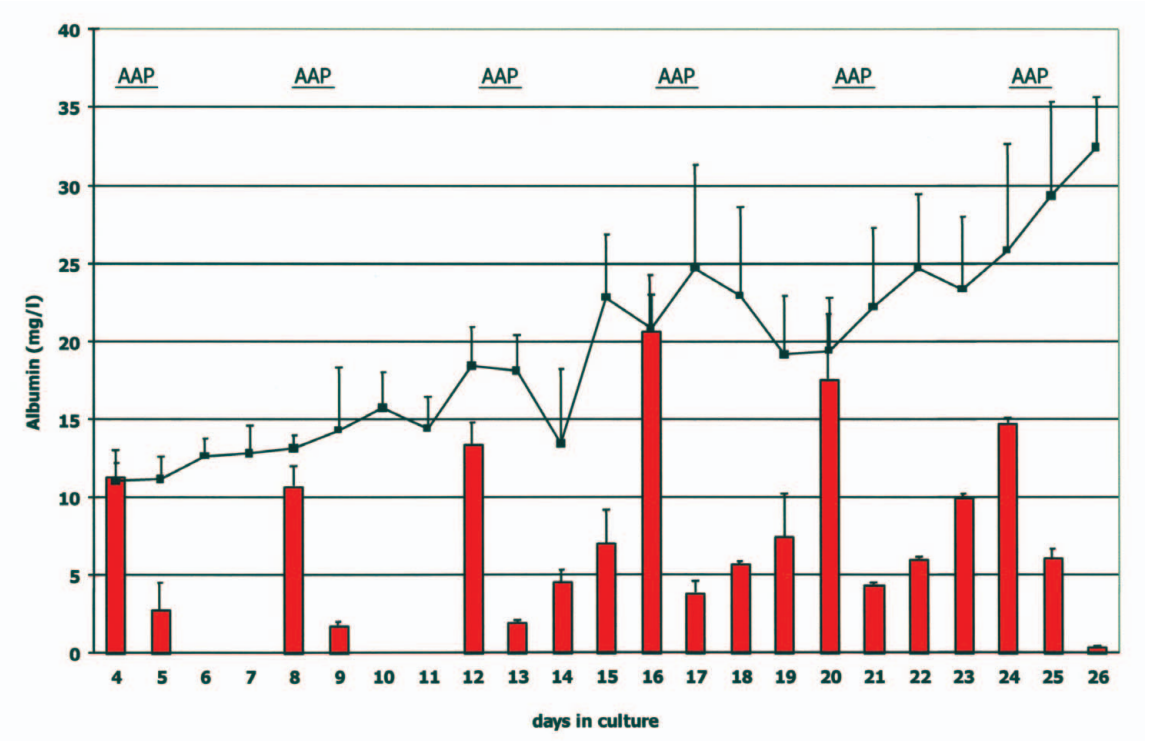

Fig. 6: Acetaminophen reduces albumin release in human hepatocytes.
Human hepatocytes were cultured in HHMM with growth factors. Medium was changed every 24 h. Albumin was detected in cell culture medium (control, black line). On the days indicated, AAP was added for 24 h. Albumin release before, during and after incubation with AAP was determined in the culture medium (red bars). Data represent means ± SD from two determinations of one representative donor out of a total of four different donors.

Dose 28-day Oral Toxicity Study in Rodents) and 408 (Repeated Dose 90-day Oral Toxicity Study in Rodents). Up to 60 animals are required for each study. Often, the results obtained with these animal studies are not in line with data obtained from human studies. HEPAC^2 allows us to culture differentiated human hepatocytes for 28 days or longer. This should give us a tool to analyse acute or repeated dose effects that chemicals may exert on human hepatocytes. If other human cells can be maintained in a comparable fashion as well, it might be possible to reduce the number of animal studies for the evaluation of repeated dose effects of chemicals.

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