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Spontaneous apoptosis, necrosis, energy status, glutathione levels and biotransformation capacities of isolated rat hepatocytes in suspension: Effect of the incubation medium

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Abstract

Isolated hepatocytes in suspension express most of the functional activities of the intact liver and offer an easy-to-handle in vitro system for investigating both the biotransformation and damaging effects induced after a single exposure to xenobiotics upto 3–4 h. There is, however, a general lack of consensus with respect to the choice of a suitable suspension medium. This motivated us to perform a comparative study of the effects of five frequently used bicarbonate-based media (Ca^{2+}-containing Krebs–Henseleit buffer (KHB) with or without 25 mM HEPES, 10 mM glucose and 2% (g/v) BSA supplements, and Williams' E culture medium) on the viability (LDH leakage, caspase-3 processing and activity, Bid/Bax expression) and functionality (energy status, glutathione content, phases I and II biotransformation) of freshly isolated rat hepatocytes in suspension upto 3 h. Also included was the bicarbonate-free HEPES buffer that does not require carbogen gassing, and is therefore handled more easily. The results clearly demonstrated that the type of incubation medium profoundly affected the functionality of the suspended hepatocytes, changing their sensitivity and response to exogenous damaging effects. While HEPES buffer and Williams' E medium offered the lowest background of spontaneous cell death, bicarbonate-based buffers and media seemed more suitable for obtaining both phases I and II biotransformation. Williams' E medium ensured a constant glutathione content of the cells and a lower level of oxidative stress.

Keywords: Isolated rat hepatocytes in suspension; Necrosis; Apoptosis; Cellular energy status; Glutathione content; Phase I and II xenobiotic biotransformation capacities

1. Introduction

Toxicological testing of new chemical entities is traditionally performed in experimental animals, according to strict regulatory guidelines. In vitro tests are present in regulatory dossiers but usually as 'value-added', for screening or assisting in the detection of a specific biological or toxicological effect [1,2]. Recent changes in the European policy on chemicals imposing testing requirement of more than 30,000 existing chemicals (REACH (Registration, Evaluation and Authorization of Chemicals), 2003 [3]), however, strongly encourage the standardization and validation of in vitro tests in order to replace and reduce animal methods.

Commonly, information on the biotransformation of chemicals in support of in vivo toxicology and clinical safety studies is provided by liver-derived in vitro systems [2]. Indeed, the liver (hepatocytes) is the major site for biotransformation of xenobiotics, converting them into biologically inactive, active or sometimes toxic metabolites. Moreover, it is a frequent target organ for toxicity induced by foreign compounds in vivo, as it is interposed between the digestive tract and the rest of the body, and selectively takes up xenobiotics in an attempt to prevent them from entering the blood stream [4,5]. In contrast to liver microsomes and homogenates, isolated hepatocytes

Abbreviations: BSA, bovine serum albumin; CYP, cytochrome P450-dependent monooxygenase; 7-EC, 7-ethoxycoumarin; GSH, reduced glutathione; GSSG, oxidized glutathione; 7-HC, 7-hydroxycoumarin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; KHB, Krebs–Henseleit buffer; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline solution

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represent a physiologically relevant, yet experimentally controllable in vitro model, in which all biotransformation enzyme systems are coupled and enzyme cofactors are present at in vivo levels. They are now frequently used to generate qualitative (metabolite pattern, enzymes involved) and quantitative biotransformation (clearance) data in both animals and humans. Hence, not only rank-ordering of hepatic clearance is possible, which is considered particularly important in the early drug discovery stage where emphasis is placed on finding compounds that are biotransformation 'resistant', but also relatively accurate predictions of in vivo clearance and metabolite formation have been obtained (reviewed in [2]). In addition, as cellular integrity is intact, experiments with isolated hepatocyte preparations generate preliminary data on acute toxicity and potential interrelationships with xenobiotic biotransformation. The mechanisms and biochemical alterations underlying the observed toxicity, such as glutathione depletion and free radical formation [6,7], can be investigated more thoroughly, and often mechanisms of toxicity similar to in vivo are detected [4,8,9].

Suspensions of isolated hepatocytes can be used for relatively short-term incubations of upto 4 h. Two major advantages in the light of their potential use in routine high-throughput testing, include cryopreservability and their easy-to-handle nature [9,10]. Their use in pharmaco-toxicological studies is not well standardized, and diverse incubation media have been used by different laboratories. The potential effects of these media on hepatocyte viability and functioning have been discussed only to a limited extent, at the early start of in vitro technology. Usually, it was and is still considered as less important [4,8,9]. The present study demonstrates quite the opposite. The effects of six frequently used media with an increasing degree of complexity have been studied on the following parameters; hepatocyte viability, phase I and II biotransformation capacities, and intracellular ATP levels and glutathione contents. The latter two parameters are known as early indices of toxicity preceding irreversible cell injury. As bicarbonate buffers are recognized to have an important nutritional value to mammalian cells, the solutions and media most frequently used for handling hepatocytes are bicarbonate-based [11,12]. They require gassing with carbogen (95:5 (v:v) air:CO_2), implying the need for either a shaking waterbath with regular manual gassing or a rotating air/CO_2 incubator. However, pH fluctuations often occur and frequent sampling is difficult. In this study, the effects of some of the commonly used bicarbonate-based buffers as well as an isotonic HEPES-based bicarbonate-free buffers were compared. The latter does not require carbogen gassing, but instead can be exposed to atmospheric levels of about 300 ppm CO_2 in a water bath at 37 °C. The results emphasize that a proper choice of the suspension medium is of key importance for any in vitro test, in particular in prospective studies.

2. Materials and methods

2.1. Chemicals

Aprotinin, BSA fraction V, β-glucuronidase from bovine liver B-3, β-glucuronidase-sulphatase mollusk H-1, β-NAPDH, CHAPS (3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate), crude collagenase type I (clostridiopeptidase), d-glucose, DTNB (5,5′-dithiobis-2-nitrobenzoic acid), EDTA (ethylenediaminetetraacetic acid), 7-ethoxycoumarin, 7-HC, HEPES, leupeptin, metaphosphoric acid ($(HPO_3)_n$), pepstatin A, PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), PMSF (phenylmethylsulfonyl fluoride), and 2-vinylpyridine came from Sigma–Aldrich. Glutathione reductase (GR), reduced (GSH) and oxidized (GSSG) glutathione were purchased from Roche Diagnostics GmbH. Wiliams' E medium was purchased from Gibco BRL, and Ac-DEVD-AFC (acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylacoumarin) came from Calbiochem. All other chemicals were readily available commercial products.

2.2. Cell isolation and incubation procedures

Procedures for housing of the rats, and isolation and culture of isolated rat hepatocytes were approved by the local ethical committee of the Vrije Universiteit Brussel (Brussels, Belgium). Rat hepatocytes were isolated under aseptic conditions from outbred adult male Sprague–Dawley rats (200–300 g, Charles River Laboratories) using a two-step collagenase perfusion method [13]. Briefly, a first perfusion of the excised liver with Ca^{2+}-free KHB (pH 7.4, 37 °C) attacks the Ca^{2+}-containing bridges between the cells. Subsequently, the liver is perfused with Ca^{2+}-containing KHB (pH 7.4, 37 °C) supplemented with 115 collagenase digestion units/ml. The rats were kept under controlled environmental conditions (12 h light/dark cycle) with free access to food (Animalabo A04) and water. Cell integrity was assessed by trypan blue exclusion and only suspensions with a minimum of 90% observed viability were used. The cells were incubated in six different media (Table 1) at a final cytocrit of 10^6 hepatocytes/ml (30 ml Teflon sealed screw-capped glass vials; 5 ml final volume). For determination of the phase I and II xenobiotic biotransformation capacities, the hepatocytes were incubated with 0.5 mM 7-EC 2. Incubation was started 10 min after resuspending the cells in the different media (37 °C, pregassed with carbogen during 30 min) with or without 7-EC, by transferring the suspension to a shaking (90 oscillations/min) water bath at 37 °C. All media except **M1** were gassed with carbogen for 10 s every hour or upon opening of the incubation flask.

2.3. Protein content

Cellular protein concentrations were determined using a Bradford protein assay kit (Bio-Rad) with BSA as a standard [14].

Table 1
An overview of the most important differences in media composition

Ingredient	**M1** (pH 7.6) (mg/l)	**M2** (pH 7.4) (mg/l)	**M3** (pH 7.4) (mg/l)	**M4** (pH 7.4) (mg/l)	**M5** (pH 7.4) (mg/l)	**M6**[a] (pH 7.4) (mg/l)
BSA	–	–	–	–	2% (w/v)	–
$CaCl_2 \cdot 2H_2O$	–	384	384	384	384	200
d-Glucose	–	–	–	1802	1802	2000
HEPES	380	–	105	105	105	–
KCl	200	362	3621	362	362	400
KH_2PO_4	–	165	165	165	165	–
$MgSO_4 \cdot 7H_2O$	–	299	299	299	299	97.67
NaCl	8000	7087	7087	7087	7087	6800
$NaHCO_3$	–	2140	2140	2140	2140	2200
$Na_2HPO_4 \cdot 12H_2O$	100	–	–	–	–	–

Osmolarities of the media were between 280 and 310 mOsm. pH values were measured at 37 °C. Abbreviations used are: M = medium, KHB = Krebs–Henseleit buffer, BSA = bovine serum albumin.

[a] **M6** is a commercially available medium, and also contained 0.05 mg/l glutathione, 0.03 mg/l methyl linoleate, 10 mg/l phenol red, 25 mg/l Na pyruvate, several vitamins, amino acids, and anorganic salts.

2.4. Cell death

2.4.1. Membrane integrity

The LDH index was determined with a Merckotest (LDH index = 100 × LDH activity in the supernatant divided by the sum of LDH activity in the supernatant and in the cells (%)), (VWR International).

2.4.2. Apoptosis

At selected time intervals, samples containing 4×10^6 hepatocytes were centrifuged ($120 \times g$, 4 °C, 2 min) and washed twice with ice-cold PBS. Cells were lysed with 100 μl lysis buffer pH 7.0 (10 mM HEPES, 2 mM EDTA, 0.1% (w/v) 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiotreitol (DDT), 1 mM phenylmethane sulfonylchloride (PMSF), 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) by five freeze-thawing cycles, and centrifuged at $10{,}000 \times g$ for 30 min at 4 °C. Caspase-3-like activity in the cell lysates was measured using the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC). Western blot analysis of caspase-3 processing, Bid and Bax expression was performed as described earlier [15]. Equal loading was confirmed by reversible 0.1% Ponceau Red staining of the membranes.

2.5. Cellular energy status

ATP/ADP levels were measured by bioluminescence using an Apoglow™ assay kit (BioWhittacker), based on the firefly–luciferase–luciferin system and resulting in the generation of measurable light at a wavelength of 565 nm [16]. The experiments were set up in triplicate in 96-well, white-walled, clear-bottomed, sterile luminometer plates (Sanbio). Samples containing 1×10^6 hepatocytes were centrifuged at $120 \times g$ (4 °C) for 2min, washed twice with ice-cold PBS, and resuspended in 250 μl ice-cold PBS. The adenylates were extracted from the hepatocytes by the addition of an equal volume of cell nucleotide-releasing reagent, and the released ATP was measured using a luminometer (Victor$_2$™ Multilabel Counter, Wallac, Perkin-Elmer). After a decay period of 20 min, 20 μl of ADP converting reagent was added. An immediate reading determined the baseline ADP relative light units, while the reading after 5-min incubation allowed for conversion of ADP to ATP. Results are expressed as pmol/mg total protein.

2.6. Intracellular glutathione content

Intracellular GSH and GSSG contents were determined spectrophotometrically (412 nm) by the enzymatic recycling method using glutathione reductase and 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [17]. GSSG was determined after derivatization of GSH with 2-vinylpyridine (5 μl/300 μl sample) [18]. Harvested and washed cells (4×10^6) were resuspended in 500 μl ice-cold PBS, sonicated, and deproteinated with 10% $(HPO_3)_n$ for 15 min. The lysate was then centrifuged for 30 min at $10{,}000 \times g$ and 4 °C, and the supernatant was diluted in 125 mM sodium phosphate—6.3 mM EDTA buffer pH 7.5. The total GSH and GSSG contents were measured in triplicate in the presence of 0.21 mM NADPH, 0.6 mM DTNB, and 0.5 U/ml glutathione reductase at 30 °C.The concentration of GSH was calculated from the difference between concentrations of total glutathione (GSH + GSSG) and GSSG. Results are expressed as nmol/mg total protein.

2.7. Phase I and II xenobiotic biotransformation capacity

Five hundred micromoles of 7-EC was incubated with 1×10^6 hepatocytes/ml during a total incubation period of 120 min. Two mililiters of samples were taken after 30, 60 and 120 min of incubation and stored at −80 °C. After thawing, they were sonicated and homogenized. In order to determine free and conjugated (glucuronidation, sulfation)

7-HC [19], 500 μl homogenate was incubated with 700 μl of either 10 mM saccharonolactone, 540 IU/ml β-glucuronidase-sulphatase H1 or 580 IU/ml β-glucuronidase B3 in 60 mM acetate buffer containing 0.11 M NaCl at 37 °C during 12 h. Free 7-hydroxycoumarin was extracted [20] and measured fluorimetrically at excitation wavelength 370 nm and emission of 457 nm. Results are expressed as μmol/mg total protein.

2.8. Statistical analysis

Data are presented as mean ± S.D. of at least three independent experiments. Three-way (xenobiotic biotransformation capacity) or two-way (LDH index, caspase-3 activation, glutathione content, ATP level) ANOVA tests were performed in order to identify the factors with a significant influence. They were followed by post-hoc tests (Scheffé and Student–Newman–Keuls) to identify deviating groups and by a one-way ANOVA with Bonferroni correction (statistical analysis for multiple comparisons) to study differences amongst individual means. All statistical analysis were done with SPSS for Windows, Release 11.5.1 (2002).

3. Results

3.1. Cell death

The hepatocytes were suspended in media **M1**–**M6** for 3 h under continuous shaking and regular gassing (with exception of **M1**). LDH leakage was determined every hour (Fig. 1). Analysis of caspase-3-processing and activity in the same samples provided information on hepatocyte apoptosis (Fig. 2A and B) [22]. In addition, an evaluation of changes in the expression levels of the pro-apoptotic members of the Bcl-2 family Bid and Bax reflect the induction of both mitochondrial, and/or endoplasmatic reticulum mediated apoptosis, including caspase-independent pathways (Fig. 2C) [23].

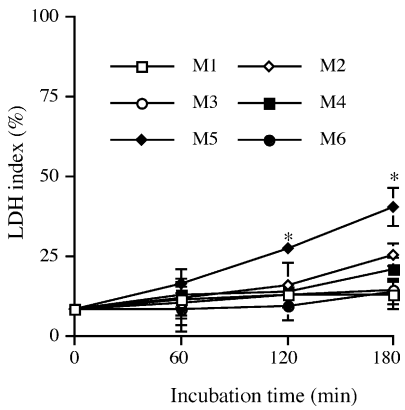

Fig. 1. Time-course analysis of the LDH indices of rat hepatocytes suspended in **M1**–**M6**. The results are expressed as % and represent the mean ± S.D. of at least three independent experiments. *Significantly different from values in other media ($p < 0.05$). The LDH index measured at the start of the incubation period equalled 8.4 ± 2.8%.

Whereas the LDH index remained unaltered in BSA-free media (**M1**–**M4**, **M6**), a significantly increased ($p < 0.05$) LDH index was measured after 120 and 180 min for **M5** (27 and 40%, respectively). Incubations beyond the conventional period of 3 h led to highly augmented LDH indices in cells suspended in simple buffers, such as **M1** (51 and 78% in 5- and 6-h samples, respectively), while those in the complex medium **M6** maintained their integrity for longer periods (26% and 54% after 5 and 6 h, respectively) (results not shown).

Hepatocytes in **M2**–**M5** showed a similar, significant increase ($p < 0.001$) in caspase-3-like activity during incubation (Fig. 2A). Surprisingly, not only the hepatocytes suspended in the rich cell culture medium **M6**, but also those in the very simple HEPES buffer **M1** showed no significant changes in caspase-3-like activity during the entire 3-h incubation period. Western blot analysis of the processing of caspase-3 at the start of the experiment (e.g. after a pre-incubation period of 10 min, T0) and after 3 h of incubation (T3) of the hepatocytes in the different media confirmed these results (Fig. 2B). Caspases are synthesized as inactive procaspases (30–50 kDa) that contain an N-terminal prodomain, a large subunit (about 20 kDa) and a small subunit (about 10 kDa). Upon proteolytic cleavage, two large and two small subunits reassemble in order to form an active heterotetramer [24]. The large subunit (p18) was already found at the start of the incubation and was continuously present thereafter. This suggests a process of ongoing caspase-3 activation in agreement with the observed increase in caspase-3-like activity in **M2**–**M5**. A short pre-incubation period of the hepatocytes in the different media was enough to induce clear differences in the expression levels of procaspase-3 and its subunits between the KHB-based media and **M1** and **M6** at time zero. A delay in (pro)caspase-3 processing appears to underly the differences in caspase-3-like activity, as the expression levels of (pro)caspase-3 tended to be higher in **M1** and **M6** after 3 h of incubation. At that moment, cells suspended in **M2**–**M5** enter a late apoptotic, secondary necrotic stage due to the absence of heterophagic cells, and the subsequent lysis of the apoptotic bodies [24]. Indeed, (pro)caspase-3 protein expression and processing is strongly reduced, and the measured LDH indices show slight increase (significant from 4 h on (results not shown)). In analogy with the results on caspase-3 processing, the increase in Bid expression is delayed in hepatocyte suspensions in **M1** (Fig. 2B). Bax expression, in contrast, shows little medium dependency at time zero. The lower levels of Bid and Bax proteins in media **M2**–**M5** and **M4** and **M5**, respectively, after 3 h of incubation correlate with the decreased (pro)caspase-3 processing, and indicate a state of secondary necrosis. The anti-apoptotic protein Bcl-XL [23] was found to be expressed

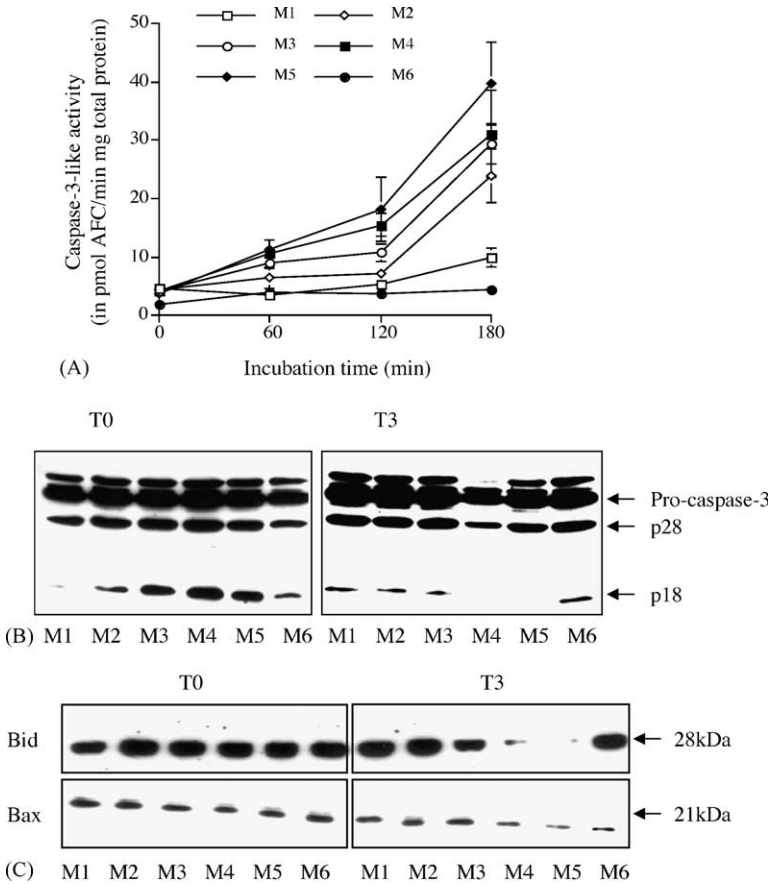

Fig. 2. Apoptotic cell death of rat hepatocytes suspended in **M1**–**M6** for 3 h. (A) Caspase-3-like activity of intact cells was measured every hour. Results are expressed as pmol AFC formed per minute and per milligram of total protein, and represent the mean ± S.D. of at least three separate experiments done in triplicate. Symbols indicating statistical significant differences were omitted for clarity reasons. (B) Expression and processing of pro-caspase-3 in freshly isolated rat hepatocytes (T0) and in 3 h old rat hepatocyte suspensions (T3) in **M1**–**M6**. The blots shown are representatives for three independent experiments. (C) Expression of Bid and Bax at T0 and T3 in **M1**–**M6**. The blots shown are representatives for two independent experiments.

at extremely low, negligible levels in all media throughout the entire incubation period (results not shown).

3.2. Cellular energy status

As the loss of cellular ATP in general precedes the leakage of cytoplasmic enzymes such as LDH [25], it is frequently measured as an early index of cytotoxicity. The ATP:ADP ratio, on the other hand, largely reflects the cellular phosphorylation potential, and has been described as a discriminator between the two modes of cell death, necrosis and apoptosis [26]. In general terms, a large drop in ATP:ADP ratios has been described to indicate necrosis, while a slightly decreased ATP:ADP ratio has been associated with apoptosis [23,26].

In Fig. 3A and B, the time-related changes in ATP:ADP ratios and ATP levels of isolated rat hepatocytes suspended in **M1**–**M6** are depicted. At the start of the incubation, no significant differences in intracellular ATP could be detected, although average energy levels appeared slightly higher in **M4**–**M6**. ATP:ADP ratios were significantly lower in **M3**–**M5**, compared with **M1** and **M6** ($p < 0.05$, $p < 0.01$, $p < 0.05$, respectively). These data correlate with the previous results, as hepatocytes in **M3**–**M5** also expressed higher (pro)caspase-3 protein levels at time zero and exhibited significantly higher caspase-3-like activities after 120 min of incubation. In each medium, the ATP:ADP ratio decreased during the first 2 h of incubation (**M5**, $p < 0.05$), indicating the continuation of the apoptosis, followed by a retrieval of the start values by 180 min of incubation as the self-destruction process extinguishes and secondary necrosis takes the upper hand. In **M1**–**M3**, hepatocyte ATP levels were kept at a constant level for 120 min, and decreased ($p < 0.05$) thereafter. Only cells suspended in glucose-supplemented **M4** and in complex **M6** could significantly ($p < 0.05$) build up energy reserves as a function of time. The supplementation of **M4** with 2% (w/v) BSA (**M5**), however, completely abolished the beneficial effect of glucose, and promoted a significant loss ($p < 0.05$) of ATP after 180 min of incubation. However, no significant differences in the cellular ATP:ADP ratios of hepatocytes suspended in **M4** and **M5** could be detected.

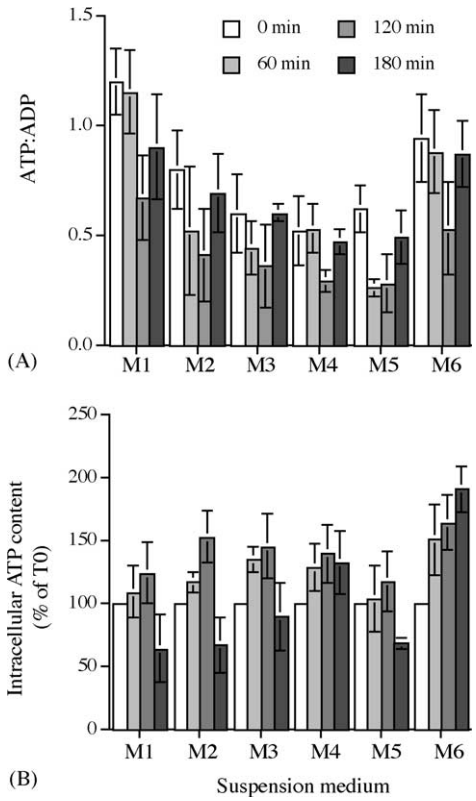

Fig. 3. Time-course study of the intracellular ATP contents and ATP:ADP ratios of rat hepatocytes suspended in **M1**–**M6** for 3 h. ATP contents were expressed as a percentage of the value measured at the start of the incubation (T0; 9.8 ± 2.1 pmol/mg total protein). Results are mean ± S.D. of three independent experiments. Symbols indicating statistical significant differences were omitted for clarity.

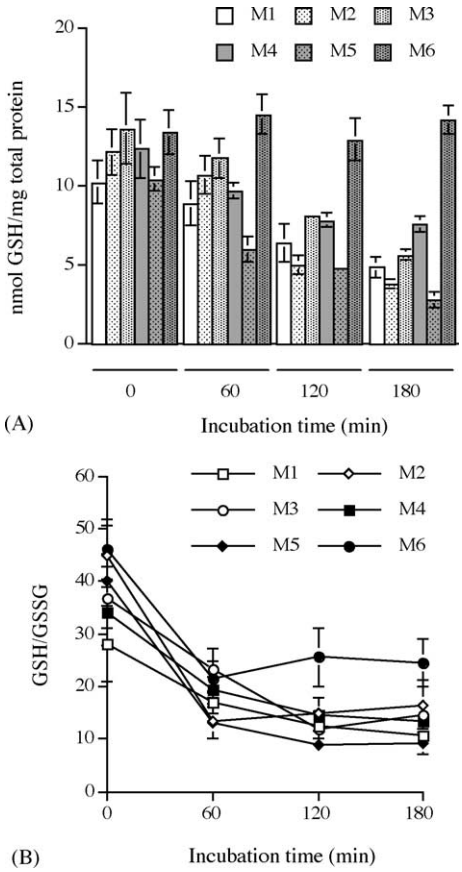

Fig. 4. Effect of the medium on time-related changes in intracellular GSH concentrations (expressed as nmol/mg total protein) (A) and calculated GSH:GSSG ratios (B) of isolated rat hepatocytes in suspension. The values are expressed as mean ± S.D. for at least three independent experiments. Symbols indicating statistical significant differences were omitted for clarity.

3.3. Cellular glutathione content

Glutathione depletion is often reported as an early cytotoxic effect, caused by many reactive organic compounds and their metabolites. Indeed, GSH may either be used as a conjugating agent (glutathione S-transferase) or be oxidized in reactions catalyzed by glutathione peroxidase after the xenobiotic-induced generation of H_2O_2 or hydroperoxides [27–30]. GSH depletion to about 20–30% can impair cell defense and may lead to cell injury and death [27].

Fig. 4A and B represent the reduced glutathione levels, as well as the calculated GSH/GSSG ratios, respectively, of the hepatocytes suspended in each suspension medium as a function of incubation time. Hepatocytes suspended in the relatively simple organic buffers, **M1**–**M5**, time-dependently lost 40–60% of their GSH levels ($p < 0.001$). The presence of glutathione precursors (0.004% (w/v) *L*-cysteïne, 0.002% (w/v) L-cystine, 0.005% (w/v) glycine, 0.005% (w/v) L-glutamate, 0.0015% (w/v) L-methionine) as well as exogenous GSH (0.005% (w/v)) in **M6**, completely annulled this loss. In concert with the loss of membrane integrity observed after 120 min of incubation (Fig. 1), hepatocytes suspended in **M5** lost their intracellular GSH more rapidly ($p < 0.05$). In all media, the intracellular GSH/GSSG ratio underwent a 50% reduction during the first hour of incubation, reflecting the oxidative stress induced during hepatocyte isolation [31]. During the following 2 h, a gradual stabilization could be observed. Due to the higher reduced glutathione content, hepatocytes suspended in **M6** achieved higher GSH:GSSG ratios after 120 min ($p < 0.001$) and 180 min ($p < 0.001$) of incubation compared to **M1**–**M5**, in which comparable ratios were measured.

3.4. Xenobiotic biotransformation capacity

While *O*-deethylation of 7-EC to 7-HC is mainly CYP-mediated (including CYP1A1, CYP1A2, CYP2B1 and CYP2E1 [20,32,33]), 7-HC is subjected to the competing phase II pathways of sulfation and glucuronidation [34]. Therefore, analysis of 7-HC formation before and after hydrolysis of 7-HC glucuronides and sulfate conjugates provide a rapid method to simultaneously measure both phase I and II biotransformation capacities in isolated hepatocytes [34] (Fig. 5). After 30 min of incubation, no statistical significant differences could be found between the amounts of non-conjugated 7-HC formed in each

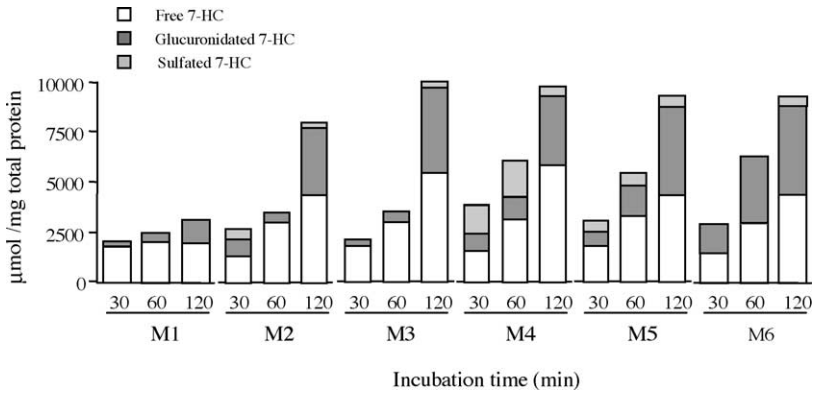

Fig. 5. Time-course analysis of phase I and II biotransformation capacities of isolated rat hepatocytes suspended in **M1**–**M6** for 3 h. 7-Ethoxycoumarin was used as a model substrate as described under Section 2 and measurements were performed for at least three independent experiments. Results are expressed as the mean quantity of 7-HC formed (µmol/mg total protein). Standard deviations and symbols indicating statistical differences were omitted for clarity reasons.

medium, while more 7-HC sulfates and glucuronide conjugates were measured in the KHB-based media **M2**, **M4**–**M6** compared to **M1** ($p < 0.05$). This difference became more striking as a function of the incubation time, as 7-EC deethylation and 7-HC conjugation steadied in **M1**. The addition of 25 mM HEPES to **M2** (**M3**) slightly inhibited 7-HC conjugation during the first 30 min of incubation ($p < 0.05$). Compared to **M2** and **M3**, the richer KHB-based media **M4**–**M6** formed 7-HC conjugates more rapidly ($p < 0.05$). The beneficial glucose effect was abolished by the addition of 2% (w/v) BSA (**M5**) ($p < 0.05$ after 60 and 120 min).

4. Discussion

The purpose of this study was to critically evaluate the influence of the incubation medium on the viability and functioning of isolated rat hepatocytes in suspension for 3 h at 37 °C under continuous shaking. The conclusions drawn from this study are important for the use of this in vitro model in predictive and retrospective studies, including mechanistic biotransformation and acute hepatotoxicity studies. Ischemia-reperfusion injury as well as cellular detachment from the extracellular matrix and neighbouring cells have been shown to be responsible for the induction of hepatocyte apoptosis during isolation (reviewed in [31]). During further cultivation of the primary hepatocytes as monolayer cultures, it has been shown that the level, the onset and the regulation of apoptosis largely depends on the medium composition [15,35,36]. For hepatocytes in suspension, such studies are not available. In a pharmaco-toxicological context, however, the (high-throughput) screening for apoptotic effects in primary hepatocytes in suspension is important [21]. In this paper, we showed that the expression and activity of caspase-3, the main end executor of both intrinsic and extrinsic apoptotic pathways [21,37] is upregulated during incubation of isolated rat hepatocytes in suspension. In correlation with their roles in the onset of apoptosis [38,39], Bid and Bax protein levels were high immediately after hepatocyte isolation, whereas Bcl-XL was expressed at negligible low levels. Compared to the bicarbonate-based buffers **M2**–**M5**, hepatocytes suspended in HEPES buffer (**M1**) and the complex culture medium Williams' E (**M6**) have a lower basal level of spontaneous cell death, and are therefore more suitable for studying apoptotic effects of xenobiotics. This does not appear related to a shortage of ATP. While **M6** has a high nutrient value, glycolytic ATP production from the hepatic glycogen stores in the absence of mitochondrial ATP synthesis can probably maintain cellular ATP levels and cell integrity during the 3-h incubation period in the Mg^{2+}-lacking HEPES buffer. Earlier observations in hepatocyte monolayers on collagen-treated dishes under an atmopshere of air using Medium SM-1 also showed that the amounts of CO_2 produced during cellular respiration were sufficient to cover the need for bicarbonate in synthetic reactions [40]. In addition, the relative high pH of the HEPES buffer (7.6 at 37 °C) has been found optimal [41,42] for hepatic respiration, glycolysis, and protein synthesis. The absence of extracellular Ca^{2+} and consequent depletion of the endoplasmatic reticulum Ca^{2+} pool probably prevents cell death of hepatocytes suspended in **M1** through a diminished calpain activation [43]. The slightly higher ATP levels in the glucose-supplemented bicarbonate-based media **M4** and **M5,** could be responsible for a more rapid onset of the apoptotic process compared to **M2** and **M3**. In the absence of phagocyting cells, the increased level of spontaneous hepatocyte apoptosis eventually results in a significant loss of membrane integrity (secondary necrosis) after 4 h of incubation, in concert with the knowledge on the period of utility of hepatocyte suspensions for pharmaco-toxicological objectives [9]. The relatively large and early increase in LDH index compared to the changes in caspase-3-like activity, as well as the early loss of cellular ATP and glutathione, suggest that hepatocytes suspended in **M5** are subjected to primary necrosis. Regular gassing with carbogen leads to the formation of flexible BSA protein

films at gas–liquid interfaces [44]. This provokes foam production that may damage the hepatocytes.

No significant differences in reduced glutathione contents were found between the buffers **M1**, **M2**, **M3** and **M4**. In contrast, the presence of GSH and its precursors in **M6** ensured a constant GSH level during the total incubation period, as well as higher GSH/GSSG ratios. This effect was only visible after 2 h of incubation, reflecting the stability of cytosolic GSH in rat liver ($T_{1/2}$ = 2–3 h) [45]. In the context of hepatotoxicity studies, differences in GSH contents can affect the susceptibility of hepatocytes to the toxic effects of xenobiotics. Molecules such as acetaminophen and bromobenzene can bind and thereby deplete glutathione after bioactivation through CYP biotransformation [6,7]. Such effects can be overlooked in media such as **M6**, as high GSH levels will stay put even in the presence of relatively high concentrations of toxic electrophiles.

7-HC phase II biotransformation capacities of hepatocytes suspended in HEPES buffer are remarkably lower than those observed in KHB, in particular after 60 min of incubation. The absence of extracellular sulfate as well as glycogen and glucose breakdown, required for the synthesis of the sulfotransferase co-factor 3′-phosphoadenosine-5′-phosphosulfate or PAPS and the cofactor UDP–glucuronic acid for glucuronidation, respectively [28,46–48], are thought to be at the origin of this observation, rather than a difference in gassing. The decreased availability of glucose by non-enzymatic attachment to BSA (‘glycoxidation’) [49], is probably in part responsible for abolishing the beneficial effects of exogenously added glucose on 7-HC conjugation. In addition, BSA could inhibit conjugation by binding the produced 7-HC that has left the hepatocytes [50]. Although a more extensive phase I and subsequent phase II biotransformation of 7-HC was observed in hepatocyte suspensions in **M6,** it is clear that its complex nature may complicate HPLC–MS and tandem MS analyses. In addition, metabolites formed in minor amounts or rapidly converted to secondary metabolites could be difficult to detect. Besides the limited availability of fresh human hepatocytes, these are some of the reasons why microsomes have often been used for identification of phase I biotransformation pathways in the past [28]. Isolated hepatocyte suspensions in HEPES buffer, however, represent a valuable alternative providing both microsomal enzymes as well as cytosolic-mediated phase I biotransformation pathways. In addition, toxicity mediated through phase I bio-activation is detected more easily as phase II detoxification (e.g. glucuronidation and sulfation rates, and intracellular glutathione content) is low. A bicarbonate-based medium, on the other hand, seems more appropriate for an additional investigation of phase II biotransformation pathways. In this respect, supplementation of the medium with 10 mM glucose was shown to enhance both 7-HC sulfation and glucuronidation. Carbogen gassing, however, is required, making the incubation procedure and sampling more complicated.

In conclusion, while HEPES buffer and Williams’ E medium offer the lowest background of spontaneous cell death, hepatocytes suspended in the latter medium might be less sensitive to xenobiotic-induced toxicity due to the presence of exogenous glutathione and antioxidants. Although bicarbonate-based buffers and media seem more suitable for investigating both phase I and II biotransformation, they require regular gassing with carbogen and have a higher background level of apoptosis. The addition of 2% (w/v) BSA induces primary hepatocyte necrosis and abolishes the enhancing effects of supplemented glucose on phase II biotransformation, while the use of commercially available culture media might complicate metabolite detection and identification. Importantly, suspensions in HEPES buffer provide a valuable and more easily handled alternative for investigating the phase I biotransformation of xenobiotics, ranking them according to phase I metabolic stability, and/or investigating phase I-mediated xenobiotic bio-activation.

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