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Application of Alamar blue/5-carboxyfluorescein diacetate acetoxymethyl ester as a noninvasive cell viability assay in primary hepatocytes from rainbow trout

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Abstract

We have adopted the application of two fluorescent indicator dyes to studying the viability of monolayers of primary rainbow trout hepatocytes. The two fluorescent dyes—Alamar blue, which indicates metabolic activity of a cell, and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), which is an indirect measure of cell membrane integrity—are noninvasive and can be monitored conveniently directly in multiwell plates. According to these dyes, L-15 culture medium supported hepatocyte viability over 96 h more stably than did M199. The two dyes proved to be capable of detecting a concentration-dependent toxic insult to hepatocytes caused by the model compound, pentachlorophenol. In contrast, a lack of impact on cell viability was indicated for up to 10^{-5} M 17β-estradiol, and that observation was supported by the induction of vitellogenin (VTG) mRNA/protein as indicator of hepatocyte-differentiated function. Application of the Alamar blue/CFDA-AM for 30 min did not alter gene expression either specifically as reflected by VTG or generally as reflected by a random selection of gene sequences that were amplified by differential display reverse transcription PCR (dd-rt-PCR). Thus, the assay represents a resource-efficient way of integrating measures of cell viability and gene expression that should aid in the interpretation of in vitro results. The assay can be applied repeatedly to the same set of cells and can be performed just prior to analysis of gene expression.

Keywords: Primary rainbow trout hepatocytes; Cell culture; Cell viability; Gene expression; Toxicity; Fluorescent indicator dyes

Primary fish hepatocyte cultures are indispensable tools for studying metabolic activity [1,2] as well as mechanisms of toxicity in vitro [3,4]. An important incentive to the use of fish hepatocytes as model systems is the role of the liver as a major site of metabolism and as a hormone target site. One significant advantage of hepatocyte cultures over the use of whole fish is that the in vitro cultures allow the role of selected parameters to be investigated in a well-defined, controlled environment. In addition, a single fish can yield millions of cells, allowing numerous parameters to be investigated without needing to sacrifice large numbers of animals. Among the donor species used most prominently for hepatocyte isolation is rainbow trout.

Primary hepatocyte cultures from rainbow trout are widely applied for detecting and understanding adverse effects elicited by toxicants. For example, they have frequently been employed to study the induction of cytochrome CYP1A on exposure to dioxin and related compounds, acting as a ligand of the aryl hydrocarbon receptor [5–8]. In addition, rainbow trout hepatocytes have been suggested for studying estrogenic effects due

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to the in vitro expression of an active estrogen receptor [9] and the inducibility of typical downstream effects such as the synthesis of vitellogenin (VTG)[1] [10,11]. Initially, potentially harmful effects of toxicants were studied on the level of protein abundance or enzyme activity, but more recently they are increasingly being explored on the level of gene expression [12,13]. Central to all of these investigations is the determination of the state of viability of the hepatocyte cells either alone or in the presence of the toxicant(s) under study. This is important not only because changes to cell viability can affect gene expression and/or protein function but also because changes in gene expression and/or protein function can eventually lead to alterations in cell viability. Thus, correlating cell viability to specific gene expression or protein function analysis can add valuable information regarding toxicant effects and is important for interpreting the in vitro results.

Several assays have been employed to assess primary fish hepatocyte viability. Neutral red, which accumulates in lysosomes of viable cells, has been applied in primary cultures of trout hepatocytes [14]. The use of propidium iodide, which enters cells on membrane damage and intercalates with the DNA, was particularly advanced by Gagne and Blaise [15,16]. Furthermore, reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) to the blue formazan by viable cells was employed in isolated trout hepatocytes [17–19]. Lilius and coworkers [20] presented a radioactive method based on the release by damaged cells of preloaded ^{86}Rb molecules. Leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium has been used as another indirect measure of hepatocyte viability [7,17,18,21]. Finally, a set of four fluorescent dyes—calcein-AM, 5-chloromethylfluorescein diacetate (CMFDA), rhodamine 123, and JC-1—was evaluated by Lilius and coworkers [22] for the suitability of identifying toxic mechanisms elicited by toxicants in freshly isolated rainbow trout hepatocytes. Among all of these viability assays, the fluorescent assay with calcein-AM and the LDH assay are the only ones that can be used without the necessity to pretreat or sacrifice the cells. However, the LDH test evaluates cell viability in an indirect and nonspecific way, and the procedure is rather cumbersome if applied to multiwell tissue culture plates. Thus, fluorescent dyes such as calcein-AM represent a valuable alternative for noninvasive cell viability assessment. To this point, however, such dyes have not been applied to monitor monolayers of rainbow trout hepatocytes in microtiter plates, particularly in conjunction with downstream applications such as analysis of gene expression.

Thus, it was the goal of this work to adopt a simple, directly applied, noninvasive cell viability assay to primary cultures of rainbow trout hepatocytes. The method of choice was a combination of two fluorescent indicator dyes. Alamar blue (BioSource, USA) was used as a measure of cellular metabolic activity, and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, Molecular Probes, USA) was used as an esterase substrate indicative of cell membrane integrity. The two dyes had previously been shown to work well together for observing viability of fish cell lines [23–25] and long-term cultures of porcine lenses [26].

Three steps were pursued to achieve the goal of establishing the use of Alamar blue and CFDA-AM in the primary rainbow trout hepatocytes. First, the two dyes were used together to repeatedly monitor the viability of hepatocyte cultures over 96 h in serum-free L-15 or M199 culture medium. Second, to examine whether the two dyes were capable of detecting an insult to the hepatocytes by a toxicant, cultures were exposed to pentachlorophenol (PCP), a common biocide and wood preservative, and were subsequently monitored using Alamar blue/CFDA-AM. Finally, 17β-estradiol (E_2)-treated hepatocytes were investigated for their viability prior to RNA extraction, and gene expression in these cells was compared with that in cells not previously analyzed with Alamar blue/CFDA-AM. This was done to determine whether the fluorescent indicator dyes could be applied to monitor primary rainbow trout hepatocyte viability just prior to analysis of gene expression without altering mRNA abundance.

Materials and methods

Cell culture

Primary cultures of rainbow trout hepatocytes

Animals. Immature rainbow trout (*Oncorhynchus mykiss*), purchased from a local fish farm, were kept in 540-L tanks with a permanent flow of fresh air-saturated water. Fish were fed regularly with commercial Aquavalent fish food (Kraftfutterwerk Beeskow, Germany) and kept under a 12-h light/dark cycle for at least 3 weeks before they were used for hepatocyte isolation.

Cell isolation. Cells were isolated from fish weighing 150 to 250 g using a two-step collagenase perfusion technique as described previously for mammals [27] with modifications for fish. The fish were not fed for 1 day

[1] *Abbreviations used:* VTG, vitellogenin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide; LDH, lactate dehydrogenase; CMFDA, 5-chloromethylfluorescein diacetate; CFDA-AM, 5-carboxyfluorescein diacetate acetoxymethyl ester; PCP, pentachlorophenol; E_2, 17β-estradiol; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; FBS, fetal bovine serum; dd-rt-PCR, differential display reverse transcription PCR; ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative rt-PCR; FITC, fluorescein isothiocyanate; CT, threshold cycle; RFU, relative fluorescence units; ANOVA, analysis of variance; CF, 5-carboxyfluorescein.

before hepatocyte isolation. They were anesthetized with 0.8 g MS 222 (ethyl 3-aminobenzoate methanesulfonic acid salt from Aldrich, USA) in 10 L water for 10 min. A detailed description of perfusion and isolation procedures for fish can be found in Ref. [28]. In the current study, the preperfusion buffer consisted of 169 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.63 mM Na_2HPO_4, 26 mM $NaHCO_3$ (all from Merck, Germany), 5.5 mM glucose (Sigma, Germany), 25 mM Hepes (Serva, Germany), and 1.1 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Sigma). The perfusion buffer was of the same composition but also contained 4000 U collagenase (Worthington type 2) and 0.2% $CaCl_2$ (Merck) instead of EGTA. The hepatocyte wash solution was medium M199 (composition mentioned below) containing 1% (w/v) bovine serum albumin (BSA, Sigma). The number of viable cells in the final cell suspension was estimated by Trypan blue exclusion using a hemocytometer according to Seglen [27]. Only hepatocyte preparations that had greater than 85% viability were used in the experiments. Our hepatocyte preparations have routinely had greater than 90% viability. In accordance with others [20,22], we have found the inclusion of BSA in the wash solution to be important for the preparation of rainbow trout hepatocytes with high viability.

Cells were plated at a density of 1×10^6 viable cells/ml/well of serum- and phenol red free culture medium in 24-well plates (Falcon 3847 Primaria plates, Becton Dickinson, USA) previously coated with Matrigel (basement membrane matrix, phenol red free, BD Biosciences, USA) as recommended by Behrens and coworkers [29]. Matrigel is a solubilized basement membrane preparation extracted from a mouse sarcoma. Its major component is laminin, followed by collagen IV and heparan sulfate proteoglycans [30], a matrix shown by Lipsky and coworkers [31] to greatly enhance attachment of rainbow trout hepatocytes. The culture medium was either Leibovitz's L-15 (Gibco, USA) or M199 (ICN Biomedicals, USA), the latter of which was supplemented with L-glutamine (Sigma), 3.7 mM Hepes (Serva, Germany), 4.2 mM $NaHCO_3$ (Merck), and 0.9 mM $CaCl_2$ (Merck). Both culture media contained 1% of a mixture of penicillin (10,000 U) and streptomycin (10 mg/ml) (Sigma) to prevent microbial contamination. For the coating of 24-well plates with Matrigel, a 100 × dilution was prepared in L-15 media, and 200 µl of this solution was added to each well for coating. After 2 h, the wells were rinsed with 300 µl L-15 medium and stored dry in the dark at 4 °C for no more than 4 days. Alternatively, the plates were stored for up to 2 weeks in the presence of 200 µl medium. In addition, a row of wells in each plate was filled with culture medium alone so as to be able to identify potential interferences by the culture media or the experimental conditions with the fluorescence viability assays.

Cells were incubated in the dark in an atmosphere of air at 19 °C. After 18 h of incubation, the success of the hepatocyte culture preparation was judged visually based on attachment and aggregation before cells were exposed and then processed as described below.

Liver cell line RTL-W1

The fish liver cell line was used in a few experiments as an additional model for comparing cellular responses on toxicant exposure and gene expression patterns with or without a prior assessment of cell viability. The RTL-W1 culture is an immortalized cell line from a rainbow trout liver [32]. It was cultured in L-15 medium supplemented with 5% fetal bovine serum (FBS, Biochrom, Germany) and 1% penicillin/streptomycin (10,000 U penicillin and 10 mg/ml streptomycin in 0.9% NaCl, Sigma) in an atmosphere of air in the dark at 19 °C.

For toxicant exposures, 7.5×10^4 cells were plated in 48-well tissue culture plates 24 h prior to adding the toxicant (PCP). The combined Alamar blue/CFDA-AM cell viability assay was performed on these cells as described previously by Schirmer and coworkers [23,33]. Thus, wells were rinsed free of the toxicant solution before the two indicator dyes were added to the wells.

For comparison of gene expression patterns, culture flasks (75 cm^2, Nalge Nunc, Belgium) containing confluent monolayer cultures of RTL-W1 either were directly processed for differential display reverse transcription PCR (dd-rt-PCR) or were exposed to the mixture of fluorescent indicator dyes as described below. After 30 min of incubation, the cells were scraped from the flask, washed with PBS buffer, and frozen at −20 °C in RNAlater solution until RNA isolation and application to dd-rt-PCR.

Exposure

Exposure to various experimental conditions was initiated on culture of primary hepatocytes for 18 h post-isolation. Prior to exposure, half of the volume of the medium in each well was replaced by fresh culture medium. Exposures consisted of a medium control (blank), a solvent control, and varying concentrations of the test compounds, PCP, and E_2. PCP was selected as a test compound because it is a known cytotoxicant. It has also been described as able to uncouple oxidative phosphorylation, which may potentially be detected as reduced cellular metabolism in the Alamar blue assay prior to an impact on cell membrane integrity as measured with CFDA-AM. E_2 was chosen due to its well-described regulation, by means of the estrogen receptor, of gene expression. This was an important criterion for studying differential gene expression in the absence or presence of Alamar blue/CFDA-AM. PCP (Sigma) was dissolved in DMSO (Merck–Schuchardt, Germany) and added directly to the cells using a glass capillary pipette,

yielding final PCP concentrations of between 1.6 and 100 μM with a DMSO content per well of 0.5% using 1:2 dilution steps. PCP exposure was pursued for 24 h. E_2 (Sigma) was dissolved in ethanol (Merck–Schuchardt) and added directly to the cells, yielding final E_2 concentrations of between 10^{-8} and 10^{-4} M, with an ethanol content per well of 0.5 or 1% using 1:10 dilution steps. Exposure to E_2 and the appropriate controls was sustained for up to 4 days (78 h). Half of the medium in each well was changed daily, and the appropriate amount of E_2 was readded to maintain nominally constant E_2 concentrations.

Exposure was terminated by first removing half of the medium of each well. In the case of exposure to E_2, the removed medium was frozen at −20 °C for subsequent VTG enzyme-linked immunosorbent assay (ELISA) as described below. Cells were then either used for cell viability analysis with the fluorescent indicator dyes or harvested for subsequent analysis of gene expression. Cells were harvested by pipetting up and down with a wide-mouth tip (Thermo Life Sciences, Germany) until all cells were suspended. The cells were then transferred into a tube and centrifuged 5 min at 180*g*. Afterward, the pellets were rinsed with PBS and frozen in RNAlater solution (Ambion, UK) according to the manufacturer's instructions until the hepatocytes were used for rt-PCR examinations.

Cell viability assay

Cell viability was assessed using a combination of two fluorescent indicator dyes: Alamar blue and CFDA-AM. The procedure followed the detailed description presented by Schirmer and coworkers [23,33] with some modifications for application to primary hepatocytes. Briefly, CFDA-AM was dissolved in DMSO to a 4 mM stock solution, which was kept frozen until use. Alamar blue was purchased as a ready-to-use solution and kept refrigerated. A mixture of these two dyes was prepared in serum-free L-15 medium immediately prior to application to the cells such that the final dye concentrations were 10% Alamar blue (v/v) and 8 μM CFDA-AM. The ability of the two dyes to be used together without interference in fluorescent measurements had previously been verified by the derivation of the relationship of cell number versus fluorescent units. Resulting standard curves were not statistically different for both dyes regardless of whether they were used individually or in combination [23,34]. An aliquot of 300 μl of the mixture of dyes in L-15 was added to each well, which still contained 300 μl of the exposure medium. After 30 min of incubation at 19 °C, fluorescence of Alamar blue (excitation 530 nm, emission 595 nm) and CFDA-AM (excitation 493 nm, emission 541 nm) was measured using a fluorescence plate reader (SpectraMax Gemini, Molecular Devices, USA). Fluorescence was measured as arbitrary units and expressed as percentage of the readings in the appropriate control wells. Prior to these calculations, fluorescence readings for wells without cells were subtracted from those for the experimental wells to account for background fluorescence. The average coefficients of variation were determined to be 6.1 and 6.2% for Alamar blue and CFDA-AM, respectively.

Analysis of gene expression

Cells that were frozen and kept in RNAlater solution were used for RNA isolation and subsequent PCR to study whether the fluorescent cell viability assays could be performed just prior to gene expression analysis without altering the gene expression results.

RNA isolation

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Germany), and the concentration was determined spectrophotometrically. Both the quality and quantity of the RNA were verified using electrophoresis on a 1% agarose gel. Potential DNA contamination was removed by DNase treatment (Roche, Germany). For hepatocyte samples used for dd-rt-PCR, mRNA was extracted using the Oligotex Kit (Qiagen) and no DNase treatment was performed.

dd-rt-PCR

To investigate whether general differences in gene expression could be observed in cells subjected to the fluorescent cell viability assays, a fluorescent-based modification of the Stratagene differential display protocol (RAP PCR) was applied. First-strand cDNA synthesis (Reverse Transcriptase Kit, Qiagen) of the hepatocyte mRNA was done with the same primer(s) (final concentration 1 μM) as was subsequent PCR except that the primers used in reverse transcription were unlabeled. For the samples from the RTL-W1 cell line, oligo dT primer was used for cDNA synthesis. We selected the random primers A2 (5′-AAT CTA GAG CTC CAG CAG-3′), A3 (5′-AAT CTA GAG CTC TCC TGG-3′), and A4 (5′-AAT CTA GAG CTC TCC AGC-3′) available from a variety of primers suggested by Stratagene. These primers were labeled with a fluorescent Texas red tag at the 5′ site (Metabion, Germany) for dd-rt-PCR. For PCR amplification, 5 μl of the cDNA template were used in 25-μl reactions containing 2.5 μl of the labeled random primer (final concentration 10 μM), 3 mM $MgCl_2$, and Ready-to-Go PCR beads (Amersham, USA). PCR conditions were as follows: 1 min at 94 °C, 5 min at 36 °C, 5 min at 72 °C, and 40 cycles of 1 min at 94 °C, 2 min at 50 °C, and 2 min at 72 °C. dd-rt-PCR was performed in a Thermocycler (T3, Biometra, Germany). The results of this PCR procedure are fluorescently labeled DNA fragments of various sizes. The fluorescently labeled PCR products were separated on a 6%

polyacrylamide gel prepared in 1 × TBE buffer using the Sequi Gen GT gel apparatus (Bio-Rad, USA). PCR products were visualized using the Molecular Imager FX (Bio-Rad).

Quantitative rt-PCR

Quantitative rt-PCR (qPCR) was done to specifically compare amounts of mRNA for VTG and actin in cells that had been subjected to the fluorescent cell viability assays with those that had not. The PCR was performed in an iCycler (Bio-Rad, Germany) with the chemicals of the Taq PCR Core Kit from Qiagen using the following reagents and final concentrations in a total volume of 50 µl: 10 nM fluorescein isothiocyanate (FITC, Bio-Rad, Germany, used to adjust the background fluorescence), the fluorescent dye SYBR green I (Bio-Rad, USA, used at 1×), 5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM primers, and 1U Taq polymerase. Primers were as follows: β-actin (GenBank Accession No. AF157514): 5′-CCAC CGTAAATGCTTCTAAACAG-3′, 5′-AATCTTTAA TCCGCTGCTTCAC-3′; and VTG (GenBank Accession No. M27651): 5′-AACCAAGTCAGCCAGGTAA TATG-3′, 5′-AGAACGACAACTGGAAACTGTGT-3′. The following PCR conditions were used: 3 min at 95 °C and 45 cycles of 20 s at 55 °C, 20 s at 72 °C, 20 s at 95 °C, and 30 s at 60 °C. SYBR green fluorescence was determined during the elongation phase. A melting curve was generated to evaluate the quality of the PCR product. For calculating the threshold cycles (CTs), the threshold position was fixed at 40,000 relative fluorescence units (RFU). Samples with CTs greater than 30 were considered below the level of detection and not included in data analysis. All samples were measured in triplicate ($n = 3$). Results of the qPCR were calculated relative to the housekeeping gene β-actin according to the normalization procedure of the Q-Gene Core Module [35]. This normalization procedure takes into account varying PCR amplification efficiencies (β-actin: 96%; VTG: 88%). In brief, CTs for triplicate determinations for the target and the reference gene (CT_{tar} and CT_{ref}, respectively) were averaged. Next the PCR amplification efficiency values for each gene were taken to the power of their respective mean CT_{tar} and CT_{ref}. Based on these values, the ratio of the target gene versus the housekeeping gene was calculated to yield the mean normalized gene expression. Thus, the mean normalized gene expression is representative of the relative expression level, or mRNA abundance, of the target gene compared with the housekeeping gene. In addition, the standard error of the mean normalized gene expression was calculated based on the differential equation of Gauss [35].

VTG ELISA

VTG protein in the culture medium of hepatocytes exposed to E_2 was determined by ELISA (Biosense, Norway) according to the instructions provided by the manufacturer.

Statistics

Statistical analysis began with a check for equal variances using the Bartlett test. If variances were found to be unequal, data were log-transformed. A one-way analysis of variance (ANOVA) was pursued to detect significant variances between treatments, followed by a Dunnett test to determine whether the treatment caused a significant change in fluorescent unit readings compared with the appropriate control. Significance was accepted at $P < 0.05$. These analyses were performed using GraphPad software (version 4.0, USA).

Results

A combination of two fluorescent indicator dyes, focusing on cellular energy metabolism and cell membrane integrity, was established as a versatile means of quantifying cell viability in primary rainbow trout hepatocyte cultures. In what follows, the usefulness of the cell viability assay is presented according to the different scenarios of application explored in the course of this work.

Cell viability in different culture media

Two media commonly used for culturing primary rainbow trout hepatocytes, M199 and L-15, were studied with regard to their ability to support cell viability over up to 5 days. Cell viability was judged based on microscopic observations and quantitative evaluation using the fluorescent indicator dyes. Both media were found to support the formation of aggregates within 24 h of plating, although the morphology of the aggregates differed somewhat. Cells could no longer be differentiated in M199 medium, whereas they remained more distinct in L-15, an observation that was made throughout the remaining hepatocyte culture period.

The 24-h time point was also used as the reference point for quantitative cell viability assessment. In five independent hepatocyte isolations stemming from four female fish and one male fish, both Alamar blue and CFDA-AM yielded fluorescent unit readings that were 10- to 20-fold above the background values without cells. In general, the fluorescent unit readings for each dye were similar in the two media, and variation among replicate culture wells was less than 10%. Past the 24-h time point, aggregation continued until the termination of culture at 96 h, implying that the cells stayed viable. This was also confirmed by the cell viability assays, although some general differences were noticeable between the two media and the two fluorescent indicator dyes. With regard to the media, the cell viability assays

suggested L-15 as a medium that supported hepatocyte viability more stably in independent culture preparations. This was indicated by a lower standard deviation between hepatocyte preparations, particularly for the longer exposure periods. Therefore, the L-15 medium was used in all subsequent experiments. In terms of the indicator dyes, Alamar blue appeared to respond more sensitively to changes in cellular function, as judged by the greater range of fluorescent unit readings observed for this dye in independent hepatocyte preparations cultured in M199.

In a separate experiment, hepatocytes were plated in L-15 in non-Primaria plates coated with Matrigel. In this culture environment, cells initially attached but dislodged from the surface after 24 h and did not form aggregates. Indeed, whereas the cell viability assays yielded fluorescent units that were approximately 10-fold above background for both Alamar blue and CFDA-AM at the 24-h time point, fluorescent units for Alamar blue dropped to background levels 24 h later (48 h of culture). For CFDA-AM, the drop in fluorescent units was approximately 40% at the 48-h time point. These results confirmed the ability of the fluorescent indicator dyes to distinguish intact hepatocytes from impaired ones and the greater sensitivity of Alamar blue in detecting changes to cellular function.

Cell viability on exposure to PCP

To explore the ability of Alamar blue and CFDA-AM to identify a decline in cell viability due to a toxic insult, hepatocytes of three fish were incubated for 24 h up to 25×10^{-6} M of PCP (Fig. 1). Alamar blue indicated a significant impairment of cellular metabolic activity at 12.5 and 25×10^{-6} M PCP with an EC_{50} value of 16×10^{-6} M PCP (95% confidence interval from 14.5 to 17.5×10^{-6} M PCP). In contrast, CFDA-AM revealed only a slight impact on the cells and EC_{50} values could not be calculated. Exposure of subsequent hepatocyte cultures to a concentration of 10^{-4} M PCP confirmed the differences in Alamar blue and CFDA-AM readings. In these experiments, fluorescent unit readings were, compared with the control, $7 \pm 0.3\%$ for Alamar blue and $62 \pm 17\%$ for CFDA-AM (average of four independent hepatocyte preparations). Finally, when the two fluorescent indicator dyes were applied to PCP-exposed cells of the rainbow trout liver cell line RTL-W1 instead of the primary hepatocytes, both dyes indicated cellular damage to the same extent. For the cell line, EC_{50} values were $19 \pm 3 \times 10^{-6}$ M PCP and $22 \pm 5 \times 10^{-6}$ M PCP (average of five independent experiments) for Alamar blue and CFDA-AM, respectively, and thus were in the range of those observed for Alamar blue in the primary rainbow trout hepatocytes.

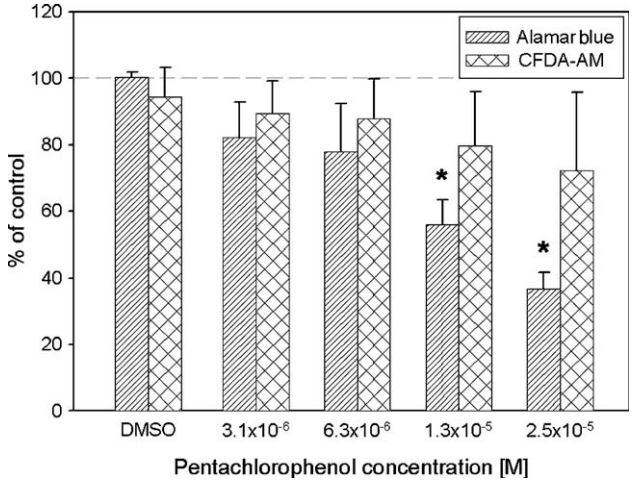

Fig. 1. Effect of PCP on cell viability in primary hepatocyte cultures of three female rainbow trout. Data are given as means and standard deviations of independent isolations in percentages relative to the controls for the fluorescent dyes Alamar blue and CFDA-AM. Asterisks indicate significant decreases in cell viability compared with the controls (one-way ANOVA followed by Dunnett's test).

In addition to the quantification of cell viability using the fluorescent indicator dyes, cells were observed microscopically. In general, PCP led to distinct cell lifting, but concentration-dependent morphological differences were not visible by eye.

Cell viability on exposure to E_2

Exposure of hepatocytes to E_2 for up to 78 h led to little or no change in fluorescent unit readings, compared with the control, for Alamar blue and CFDA-AM for up to 10^{-5} M E_2, but a notable change in viability was observed beyond 10^{-5} M E_2 (Fig. 2).

The viability of cells on exposure to concentrations of E_2 up to 10^{-6} M was confirmed by the secretion of VTG protein into the culture medium as an indicator of hepatocyte differentiated function. Maximal levels of induction were in the range of 1500 ng/ml (up to ~20-fold above background). They were found between 30 and 78 h of exposure and at 10^{-7} M E_2 for female rainbow trout and 10^{-8}–10^{-7} M E_2 for male rainbow trout.

Potential impact of cell viability assays on gene expression (mRNA abundance)

Because of its importance as a marker of hepatocyte differentiation and its role in endocrine function, VTG was subsequently used as a specific target gene for exploring the suitability of carrying out the Alamar blue/CFDA-AM cell viability test just prior to the analysis of gene expression using quantitative real-time rt-PCR. Investigations were further supported by a nontargeted, random gene expression analysis approach, namely dd-rt-PCR. For these studies, parallel sets of cells were treated as desired before they either were directly processed for PCR analysis or underwent a prior 30-min Alamar blue/CFDA-AM exposure.

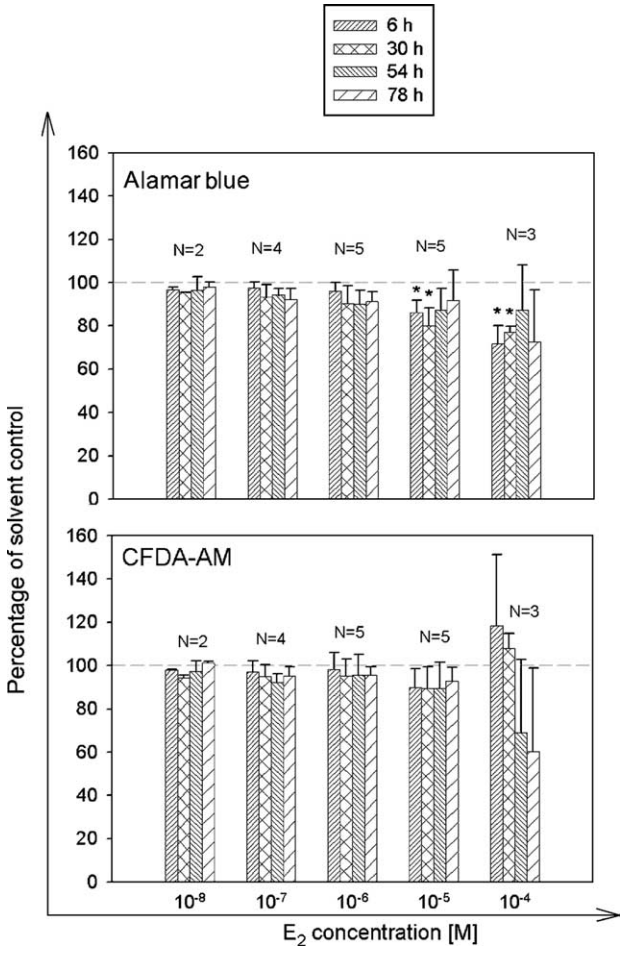

Fig. 2. Cell viability of hepatocytes from female trout exposed after 18 h of attachment for up to 78 h to E_2 in L-15 medium. Numbers of independent hepatocyte preparations (*N*) are indicated in the diagram. The reference line represents 100% cell viability measured in the solvent control. Data are given as means and standard deviations of independent isolations in percentages relative to the solvent control. One-way ANOVA yielded no significant differences for the CFDA-AM measurements. Asterisks in the Alamar blue graph indicate a significant decrease in cell viability compared with the control (one-way ANOVA followed by Dunnett's test).

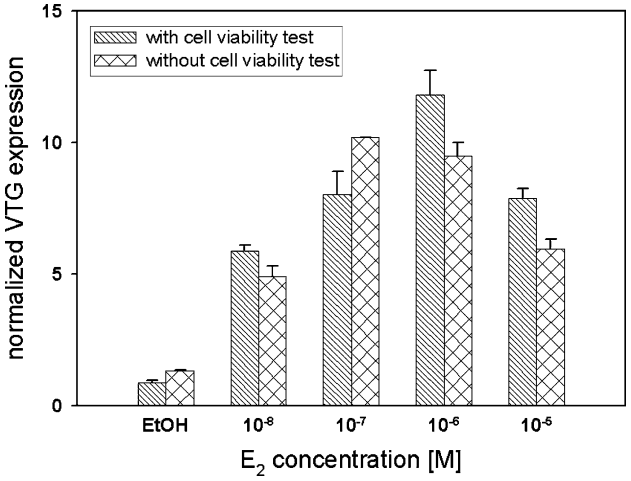

Fig. 3. Comparison of VTG mRNA abundance on exposure of primary hepatocytes from a female rainbow trout for 78 h to various levels of E_2 with or without a 30-min Alamar blue/CFDA-AM exposure. mRNA abundance was derived for the target gene (VTG) and the β-actin gene (the control gene) using the Q-Gene Core Module [35] and taking into account the PCR amplification efficiencies for each gene. mRNA abundance of the target gene was normalized based on β-actin. Thus, the normalized gene expression is representative of the relative expression level, or mRNA abundance, of the target gene (VTG) compared with the housekeeping gene (β-actin). Each bar displays the mean and standard error of triplicates in one representative qPCR. Detectable VTG mRNA levels in the control indicate a beginning vitellogenesis in this female.

Targeted gene expression analysis

Hepatocytes exposed from 10^{-8} to 10^{-5} M E_2 for 78 h and then either treated with Alamar blue/CFDA-AM or not treated yielded comparable VTG mRNA abundance. One example of this is shown in Fig. 3. Differences generally were less than 30%, and no pattern of under- or overestimation for Alamar blue/CFDA-AM-treated or -untreated cells was observed.

Random gene expression analysis

dd-rt-PCR using different combinations of fluorescently labeled primers and several exposure conditions revealed no apparent differences in the expression patterns of cells that either were or were not exposed to Alamar blue/CFDA-AM for 30 min prior to RNA extraction (Fig. 4). Thus, the random differential display analysis approach confirmed that Alamar blue/CDFA-AM did not affect the pattern of gene expression as had been observed for the target gene analysis.

Discussion

A combined Alamar blue/CFDA-AM assay was adopted as a simple means to noninvasively monitor cell viability of primary hepatocytes from rainbow trout with or without subsequent analysis of gene expression. Alamar blue is a commercial preparation of the dye resazurin [36]. On diffusion into living cells, it is reduced to resorufin, which is water soluble and fluorescent, by reductases localized in mitochondrial membranes as well as in the cytosol [37]. Alamar blue is increasingly being used in pharmacology for repeated measurement and high-throughput screening with mammalian cell lines [38,39]. In addition, it has recently been validated for primary rat hepatocytes [39,40] but not yet for primary hepatocytes of fish. Along the lines of Alamar blue, CFDA-AM diffuses into cells rapidly but is converted by nonspecific esterases of living cells from a nonpolar nonfluorescent dye into a polar fluorescent dye, 5-carboxyfluorescein (CF), which diffuses out of cells slowly. Esterase substrates have been used as a measure of cell membrane integrity since the 1960s [41], but CFDA-AM

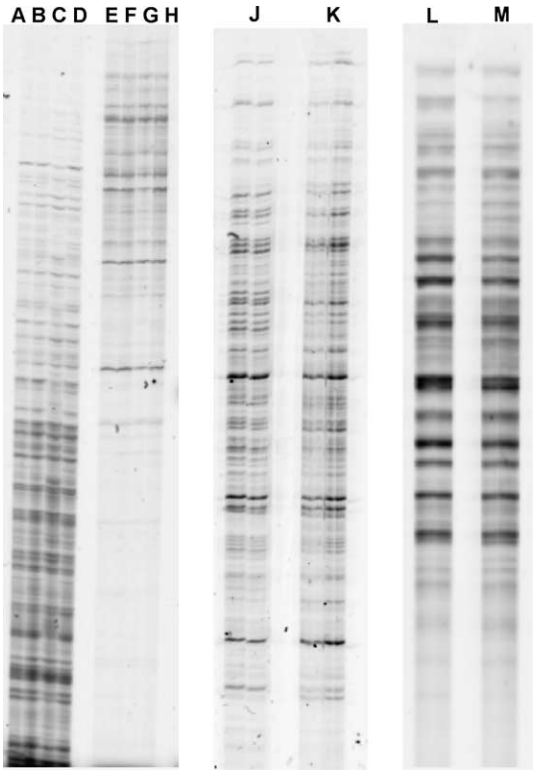

Fig. 4. Comparison of gene expression patterns in RTL-W1 cells (lanes A–H) and primary rainbow trout hepatocytes from a female fish (lanes J–M) with or without a 30-min Alamar blue/CFDA-AM exposure just prior to processing for dd-rt-PCR. PCR products were separated on a polyacrylamide gel and visualized using the Bio-Rad molecular imager. Representative portions of the gels, showing the detectable band patterns, were selected for respective comparisons. PCR was carried out with fluorescently labeled primers as follows. Lanes A–D (blank cells): primers A2 and A4, with A and B representing RTL-W1 cells without Alamar blue/CFDA-AM exposure and C and D representing RTL-W1 with Alamar blue/CFDA-AM exposure; lanes E–H (blank cells): primer A3, with E and F representing RTL-W1 cells without Alamar blue/CFDA-AM exposure and G and H representing RTL-W1 with Alamar blue/CFDA-AM exposure; lanes J and K (EtOH-exposed 0.5%): primer A3, with J representing primary hepatocytes with Alamar blue/CFDA-AM exposure and K representing primary hepatocytes without Alamar blue/CFDA-AM exposure; lanes L and M (10^{-6} M E_2): primer A3, with L representing primary hepatocytes with Alamar blue/CFDA-AM exposure and M representing primary hepatocytes without Alamar blue/CFDA-AM exposure.

is an example of one development to improve this application [42]. In the study presented here, Alamar blue and CFDA-AM were applied for the first time in primary fish hepatocytes. Both dyes elicited by themselves no impact on the cells and thus allow multiple analyses of the same set of cells over the course of an experiment.

Based on the two fluorescent indicator dyes, L-15 cell culture medium was superior to M199 in supporting less variable in vitro cell viability of the rainbow trout hepatocytes. This is reminiscent of Mommsen and coworkers [28], who noted most consistent results in fish hepatocyte cultures by using L-15. Both M199 (e.g. [5,43]) and L-15 (e.g. [18,44,45]) have frequently been used in primary hepatocyte cultures from rainbow trout. M199 contains more vitamins and a higher number of amino acids but in a lower concentration than L-15. The latter is commonly used for maintaining fish cell lines (e.g. [32]). Aside from the greater consistency of cell viability in L-15, slight differences in the morphology, but not in the speed of formation, of monolayers of rainbow trout hepatocyte in L-15 and M199 media were seen. Recently, Kim and Takemura [46] reported a more rapid monolayer formation in the beginning of culture of tilapia hepatocytes in M199 and in Williams' medium E compared with L-15. However, VTG synthesis as an indicator of hepatocyte differentiated function was highest with L-15 in the tilapia cells. This again is in support of the better sustainability of fish hepatocyte cultures in L-15.

Alamar blue and CFDA-AM were capable of distinguishing the presence or absence of an insult on rainbow trout hepatocyte viability on exposure to potentially toxic compounds. For example, E_2 did not cause a considerable decline in cell viability up to 10^{-5} M, as indicated by both Alamar blue and CFDA-AM. A decline in fluorescent unit readings, compared with the control, was observed for both dyes on culture of hepatocytes in non-Primaria plates and on exposure to PCP. However, the Alamar blue assay reacted more sensitively than CFDA-AM in both applications. This difference could point, at least in part, to a methodological shortcoming of the assay as applied to the hepatocytes. To reduce cell loss, only half of the medium had been removed prior to the addition of the fluorescent dyes. It is conceivable that esterases leaking out of damaged cells were still present in the portion of the medium not exchanged prior to dye application. Thus, these enzymes may have transformed CFDA-AM to its fluorescent form, CF, thereby leading to an overprediction of hepatocyte cell viability. A similar hypothesis was put forward by Dayeh and coworkers [47], who found elevated CF fluorescent unit readings in Triton X-exposed *Tetrahymena thermophila* if the exposure medium was not completely removed prior to CFDA-AM application. Support for this hypothesis also comes from the finding that a difference in fluorescent unit readings was not observed for Alamar blue/CFDA-AM on PCP exposure of RTL-W1 cell cultures. In these cultures, the exposure medium was completely removed prior to dye application. Thus, future investigations should explore whether a more complete medium removal prior to the addition of fluorescent indicator dye is feasible for the hepatocytes and whether this methodological change would remedy the differences in sensitivity observed for PCP.

Despite the possibility of the dissimilarity in Alamar blue/CFDA-AM fluorescence being due to the method applied, other causes accounting for the differences must be considered as well. Dayeh and coworkers [24] found that Alamar blue reacted much more sensitively than

CFDA-AM when a gill cell line of rainbow trout, RTgill-W1, was exposed to PCP for 24 h. Early in vitro studies have shown that PCP can uncouple oxidative phosphorylation, inactivate respiratory enzymes, and cause mitochondrial damage [48]. In metabolic studies using mammalian liver homogenates, PCP was found to undergo oxidative dechlorination to yield tetrachlorohydroquinone [49,50]. A quinone metabolite of benzo[*a*]pyrene led to a metabolic impairment in fish liver cells, as indicated by a much greater decline in fluorescent unit readings for Alamar blue than for CFDA-AM. A direct inhibition of reductases in the respiratory chain or the futile redox cycling caused by the quinone, accompanied by the formation of reactive oxygen species, was discussed as the cause of the apparent decline in cellular metabolism as assessed with Alamar blue [51]. Similar mechanisms may have contributed to the differences in Alamar blue and CFDA-AM fluorescence in the study presented here. Thus, the application of the two dyes together has the potential of revealing the mechanism(s) behind a change in fluorescent unit readings beyond measuring cell viability. As the Alamar blue/CFDA-AM assay is applied more widely to different cellular models under different exposure conditions, more possible scenarios for cellular responses to toxicants will be identified and help in the interpretation of the specific functions monitored by the dyes [25].

Application of the two fluorescent indicator dyes did not affect gene expression patterns in the hepatocytes. This was confirmed by monitoring VTG/β-actin mRNA abundance on E_2 exposure in primary hepatocytes specifically and by observing differential gene expression patterns in RTL-W1 and primary hepatocytes in control or E_2-treated cells using random primers generally. The two dyes were applied to the cells for 30 min, a time frame sufficient for significant mRNA synthesis if transcription were initiated. Thus, the results presented here indicate that the two indicator dyes do not regulate gene transcription directly and thus are valuable for assessing cell viability just prior to gene expression due to a toxic insult in the primary rainbow trout hepatocytes. Nevertheless, despite the use of the random gene expression monitoring approach, in addition to analyzing specific target genes, not all possible gene regulations can be accounted for with the number of random primers applied. In the future, whole genome array analysis, comparing cells with or without prior Alamar blue/CFDA-AM application, would be the method of choice to fully confirm the lack of impact on gene expression by the fluorescent indicator dyes as demonstrated here.

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