Profiling the Impact of Medium Formulation on Morphology and Functionality of Primary Hepatocytes in vitro

Leonard J. Nelson1, Philipp Treskes1, A. Forbes Howie2, Simon W. Walker2, Peter C. Hayes1 & John N. Plevris1

1Hepatology Laboratory, University of Edinburgh, Chancellor’s Building, Royal Infirmary of Edinburgh, EH16 4SB, Scotland, UK, 2Dept of Clinical Biochemistry, University of Edinburgh, Chancellor’s Building, Royal Infirmary of Edinburgh, EH16 4SB, Scotland, UK.

The characterization of fully-defined in vitro hepatic culture systems requires testing of functional and morphological variables to obtain the optimal trophic support, particularly for cell therapeutics including bioartificial liver systems (BALs). Using serum-free fully-defined culture medium formulations, we measured synthetic, detoxification and metabolic variables of primary porcine hepatocytes (PPHs) - integrated these datasets using a defined scoring system and correlated this hepatocyte biological activity index (HBAI) with morphological parameters. Hepatic-specific functions exceeded those of both primary human hepatocytes (PHHs) and HepaRG cells, whilst retaining biotransformation potential and in vivo-like ultrastructural morphology, suggesting PPHs as a potential surrogate for PHHs in various biotech applications. The HBAI permits assessment of global functional capacity allowing the rational choice of optimal trophic support for a defined operational task (including BALs, hepatocellular transplantation, and cytochrome P450 (CYP450) drug metabolism studies), mitigates risk associated with sub-optimal culture systems, and reduces time and cost of research and therapeutic applications.

There has been a resurgence of interest in the use of PPHs for biotechnology applications including cellular xeno-transplantation and BAL therapies1–3. Recent insights also support the use of PPHs as authentic surrogates for PHHs, given their similar phenotype and function4,5. Availability of PPHs for cell-based approaches to treat acute liver failure (ALF) is severely restricted due to a shortage of donor organs. As such, harnessing the potential of PPHs could herald a significant stimulus to the biotechnology sector7. In fact, BAL devices representing the most sophisticated approaches for treating ALF in a clinical setting, mostly utilize pig hepatocytes as their functional component4, whilst intracorporeal liver replacement using PPHs targeting diseased liver foci, is feasible through 3D scaffold-based tissue engineering strategies5,9–11. Critically, in all systems up to date, no transmission of porcine endogenous retroviruses was detected12.

For such diverse applications, maintenance of liver-specific functions will require appropriate microenvironmental and hepatotrophic support.

PPHs offer many advantages for BAL systems and hepatocellular transplantation (HTx) strategies for treatment of human ALF in terms of sufficient yield, viability and biocompatibility13. Indeed the pig is the most suitable non-primate species available to provide the estimated 20% (of whole liver) functional liver cell mass required for support; equivalent to approximately 20 billion hepatocytes14,15. PPHs exhibit differentiated function in culture and have a higher intrinsic metabolic activity compared to other mammalian hepatocytes16–18. Furthermore, several studies have shown that pig hepatocytes are similar to human in form, phenotype and function including biotransformation potential and hepatic phase I drug metabolism via CYP450 substrates4,5.

The primary requirement of any therapeutic application of hepatocytes is the preservation of in vivo-like metabolic functions. Replacement of impaired liver functions on a temporary basis may sustain life by preventing and/or decreasing the progress of hepatic encephalopathy and/or create conditions for native liver regeneration of functional tissue and full recovery19,20. Whilst the precise metabolic functions required of hepatocytes to treat ALF
are unknown, it is likely that a full repertoire of in vivo-like differentiated functions including synthetic, detoxifying and metabolic capabilities are prerequisites of xenogeneic hepatocytes in order to support the failing liver.

Deterioration of both liver-specific metabolic activities and hepatocyte morphology occurs within days of culture\(^9\). Since hepatocyte shape and structure are considered to be intimately related to functional activity\(^{12-25}\), various strategies have been adopted to address this problem, such as the use of complex biomatrices, microcarriers or synthetic hollow-fibre biomembranes to improve attachment and function of cells\(^5\). An important consideration in this regard is the optimization of the PPH culture system given that the use of xeno-based serum-supplements or biomatrices (e.g. mouse sarcoma-derived Matrigel) to retain hepatic-specific functions may be unnecessary or even detrimental to cells and therapeutic efficacy of BAL devices. Indeed, non-autologous (bovine) serum is heterogeneous in composition, promotes rapid cellular dedifferentiation and selective growth of non-parenchymal cells and may contain zoonotic endotoxins\(^{20,27}\).

Interestingly, it was previously shown that PPHs cultured on tissue culture plastic perform equally well compared with cells grown on a variety of exogenous attachment substrates\(^{20,28}\) or under defined, serum-free conditions on unmodified tissue culture plastic\(^4\).

Systematic investigations of the optimal medium requirements for PPH culture in BAL devices are few, perhaps reflecting the absence of standardization and diversity of culture conditions used by different laboratories, as well as extrapolation of data from rat hepatocyte studies to evaluating PPH culture systems. Previous studies have shown that the type of medium formulation modulates morphology and function of rat and sheep hepatocyte cultures\(^{29,30}\), as well as porcine hepatocytes on biomatrix-coated culture dishes with or without serum\(^1\).

Surprisingly, given the importance, and interdependency of cellular form, phenotype and function, none of these studies addressed detailed morphological parameters in parallel, to assess culture stability.

To permit a more rigorous assessment of the effects of different media formulations on differentiated biochemical functionality and morphology, PPHs were cultured on biomatrix-free (unmodified) tissue culture plastic in four separate serum-free chemically-defined media formulations: William’s E (WE) Medium\(^{32,33}\), Medium 199 (M199)\(^9\) and Medium 1640 (M1640)\(^9\) are commonly used in BAL culture systems; whereas L-15 (Leibowitz) medium maintains in vitro differentiated function in a variety of mammalian species\(^9\) and has been used in its modified formulation (ML-15)\(^9\). Identifying the optimal medium which preserves hepatic metabolic support function and morphology would be advantageous for BAL devices. This culture system also eliminates the confounding variables introduced when using serum and/or biomatrix components.

We investigated hepatic-specific synthetic, detoxifying and metabolic capabilities of PPH to show their clinically-relevant potential to fulfill the hepatic replacement function required of BAL systems, including: (i) Albumin production as an energy-requiring biosynthetic and secretory function, (ii) Urea production from ammonium chloride as a measure of detoxifying capacity, (iii) Galactose elimination to reflect the integrity of carbohydrate metabolism, and (iv) Total cytochrome P450 (TCTP450) content as an indicator of bio- transformation potential. Gross and ultrastructural features of PPHs were assessed in parallel studies.

In order to quantify global functional capacity, a scoring system (hepatocyte biological activity index; HBAI) was devised, to simplify multiple comparisons, and as an integrated measure of overall functional capability. The biochemical data reported here were used in the generation of the HBAI, whereas parallel morphological observations were used as further validation of the comparative scores of the HBAI.

### Results

**Cell isolation.** Cell viability of primary isolates was assessed by trypan blue exclusion test after isolation as 87 ± 5% with a yield of 2.2 ± 0.8 × 10\(^{10}\) viable cells from 12 ± 1 kg piglets\(^9\). The non-parenchymal fraction was 5%; as judged by the size (<10 \(\mu\)m in diameter) and morphology (non-polygonal or stellate). For details on viability and plating efficiency after primary isolation, see supplementary information. If not stated otherwise, experiments were performed \(n = 10\) times.

**Urea synthesis rates.** Between-media comparisons of USR (Urea synthesis rates) were made on each day of culture (\(n = 9\), Fig. 1). PPHs cultured in M1640 medium on day 2 of culture showed significantly higher (\(p < 0.05\)) conversion of ammonium chloride to urea (511 ± 141 nmol/h/mg protein) compared to both WE medium and M199 (292 ± 62 nmol/h/mg protein; \(p = 0.044\) and 201 ± 50 nmol/h/mg protein; \(p = 0.015\), respectively). By day 4 of culture in M1640, the USR had further and significantly increased (608 ± 102 nmol/h/mg protein) compared to WE (335 ± 79 nmol/h/mg protein; \(p = 0.01\), M199 (248 ± 31 nmol/h/mg protein) whereas a moderate increase was observed relative to ML-15 (428 ± 38 nmol/h/mg protein; \(p = 0.078\)). M1640 maintained a significantly higher USR capacity (847 ± 249 nmol/h/mg protein) over ML-15 on day 6 (359 ± 77 nmol/h/mg protein; \(p = 0.034\) and a greater conversion rate than hepatocytes cultured in WE (448 ± 90 nmol/h/mg protein; \(p = 0.074\)) or M199 (360 ± 125 nmol/h/mg protein; \(p = 0.055\)).

**Percentage galactose elimination.** For each day of culture, between-media comparisons of galactose elimination (GE) were made (\(n = 5\), Fig. 2). Percentage GE for WE, ML-15, M199 and M1640 on culture day 2 were 29 ± 4, 17 ± 2, 29 ± 3 and 22 ± 6%, respectively. Galactose metabolism was significantly lower in ML-15 compared to both WE and M199 (\(p = 0.043\)). By day 4, galactose was eliminated more slowly from M1640 (19 ± 3) than from WE (29 ± 4) or M199 (30 ± 4; \(p = 0.04\)), only reaching significance in the latter culture. Day 6 cells maintained the ability to metabolise galactose as efficiently as earlier hepatocyte cultures.
Hepatocytes Cultured in Four Separate Chemically-Defined Media Formulations. Percentage galactose elimination of primary porcine hepatocytes cultured at 10^7 viable cells per 100 mm dish in WE, ML-15, M199 and M1640 chemically-defined media for up to 6 days. 1 mM D(+)-galactose was added to each culture medium and incubated for 2 hours on days 2, 4 and 6. GE was determined as above. Results are expressed as % decrease of initial [galactose]. All values represent the means ± SEM of five experiments in duplicate. Statistics: Day 2: WE vs ML-15, p = 0.043; ML-15 vs M199, p = 0.071. Day 4: M199 vs M1640, p = 0.040.

**Total cytochrome P450 content.** Between-media evaluations of tCYP450 (Total cytochrome P450 content) were made on each day of culture (n = 3, Fig. 3). Significant differences between cells cultured in each test medium were not observed until culture days 4 and 6. tCYP450 content of ML-15 grown hepatocytes on day 4 (36 ± 3 pmol/mg protein) was significantly higher than both WE medium (24 ± 3 pmol/mg protein; p = 0.043) or M1640 medium (25 ± 5 pmol/mg protein). tCYP450 content was significantly lower in WE medium (17 ± 1 pmol/mg protein) compared to M199 (28 ± 1 pmol/mg protein; p = 0.026) and M1640 (27 ± 5 pmol/mg protein; p = 0.033); and the mean value was lower, although not statistically significant, than ML-15 cultured cells (25 ± 1 pmol/mg protein; p = 0.071).

There was a highly significant decrease in tCYP450 content, from 120 ± 37 pmol/mg protein at day 0 to ~40 ± 10 pmol/mg protein by day 2 of culture in all media formulations (p < 0.001). tCYP450 content remained quite stable (~30 ± 3 pmol/mg protein) up to day 4, but had declined significantly, when comparing day 2 values (~40 ± 10 pmol/mg protein) with day 6 values in WE (17 ± 1 pmol/mg protein; p = 0.047) and ML-15 (25 ± 1 pmol/mg protein; p = 0.021) media formulations.

**Albumin synthesis rates.** For each medium formulation, between day comparisons were analysed (n = 7, Fig. 4). Albumin synthesis rate (ASR) by PPHs cultures remained stable up to day 4 (~1.0–1.4 µg/h/mg protein) but increased significantly thereafter up to day 6 of cell culture in M1640 medium (2.5 ± 0.5 µg/h/mg protein; p = 0.024); with substantial increases to 2.3 ± 0.5 µg/h/mg protein observed in M199 (p = 0.058) versus 1.7 ± 0.4 µg/h/mg protein in WE medium during the same culture period. ASR could not be measured in ML-15 due to very high background levels in this proprietary medium formulation.

**Morphology of cultured hepatocytes.** The effects of medium formulation on gross cellular morphology and overall culture integrity of day 1 cultures as seen under phase contrast light microscopy are shown in Fig. 5. Plating efficiencies (see supplementary information for details) reflected the morphological observations in terms of the degree of confluency observed for each test medium and were not significantly different between media. Hepatocytes maintained in ML-15 (Fig. 5 B) and to a lesser extent, in M1640 (Fig. 5 D) display the formation of discrete colonies of piled up cells (denoted by asterisks in Fig. 5) as evidenced by the difficulty in obtaining an even plane of focus throughout the field. Monolayer formation is more evident in M1640. In contrast, cells grown in both M199 (Fig. 5 C) and WE (Fig. 5 A) media, establish more classical features of monolayer cultures although with some loosely attached cells still present.

Hepatocytes maintained for 2 days in both WE (Fig. 5 E) and M199 (Fig. 5 G) show characteristic morphology of cell culture monolayers. Hepatocyte cultures reached a completely confluent monolayer of densely packed cells, exhibiting a compact polygonal cell shape delimited by sharply-defined refractile borders containing bright nuclei under phase contrast. The lucent rim surrounding each hepatocyte represents the presence of bile canaliculi. Contrastingly, discrete cell colonies were conspicuous in ML-15- (Fig. 5 B) and
M1640-grown (Fig. 5 H) day 2 cultures, which persisted up to day 4 in ML-15 maintained cultures (not shown). In both media, monolayers of hepatocytes were more evident than on day 1, although with areas devoid of cells. In addition, hepatocyte monolayers cultured in ML-15 and, to a lesser extent in M1640, were generally more spread out (Fig. 5 F) on the tissue culture plastic dish compared to WE and M199 cultures often showing cellular processes including pseudopodia (Fig. 5 F, insert). These morphological characteristics were evident up to day 4 of culture (not shown).

All cultures maintained in each test medium showed distinct but varying signs of degeneration by day 6 (Fig. 5 I–L) including cell detachment, general loss or ‘blurring’ of delineating cell borders, nuclear fragmentation and the appearance of necrotic and apoptotic cells (denoted by black arrows in Fig. 5). A striking feature of WE medium was the appearance in culture of discrete foci of small cell aggregates (denoted by F* in Fig. 5 I) with a radiating cord-like arrangement of hepatocytes reminiscent of the in vivo cell microenvironment. However, although cell detachment was clearly evident, hepatocyte morphology was well preserved and similar to day 4 cultures. A distinct feature of ML-15 cultures was the presence of small rounded structures, possibly lipid droplets or small vacuoles (denoted by white arrows in Fig. 5 J).

Observing ultra-thin sections of cells cultured for either 2 or 6 days via transmission electron microscopy (TEM) showed that all day 2 cells had a non-polyhedral, rounded appearance probably as a result of the trypsinization process. Only WE (Fig. 6 B + G) and M199 (Fig. 6 D + I) cultured hepatocytes showed many of the classical ultrastructural features of fresh liver tissue (Fig. 6 A + F + K). In fact, some WE grown hepatocytes, uniquely, showed bile canaliculi-like structures (Fig. 6 B + G) (confirmed using fluorescein diacetate (FDA) and nuclear (Nu) and well-defined plasma membrane (Pm), membranes and lysosomes (L), abundant mitochondria (Mt) and intact mitochondrial cristae (Mtc) and granular endoplasmic reticulum (ger) interspersed between the mitochondria. Smooth endoplasmic reticulum and golgi apparatus (Ga), peroxisomes (P) are evident, as well as numerous dense glycogen (G) β-particles and rosettes (α-particles) between smooth endoplasmic reticulum and in close proximity to mitochondria. Bile canaliculus-like structures (Bc) are formed between adjacent hepatocytes in both fresh liver tissue and in isolated hepatocytes. Pictured hepatocytes correspond to phase contrast micrographs of day 2 and 6 displayed in Figure 5. The photomicrographs show areas of high morphological representation and were taken at the following magnifications appropriate for visualization: ×3800 (M, N), ×5000 (B, E, L), ×6500 (C, D), ×10000 (O), ×28000 (I, J) and ×75000 (G, H). Based on TEM pictures, cultures were subsequently assessed for the relative frequencies of cytoplasmic organelles and bile canaliculus-like structures on days 2 and 6 of culture for each test medium (as summarised in Table 1). Depending on the culture medium tested, a proportional reduction of intact cytoplasmic structures was observed in parallel with the occurrence of asymmetric mitochondrial swelling (aMt), the formation of lipid droplets (Ld) and extensive vacuolation (V).
The aim of this study was to assess in vitro retention and differentiated function in vitro for BALs, and to identify the optimal medium formulation required for these modalities. A previous study reported that despite widespread use of culture media for cell therapy, pharmacological parameters of high-density hepatocytes cultured in fully defined WE, Modified L-15, M199 and RPMI 1640 media formulations; integrate these datasets using the HBAI to assess global functional capacity, with parallel morphological parameters to elucidate hepatotrophic support suitability.

The rationale for examining hepatocyte morphology and functional activity over a 6 day culture period follows from the fact that most porcine-based BALs are designed to be operational within 2–4 days of seeding the bioreactor using either or both a biomatrix component and serum-supplemented medium for cell support. The majority of BALs utilise hollow fibres where porcine hepatocytes are inoculated onto various substrates which introduces unknown variables. Moreover, although hepatocyte morphology is considered intimately related to function, there are few comparative studies which evaluate and correlate both parameters.

We have demonstrated that primary porcine hepatocytes can be cultured with high plating efficiency on 2D biomatrix-free tissue culture plastic in separate serum-free, chemically-defined media formulations whilst maintaining high viability and retention of significant hepatic function over six days. Assessment of overall performance of hepatocyte cultures in each test medium during this period showed that medium 199 gave the highest mean values of plating efficiency, viability, albumin synthesis rate (ASR) and percentage galactose elimination (GE). Both M199 and M1640 show differential effects on urea synthesis capacity, the latter significantly greater than all media tested (Fig. 1), and substantially higher than previously published reports. Total CYP450 content remained stable in 2-day-old cultures, despite a 66% decrease compared with fresh cells although ML-15 maintained day 2 CYP450 levels up to culture day 6. Percentage GE was relatively stable whereas ASR tended to increase in all media up to day 6. Furthermore, parallel morphological studies indicated that different medium formulations modulate gross and ultrastructural morphology of hepatocyte cultures. The results presented here are in concordance with recent assessment of comparable porcine hepatic functionality with a human HepaRG cell line-based BAL system, considered a suitable biological surrogate to PHHSs. Taken together these outcomes may be of relevance when choosing an appropriate medium for porcine-based BAL devices, which require rapid attachment of high viability cells with significant ammonia detoxifying capacity whilst maintaining biotransformation potential and a diverse functional profile.

**Table 1** shows the relative frequencies of cytoplasmic organelles and bile canaliculus-like structures in TEM sections on days 2 and 6 of culture for each test medium. Ten areas from each of 4 copper grids per sample for 3 separate isolations were investigated and the number of organelles (nuclei, mitochondria, granular endoplasmic reticulum (Ger), glycogen granules, and Golgi complex) counted.

**Table 1** | Relative Frequencies of Cytoplasmic Organelles and Bile Canaliculi Examined Under TEM in Primary Porcine Hepatocytes on Days 2 and 6 of Culture In Chemically-Defined Serum-free Medium Formulations

|             | Nuclei | Mitochondria | Ger | Glycogen Granules | Golgi Apparatus | Bile Canaliculi |
|-------------|--------|--------------|-----|-------------------|-----------------|-----------------|
| **DAY 2**   |        |              |     |                   |                 |                 |
| WE          | +++    | +++          | +++ | +++               | +++             | +++             |
| ML-15       | +++    | +            | +   | +                 | +               | +               |
| M199        | +++    | +            | +   | +++               | +++             | +               |
| M1640       | +++    | +            | +   | +                 | +               | +               |
| **DAY 6**   |        |              |     |                   |                 |                 |
| WE          | +      | ++           | +   | +                 | +               | +               |
| ML-15       | +      | +            | +   | +                 | +               | +               |
| M199        | +++    | +            | +   | +                 | +               | +               |
| M1640       | +      | +            | +   | +                 | +               | +               |

Assessment of overall performance of test media. In order to define an index of global functional capacity, a scoring system was devised as an integrated measure of overall functional capability. Scores were generated for each test medium at each time point, by assigning an equally weighted arbitrary score of 0–10 points, for the mean values of each biochemical parameter determined from the data. Table S1 shows how comparative scores for this hepatocyte biological activity index (HBAI) were generated. The midpoint values reflect a range of values reported in the literature. Following from this, Table 2 shows a summary of comparative scores; the rank order of test media accumulated over the 6 day culture period for each parameter and the overall ranking derived from the total score thus: M199 > WE > M1640 > ML-15.

**Discussion**

Despite widespread use of culture media for cell therapeutic, pharmaceutical, and other biotechnology applications, defining the optimal medium formulation required for these modalities has not been systematically approached. The importance of identifying a culture medium which promotes hepatotrophic support, viability and differentiated function in vitro for BALs, is well recognised. The aim of this study was to assess in vitro retention of synthetic, detoxification and metabolic parameters of high-density hepatocytes cultured in fully defined WE, Modified L-15, M199 and RPMI 1640 media formulations; integrate these datasets using the HBAI to assess global functional capacity, with parallel morphological parameters to elucidate hepatotrophic support suitability.

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Table 2 | Hepatocyte Biological Activity Index: Comparative Scores

| Medium | Culture-day | UGR | ASP | CYP450 | TP | LDH | Total Score | Rank-Order of Test Media | Culture-day | Total Score |
|--------|-------------|-----|-----|--------|----|-----|-------------|--------------------------|-------------|-------------|
| ML-15  | 1           | 2   | 4   | 6     | ST | 36  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| M1640  | 1           | 2   | 4   | 6     | ST | 36  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| ML-15  | 2           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| M1640  | 2           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| ML-15  | 3           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| M1640  | 3           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| ML-15  | 4           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| M1640  | 4           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| ML-15  | 5           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |
| M1640  | 5           | 4   | 6   | 3     | 1  | 30  | 31          | M199 > ML-15 > WE > M1640| 1           | 30          |

Hepatic detoxification of ammonia to urea is considered a pre-requisite hepatic support function of BAL devices. Our data show highly efficient urea synthesising capacity, in agreement with previous studies19,20,32,33,37, exceeding those of PPHs grown on a variety of extracellular matrices34, including Matrigel35, considered the ‘gold standard’ attachment substrate promoting differentiated hepatic function. Furthermore, our values for albumin synthesis are 100× greater than those achieved using collagen gel entrapped PPHs cultured under serum-free conditions36. This reinforces the notion that (often undefined) exogenous attachment substrates, notwithstanding bio-incompatibility issues and expense, may be unnecessary for porcine-based BAL devices. Augmented urea synthesis rates may reflect: preferential cell isolation from the periporal region37; urea cycle enzyme stability; and the presence of potent stimulators of ureagenesis (arginine38, alanine, glutamine39) present in supraphysiological but variable concentrations in the test media. Indeed, arginine concentration in M1640 is 3–4 fold greater than the other media tested, and may act in synergy with released lactate39 (suggested by deteriorated gross morphology, Fig. 5) to augmentUSR. Despite the presence of vacuoles and/or lipid deposition one can speculate that M1640-cultured hepatocytes, under stress, increase USR/ammonia removal – which is conceivably a beneficial function in the BAL setting. However, such cells would be contra-indicated for other cell therapeutic applications such as HTx, given that even with isolated cells from marginal human donor livers, low quality of such transplanted cells may be a major cause of equivocal efficacy in human clinical trials40. Indeed, ultrastructural evaluation of a porcine-based HTx strategy to treat ALF mice, showed phenotypic correlation with maintained hepatic architecture and functionality41.

Galactose elimination capacity is used as a quantitative liver function test to study functional hepatic mass42 and remained stable throughout the culture period within a 20–30% range (Fig. 2) in agreement with previous reports32. Noteworthy, is that 30–35% of a galactose loading dose of 5 mmol/L is metabolized in normal individuals42. Our galactose elimination values are 2–7 fold higher (depending on the test media and culture day in our experiments) than the porcine-based BAL of Iwata et al.43.

A major complication of ALF is hepatic encephalopathy due in part to putative toxic compounds released from the injured liver, normally metabolised by the CYP450 system44,45. In agreement with previous observations in swine44,45, our data show that ICYP450 levels decreased but remained relatively stable over 6 days (Fig. 3). This concurred with values from serum-containing cultures of rat, mouse, hamster and human hepatocytes46,47. The labile nature of total P450 may be explained by observed down-regulation of CYP450 mRNA transcripts triggered during the collagenase isolation process48. Due to variations in the abundance and metabolic specificity of CYP450s between species, we utilised a species-independent approach by assessing ‘total CYP450 content’ as a measure of biotransformation potential. Total CYP450 was measured given the lack of specific antibodies/substrates for measurement of porcine CYP450. For the purpose of the HBAI more specific isoenzyme (CYP3A4 or CYP2E1) activity may be substituted for tCYP450 concentration test to study functional hepatic mass42 and remained stable throughout the culture period within a 20–30% range (Fig. 2) in agreement with previous reports32. Noteworthy, is that 30–35% of a galactose loading dose of 5 mmol/L is metabolized in normal individuals42. Our galactose elimination values are 2–7 fold higher (depending on the test media and culture day in our experiments) than the porcine-based BAL of Iwata et al.43.

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selecting a given medium for a specific SCIENTIFIC Hepatocytes were isolated from weanling piglets (with the the UK Home Office Guidelines under the Animal (Scientific Procedures) Hepatocyte isolation and culture phology – distinct nuclei and nucleoli with well-demarcated refrac-

acetaminophen-induced ALF59. However, the absence of standard-

media consisted of a serum-free, chemically-defined base medium (either Williams

air: 5% CO2 atmosphere at 37

aid dispersal of cells for attachment and placed in a humidified incubator under a 95%

acetaminophen content was determined by carbon monoxide difference spectroscopy of the sodium hydrosulphite-reduced samples, using a dual-beam spectrophotometer (Kontron, Switzerland). Results are expressed as pmol/mg protein.

Urea concentrations for each test medium and USR were assessed as previously described14. Briefly, on days 2, 4 and 6, duplicate cell culture dishes were washed twice with HBSS at 37°C and incubated in 8 ml of each test medium with either 2 mmol/L NH4Cl or 1 mmol/L D(-)-galactose for 2 hours38 at 37°C. For reference, media controls were treated under otherwise identical conditions except without cells. For the calculation of USR 0.5 ml samples were taken at day 2, 4 and 6 at t = 0 and t = +2 hours using a modified colourimetric urea nitrogen kit (Sigma, Mo, USA) according to the manufacturer’s instructions. The USR was calculated for total protein content and for pre- and post-incubation volumes for each culture dish and is expressed as pmol/hour/mg TP.

Galactose concentration was determined in 0.5 ml samples taken at day 2, 4 and 6 at t = 0 and t = +2 hours using a modified galactose kit (Roche, UK) according to the manufacturer’s instructions. Percentage GE was calculated as the amount of galactose eliminated after 2 hours expressed as a percentage of the initial value at 0 hours.

The ER was determined based on albumin concentration. Duplicate samples of culture supernatants taken on days 2, 4 and 6 using a flurometric albumin blue 580 dye-binding assay39 optimised for measurement in cell culture supernatants. Our adaption sought to exploit the use of a fluorescence reader for microtitre plates so that multiple samples could be analysed simultaneously.

1 ml samples were taken from duplicate cell culture dishes on days 2, 4 and 6. Pig albumin (Sigma) dissolved in calibrator diluent was used as the albumin standard and was prepared fresh for each assay run. Calibrator diluent was prepared by dissolving 2.7 g KH2PO4, 0.9 g K2HPO4, 4.5 g of sodium chloride, 0.5 g of EDTA disodium salt and 30 mg of pig gamma immunoglobulins (IgG, Sigma) in 300 ml of dH2O (pH 6.0) and stored at 4°C. Pig IgG was included as a stabilizer to prevent non-specific absorption of albumin to plastic surfaces.

Methods Hepatocyte isolation and culture. All animals received humane care in compliance with the the UK Home Office Guidelines under the Animal (Scientific Procedures) Act 1986 and study protocols complied with University of Edinburgh guidelines. Hepatocytes were isolated from weaning piglets (<15 kg) using our ex vivo collagenase perfusion method, previously described in detail40. They were seeded at a density of 105 viable cells per 100 mm tissue culture plastic dish (Dow Corning, MI, USA) in 8 ml of Medium. The culture dishes were shaken gently for some minutes to aid dispersal of cells for attachment and placed in a humidified incubator under a 95% air: 5% CO2 atmosphere at 37°C. Cells were kept in culture for 6 days and analyses were performed on days 1, 2, 4 and 6 as detailed in the following paragraphs. Medium was changed on day 1, 2 and 4.

The cells were tested against different from four types of culture media. Each culture medium consisted of a serum-free, chemically-defined base medium (either Williams E (WE), (Leibowitz) modified L-15 Medium (ML-15), Medium 199 (M199), RPMI 1640 (M1640)) supplemented with 50 ng/ml Long-Epidermal Growth Factor (L- EGF); 10 μg/ml porcine insulin; 1 μmol/L dexamethasone; 2 mmol/L L-Glutamine; 50 ng/ml Gentamycin; 50 mg/ml Penicillin-Streptomycin and 2.5 μg/ml Fungizone.

Biochemical and metabolic assessment. For each tested medium, hepatocytes were cultured on duplicate tissue culture plastic dishes and evaluated for markers of differentiation and biochemical function. On the day of isolation biochemical function was assessed (Figures S1, S2), and the significant influence of ML-15 (day 2) on retained LDH activity, the morphological observations, coupled with HBAI scoring, strongly favour WE and M199 media over and above the other media defined.

Using the overall performance of media formulations is crucial for effective cell-based therapies6, whilst testing efficacy of the various BAL devices under development require a large animal model of acetaminophen-induced ALE39. However, the absence of standardization for assessment of cell function between laboratories, repre-

sents a major drawback, due to the variable usage of, for example, serum-free or serum-containing media formulations, biomatrices or culture system modalities. Such diversity of approach and our limited information of the most important hepatocyte functions make direct comparisons between research groups very difficult. It may therefore be useful to assess and weight all cell functions equally. Therefore, to simplify comparisons, we propose a HBAI, which normalises each hepatic functional parameter with respect to an equally weighted scoring system (Supplementary Table S1). Additionally, the impact of media formulation on culture morphology, including ultrastruc-
ture (Table 1; Fig. 6), and gross cell morphology (Fig. 5) are taken into account. Using these criteria it is evident that M199 attained the highest HBAI score overall (Table 2), ranking the media as follows: M199 > WE > M1640 > ML-15. This utility may allow direct comparisons of any hepatocyte-based cell culture system.

Morphological and ultrastructural assessment. To examine gross morphology, cultured cells were examined under phase-contrast using a Telavaf 20 inverted microscope (Zeiss, Germany) on days 1, 2, 4 and 6 of culture for each test medium and images captured.

To examine ultrastructural features via TEM, cells were processed as previously described41. Cells cultured in each test medium, obtained from the same isolation as used for the phase-contrast study were taken on days 2 and 6 washed in HBSS buffer to remove dead or loosely attached cells and trypsinized with 0.5% trypsin-EDTA (Sigma).

Following low-speed centrifugation (20 g: 5 minutes) and washing steps in HBSS/ 5% FBS to neutralize trypsin activity, the cell pellet was resuspended and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer solution at pH 7.3 for 2.5 hours at room temperature. Fresh liver tissue and freshly isolated cells were similarly treated with 1% osmium tetroxide in 0.1 M cacodylate buffer solution at pH 7.3 for 1 hour at room temperature. Following dehydration in a graded series of acetone solutions (50, 70, 90, and 100% (3 times)) the tissue was infiltrated and embedded in araldite mixture. In order to orientate and select an area for the ultrastructural observations, semi-thin sections (1 μm in thickness) were cut with glass knives, stained with 1% toluidine blue and examined under the light microscope. Ultra-thin sections with silver gold interference colour (approximately 70-80 nm in thickness) were cut on an ultramicrotome (Ultracut OmU4, Reichert Co., Austria) and collected on 200 mesh uncoated copper grids. The sections were double stained in saturated uranyl acetate in 50% methanol for 30 minutes followed by lead citrate in water for 5 minutes. Ten areas from each of 4 copper grids were selected for investigation and representative grids examined and photographed in a scanning transmission electron microscope (CM12, Philips, UK) at 80 kV.

Statistical analysis. One-Way Analysis of Variance (ANOVA) was performed on 1) between-media statistical analysis on each single medium formulation followed by a post-hoc multiple comparisons tests of least significant differences, using the SPSS 9.0 statistical software package. The mean ± SEM was considered statistically significant at the 0.05 level and expressed as mean ± SEM (standard error mean) and the number of experiments performed (n) is indicated. Statistical data is presented where relevant (significant) p-values for multiple comparisons of either or both 1) and 2) above were calculated.
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Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Nelson, L.J. et al. Profiling the Impact of Medium Formulation on Morphology and Functionality of Primary Hepatocytes in vitro. Sci. Rep. 3, 2735; DOI:10.1038/srep02735 (2013).

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