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d-LIVER

ICT-enabled, cellular artificial liver system incorporating personalized patient management and support

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D8.4: Report on performance of progenitor-derived hepatocytes on toxic plasma

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PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including Commission Services)	
CO	Confidential, only for members of the consortium (including Commission Services)	

Document History

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1. Executive Summary

Using clinical liver disease data, a cocktail of substances and concentrations that re-capitulate their levels in patients with liver failure and effects on the viability of rodent and human HPAC-derived hepatocyte-like cells has been examined. In all cases, levels of toxins 100 times greater than the level estimated to model cirrhotic levels were not toxic to cells. These data suggest that there is minimal risk of bioreactor cells being adversely affected by patient sera and that they will be able to remain functional in the bioreactor and assist in re-compensating hepatic function.

2. Introduction

Hepatocytes are the primary defining cell of the liver, performing the vast majority of its functions. Isolation and/or culture of hepatocytes is therefore a potential source of functional cells that could be used in a bioreactor.

One important consideration for any bioreactor is the potential for the patients' sera to be toxic to the cells within the bioreactor, which likely would as a minimum, impair their function and ability to detoxify the sera. Furthermore, any significant cell death within the bioreactor would also have the potential to compromise the patient's health since the patient may then be systemically exposed to bioreactor cell proteins and other factors which could for e.g. lead to a systemic inflammatory response.

To examine this potential scenario, a consideration of the critical factors known to be altered in patients with decompensating liver failure was made and the effect of these altered factor levels on rat and prototype human progenitor and hepatocyte-like cells was examined.

Liver failure leads to a variety of physiological changes, related to the role that the liver plays in intermediary metabolism and endobiotic detoxification and excretion. The major functional role of the liver in metabolism will impact on for example:

- Glucose homeostasis
- Amino acid homeostasis
- Lipid homeostasis
- Endobiotic homeostasis

With respect to endobiotic homeostasis, the liver regulates the blood levels of:

- Ammonia
- Bile acids
- Bilirubin
- Lactic acid

This current deliverable assesses the ability of progenitor (B-13 and HPAC) and progenitor-derived hepatocytes to tolerate the main factors that are increased in patients with decompensating liver disease.

3. Report on performance of progenitor-derived hepatocytes on toxic plasma

3.1. Methods

Cell culture

Cells were cultured in low glucose Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 80 U/ml penicillin and 80 µg/ml streptomycin. Cells were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air. Dexamethasone (DEX) was purchased from the Sigma Chemical Co. (Poole, UK) and was added to medium from 1000-fold concentrated ethanol vehicle solvated stocks, control cells were treated with 0.1% (v/v) ethanol alone as control.

Preparation of "cirrhotic serum"

Cirrhotic serum (CS) was prepared directly in media as a 100-fold concentrated stock. CS- refers to media (with x1 fold CS constituents) without further addition of endotoxin (LPS); CS+ refers to media with endotoxin supplementation with 100ng LPS/ml (*E. coli* origin - Sigma L-6529).

Toxicity assessment

Cells were in addition treated with drugs and toxins known to result in toxicity. Methapyrilene (MP) is an rat-specific hepatotoxin that requires cytochrome P450 metabolism for toxicity (e.g. CYP2C11) [1-3]; chlorpromazine is a general non-specific cell toxin [4], gliotoxin is a fungal toxin that stimulates mixed necrosis and apotosis mechanisms of cell death depending on the cell type [4,5] and paracetamol is an hepatotoxin that requires metabolism by cytochrome P450s for toxicity (e.g. CYP2E1) [6].

Viability was determined by Trypan Blue dye exclusion as previously outlined [5].

3.2. Results

The basal medium employed in all studies is Dulbecco's modified Eagle's medium (DMEM) - low glucose - (Sigma D5546), with 80U/ml penicillin and 80µg/ml streptomycin plus added glutamine with the addition of up to 10% (v/v) foetal calf serum. In culture, the volume of culture medium is, for practical reasons, disproportionately greater compared with the number of cells in the well when compared with the extracellular volume to cell volume *in vivo*. Accordingly, this should be taken into consideration when cells *in vitro* are challenged, with for example, an endobiotic. The extracellular volume consists of plasma and interstitial fluid. According to Netter's Atlas of Human Physiology (2002), extracellular fluid constitutes about 33% of body water. For a 40 litre body, about 15 litres is extracellular fluid (of which 12L is interstitial fluid and 3L is plasma).

For an adult human, a 1.5kg liver (equivalent to 225 g liver protein) will therefore be required to regulate 15L extracellular body fluid = 0.067 ml/mg liver protein.

Normal B-13 / B-13/H cell culture employs approximately 0.5mg cell protein in 1.5ml culture medium = 3ml/mg B-13/H protein. Therefore, the medium load *in vitro* is 3/0.067 = 45 times greater.

The concentration of endobiotics should therefore be reduced by this factor *in vitro*, in order to model the same load *in vivo* (since reducing culture medium volume by this factor is not practicable). However, this assumes that endobiotic medium concentration plays no role in the cells' ability to metabolise it and or its toxicity, which limits the relevance of the concentrations employed *in vitro*. To at least take this limitation into some account, the medium load *in vitro* was reduced to a factor of 20.

The toxicity of the synthetic toxic sera was therefore examined in rodent progenitor (B-13) and rodent progenitor-derived hepatocytes (B-13/H) termed CS levels (predicted to be approximately equivalent to the levels of toxins present in cirrhotic sera, taking into account the practical limitations of the *in vitro* system in mimicking the *in vivo* situation) and at up to 100 times that level (10CS, 10 times the concentration; 100CS, 100 times the concentration). In addition, the effect of adding endotoxin was examined (-, no LPS; + LPS added at 100ng LPS/ml (*E. coli* origin - Sigma L-6529). Figure 1 demonstrates that concentrations up to 100CS with or without LPS were not toxic to the cells over a 24 hour period of incubation.

In contrast, toxins known to be activated by hepatocytes to produce toxic products, such as methapyrilene caused significant toxicity (Figure 1).

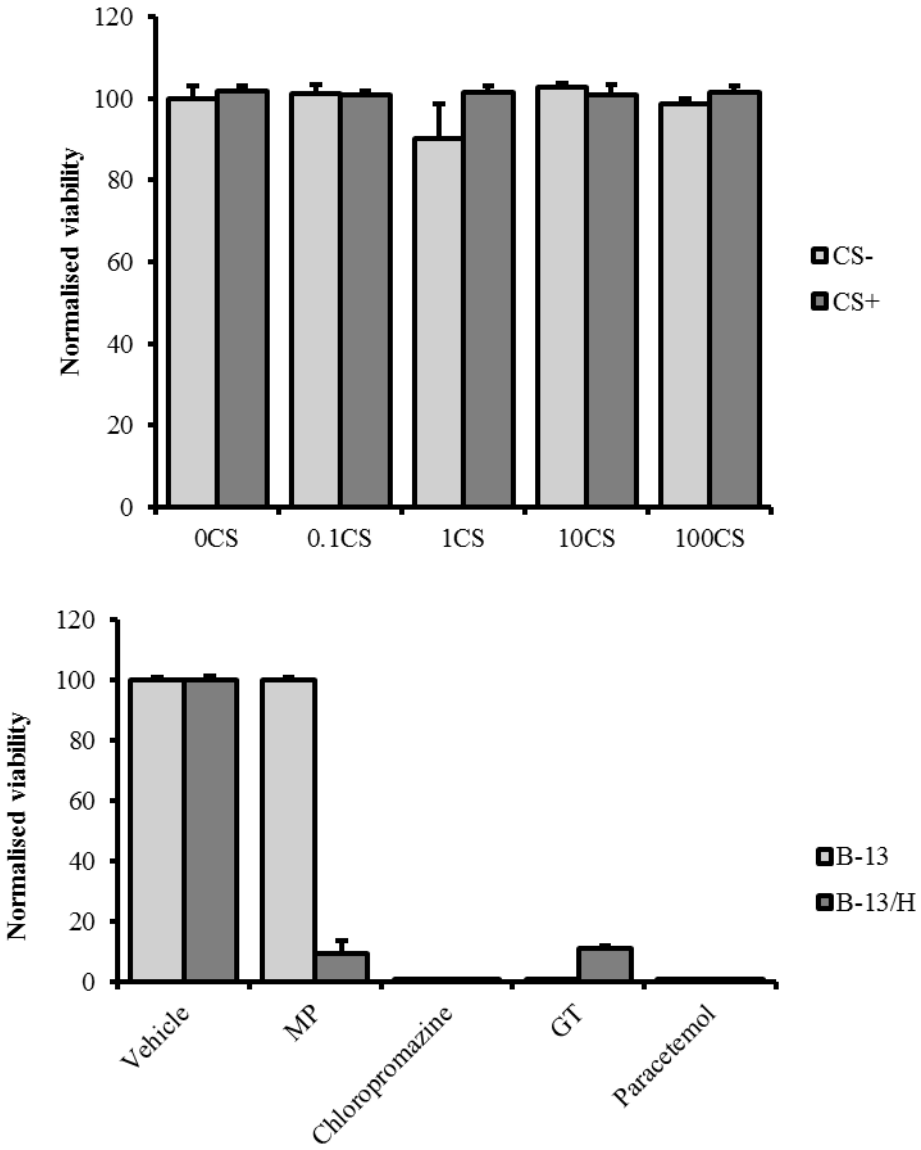

Figure 1. Viability of B-13 and B-13/H cells after treatment for 24 hours with CS medium and known hepatotoxins. Cells were treated with either the indicated dilution of CS medium with (+) or without (-) further addition of 100ng/ml endotoxin, 200µM methapyrilene (MP), 200µM chlorpromazine, 20µM gliotoxin (GT) or 20mM paracetamol. Viability was assessed by Trypan Blue dye exclusion [5] and data represent the mean and standard deviation of 3 separate assessments from the same experiment, typical of at least 3 separate experiments.

The same approach was examined in HPAC cells and HPAC cells treated with dexamethasone to promote an hepatic phenotype. Figure 2 demonstrates that concentrations up to 100CS with or without LPS were not toxic to the cells over a 24 hour period of incubation.

In contrast, toxins known to be activated by hepatocytes to produce toxic products, e.g. paracetamol caused significant but limited toxicity, likely due to the low expression of CYP2E1 after treatment with dexamethasone (Figure 5).

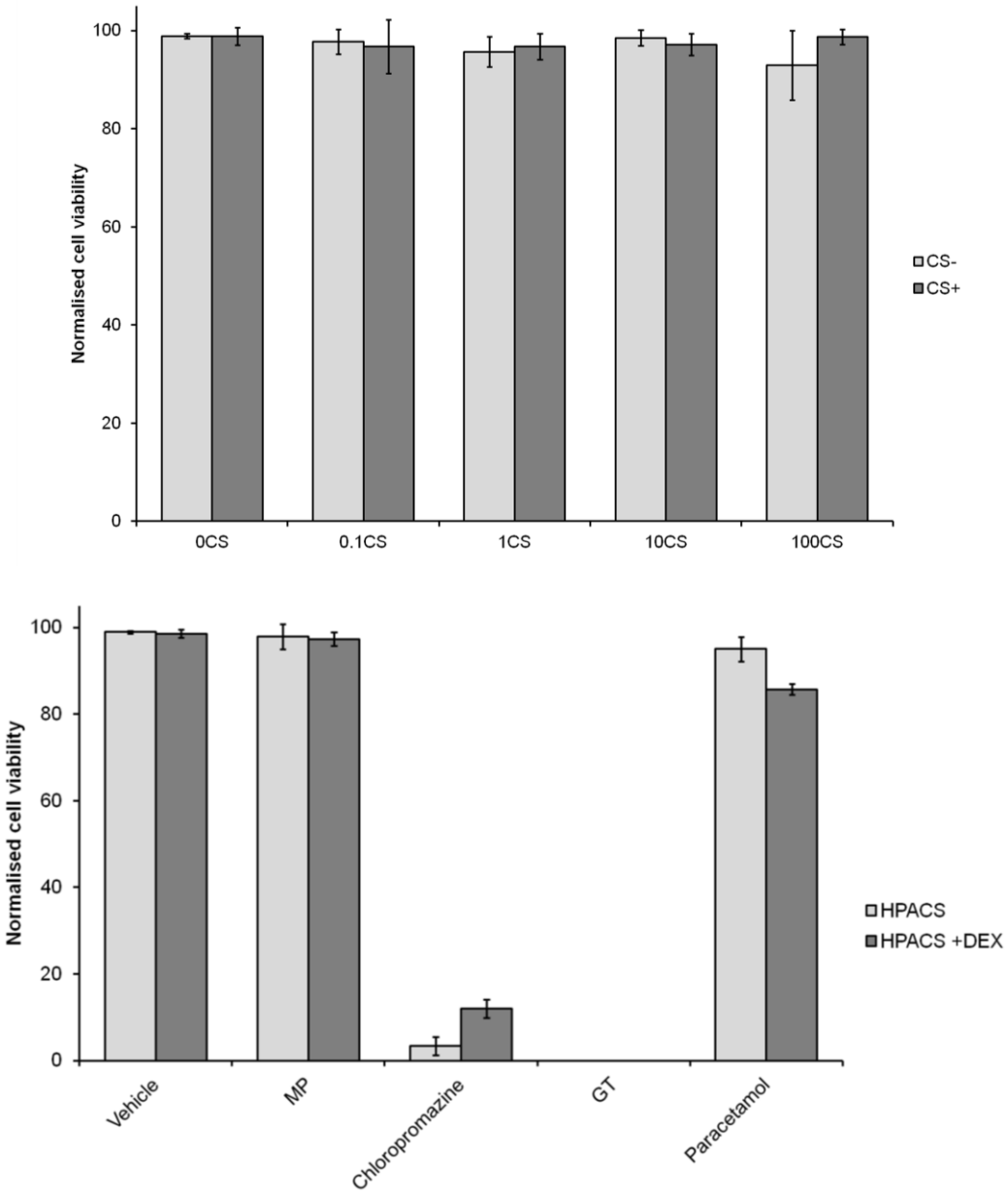

Figure 2. Viability of HPAC and dexamethasone pre-treated HPACS cells after treatment for 24 hours with CS media and known hepatotoxins. Cells were treated with either the indicated dilution of CS medium with (+) or without (-) further addition of 100ng/ml endotoxin, 200µM methapyrilene (MP), 200µM chlorpromazine, 20µM gliotoxin (GT) or 20mM paracetamol. Viability was assessed by Trypan Blue dye exclusion [9] and data represent the mean and standard deviation of 3 separate assessments from the same experiment, typical of at least 3 separate experiments.

4. Conclusions

These data suggest that sera from patients will not be toxic to the HPAC cells within a bioreactor. Future stably transfected cell lines will therefore be tested for their ability to tolerate CS media as outlined in this document. However, in addition, cells will be exposed to sera from patients with decompensating liver disease to establish whether the cells are able to modify the serum (e.g. reduce ammonia levels and metabolise endobiotics such as bilirubin and bile acids) and therefore potentially assist a patient's failing liver.

5. References

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