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Effect of oxygen concentration and selected protocol factors on viability and gene expression of mouse liver slices

Ewa Szalowska *, Geert Stoopen, Jeroen C.W. Rijk, Si Wang, Peter J.M. Hendriksen, Maria J. Groot, Jan Ossenkoppele, Ad A.C.M. Peijnenburg

RIKILT – Institute of Food Safety, Wageningen UR, PO Box 230, 6700 AE Wageningen, The Netherlands

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ABSTRACT

Precision cut liver slices (PCLSs) are widely used as a model to study hepatotoxicity. For culturing of PCLS diverse protocols are used which could affect slices viability and results.

We aimed to identify the most optimal culture protocol for mouse PCLS. Slices were cultured for 24 h under different concentrations of serum, glucose, insulin, and oxygen. Thereafter, slices viability was assessed by biochemical methods. Transcriptome analysis was performed to identify changes introduced by culture at different oxygen concentrations (20%, 40%, 60%, and 80% of oxygen).

Medium composition did not affect the slices viability. Although metabolic competence was unaffected by oxygen concentrations, culturing at 80% of oxygen yielded slices with the best biochemical characteristics. The comparison of uncultured vs. cultured slices revealed 2524 genes to be differentially expressed. Genes involved in drug metabolism, peroxisomal and mitochondrial functions were down-regulated while several adaptive/stress response processes were up-regulated. Moreover, 80% of oxygen was the most favorable condition with respect to maintenance of expression of genes involved in drug and energy metabolism.

The outcome of this study indicates that mouse PCLS are a valuable tool in research on hepatic functions and toxicity, particularly if they are cultured under a controlled oxygen concentration of 80%.

1. Introduction

Precision cut liver slices (PCLSs) are commonly used as an *ex vivo* model system in pharmaco-toxicological research to study processes related to hepatotoxicity (de Graaf et al., 2010). The major advantage of PCLS in comparison to other *in vitro* models, such as the human hepatocarcinoma cell line HepG2, is the presence of various cell types characteristic for the liver, such as hepatocytes, Kupffer cells, stellate cells, and others (May et al., 2009). Moreover, in PCLS the native liver architecture is preserved with intact cell–cell and cell–extracellular matrix interactions (de Graaf et al., 2010). Over the past years the PCLS technique was optimized with respect to different technical and biochemical factors. It was shown that dynamic culture conditions compared to static ones improve slices viability (de Graaf et al., 2010; Olinga et al., 1997) and addition of serum, insulin, and dexamethasone can be beneficial for long term culture of PCLS (de Graaf et al., 2010). However, there is no consensus regarding the culture protocol of PCLS between laboratories applying this technique. Therefore, it can be anticipated that these differences in protocols could have an impact on gene expression, metabolic functions or viability of PCLS and eventually affect experimental outcomes. For example, reported glucose concentration in PCLS culture medium ranges from 4 to 36 mM (Boess et al., 2003; Jiang et al., 1999; Toutain et al., 1998; Vickers et al., 2004). It is known that glucose concentrations higher than physiological level of 5 mM could cause oxidative stress (Leavens and Birnbaum, 2011; Yadav et al., 1994) and induce insulin resistance (Herman and Kahn, 2006). Moreover, in terms of the liver physiology high glucose concentration seems to be unnecessary. The preferential energy substrates for the liver *in vivo* are keto-acids formed by degradation of amino acids. The liver utilizes glucose as an energy source only if keto-acids are depleted (Stryer, 1997). Similarly, hyperinsulinemia and (sub)chronic exposure to insulin *in vivo* and *in vitro* were shown to induce insulin resistance which results in a starvation like state and decreased cellular viability (Lustig, 2006;Zhang et al., 2010). Another protocol factor varying considerably in the literature is oxygen concentration applied in the air that is used to oxygenate the medium. Although

* Corresponding author. Address: RIKILT – Institute of Food Safety, Wageningen UR, Akkermaalsbos, 6708 WB Wageningen, The Netherlands. Tel.: +31 317 480406; fax: +31 317 417717.

E-mail addresses: ewa.szalowska@wur.nl (E. Szalowska), geert.stoopen@wur.nl (G. Stoopen), Jeroen.Rijk@wur.nl (J.C.W. Rijk), si.wang@wur.nl (S. Wang), peter.hendriksen@wur.nl (P.J.M. Hendriksen), maria.groot@wur.nl (M.J. Groot), Jan.Ossenkoppele@wur.nl (J. Ossenkoppele), ad.peijnenburg@wur.nl (A.A.C.M. Peijnenburg).

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most often liver slices are cultured at 95% oxygen (de Graaf et al., 2010), other studies applied 40% oxygen (Clouzeau-Girard et al., 2006;Guyot et al., 2007) and occasionally 20% oxygen has been used (Toutain et al., 1998; Sarsat et al., 1998; Chelin et al., 1998).

The physiological oxygen concentration varies from 3% to 11%, depending on tissues and physiological processes (Carreau et al., 2011). Although this oxygen concentration cannot be directly compared with the oxygen concentration used to oxygenate PCLS it is still high compared to standard conditions used to culture primary cells and cell line models where 20% of oxygen is used. It is known that both hyperoxia and hypoxia could evoke stress responses, which on the long term will lead to decreased cell viability and eventually cell death (Kulkarni et al., 2007). Moreover, other *in vitro* studies showed that different oxygen concentrations exert effects on diverse cellular processes and gene expression (Allen et al., 2005;Kidambi et al., 2009;Wright and Dennery, 2009;Oller et al., 1989). *In vivo,* different oxygen tensions are known to regulate hepatic gene expression as well and to contribute to the phenomenon of liver zonation (Maronpot et al., 2010;Matsubara et al., 1982). At present, there is very limited data on the effect of oxygen tension on PCLS viability and metabolism (Drobner et al., 2000;Olinga et al., 1997; Toutain et al., 1998) and there are no studies devoted to the effect of oxygen on gene expression in PCLS at all.

Moreover, PCLS are most frequently derived from rat and, to minor degree, human (de Graaf et al., 2010). Mouse liver slices are not often used and so far no comprehensive study on their biochemical and molecular characteristics has been published.

Therefore, the aim of current study was to identify the optimal culture conditions of mouse PCLS with respect to oxygen concentration and protocol factors, such as (1) glucose concentration in preparation buffer and culture medium, (2) the presence/absence of serum and insulin in culture medium, and (3) volume of culture medium. In order to study effects of these protocol factors a fractional factorial design was used. Such a design allows studying many factors at once with relatively low number of experiments, leading to identification of the most important factors influencing the process of interest (Szalowska et al., 2007).

The quality of slices cultured at different oxygen concentrations was assessed by biochemical assays, histological analysis, and a metabolic competence assay based upon testosterone metabolism. For a genome-wide analysis of changes in gene expression related to (1) the *in vivo–ex vivo* transition and (2) culturing at different oxygen concentrations, a DNA microarray study was performed. DNA microarrays are commonly used in life sciences and allow to study changes in the total transcriptome caused by experimental conditions (Guengerich, 2011; Heijne et al., 2005). The transcriptomics data analysis was performed using open access bioinformatics tools and led to the identification of biological processes that were affected by *ex vivo* culturing of mouse PCLS at different oxygen concentrations.

2. Materials and methods

2.1. Preparation of liver slices

About 9-weeks old male C57BL/6 mice were obtained from Harlan (Horst, The Netherlands). Animals were kept for 1 week at a housing temperature of 22 °C and at a relative humidity of 30–70%. Lighting cycle was 12-h light and 12-h dark. At the age of 10 weeks animals were sacrificed by an overdose of isoflurane. The treatment protocol was approved by the Ethical Committee for Animal Experiments at Wageningen University.

Immediately after the animals were killed the liver was perfused with PBS and placed in ice–cold Krebs–Henseleit buffer (KHB) (pH 7.4, containing 11 mM or 25 mM glucose). Liver tissue was transported to the laboratory within approximately 30 min and cylindrical liver cores were produced using a surgical biopsy punch with diameter of 5 mm (KAI, SynErgo Europe, Romania). Liver cores were placed in a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) filled with ice–cold and aerated with carbogen KHB supplemented with 11 mM or 25 mM of glucose. Slices with a diameter of 5 mm and a thickness of 0.2 mm were prepared. Immediately after preparation, slices were transferred into 12-well plates filled with 1.3 or 1.5 ml of pre-warmed (37 °C) Williams E medium (WEM) supplemented with or without glucose, serum, and insulin (see Table 1). Each slice was incubated individually and the weight of one slice was about 10 mg.

Culture plates containing the liver slices were incubated for 24 h with continuous shaking (70 rpm) at 20%, 40%, 60%, and 80% of oxygen; 5% CO2 and the remaining gas volume was filled up to 95% with N2. Incubations were performed in an oxygen controlled incubator (Galaxy 48 R New Brunswick, Nijmegen, The Netherlands). After incubations, samples were snap-frozen in liquid nitrogen and stored in −80 °C for further analysis. Samples dedicated to histochemistry were stored in 4% formaldehyde at room temperature.

2.2. Fractional factorial experiment

In order to optimize liver slices culture with respect to selected culture medium biochemical parameters a fractional factorial experiment was designed using SYSTAT 11 statistical software package (SYSTAT 11 Software Inc., Chicago, USA). The design consisted of five independent variables (protocol factors) with two levels: 0 and 1 (Table 1). In total 16 protocols with different combinations of factor levels were tested using slices obtained from three mice for each of the four oxygen concentrations (Supplementary Table 1). Thus, a total of 12 mice were used for this fractional factorial experiment in which the effect of the various combinations of factor levels on the viability of slices was tested. To that end, for each of the 16 protocols (at one particular oxygen concentration), liver slices from 3 different mice were cultured individually for 24 h. Each mouse liver was represented by three slices. Upon culturing, the three liver slices were pooled and ATP and protein content of the slices was measured. Leakage of lactate dehydrogenase out of the slices into the culture medium was measured for each slice individually. Each measurement was performed in duplicate. The results of the fractional factorial experiments were analyzed by ANOVA in SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA) using a general linear model. Main effects were analyzed and were considered significant at a p-value < 0.05.

Table 1
Fractional factorial experiment.

Factor	Factor level	
Glucose concentration in preparation medium	11 mM	25 mM
Insulin in culture medium	0 nM	60 nM
Serum in culture medium	0%	5%
Volume of culture medium	1.3 ml	1.5 ml
Glucose concentration in culture medium	11 mM	25 mM

Five protocol factors were tested in a fractional factorial design. Factors were represented by two levels and in total 16 protocols with different combinations of the protocol factors levels were analyzed. All the protocols were tested at four different oxygen concentrations, with slices obtained from three mice for each oxygen concentration.