



*Original Article*

## **Cryopreserved human hepatocytes culture optimization on polymethylpentene oxygen permeable membranes for drug screening purposes**

**Mathieu Danoy<sup>1</sup>, Benedikt Scheidecker<sup>1,2</sup>, Hiroshi Arakawa<sup>3</sup>,  
Katsuhiro Esashika<sup>4</sup>, Naoki Ishida<sup>3</sup>, Hiroyasu Ito<sup>1</sup>, Hisaaki Yanai<sup>5</sup>, Jun Takahashi<sup>5</sup>,  
Masaki Nishikawa<sup>1</sup>, Yukio Kato<sup>3</sup> and Yasuyuki Sakai<sup>1</sup>**

<sup>1</sup>*Department of Chemical Engineering, Faculty of Engineering, University of Tokyo,  
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

<sup>2</sup>*CNRS UMI 2820, Laboratory for Integrated Micro Mechatronic Systems, Institute of Industrial Science,  
University of Tokyo, 4-6-1 Komaba; Meguro-ku; Tokyo, 153-8505, Japan*

<sup>3</sup>*Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University,  
Kakuma-machi, Kanazawa, Ishikawa, 920-1192, Japan*

<sup>4</sup>*New Business Solutions Department, Innovative Solutions Center for Information & Communication Technology,  
R&D Center, Mitsui Chemicals, Inc.*

<sup>5</sup>*Bio Technology & Medical Materials Department, Synthetic Chemicals Laboratory,  
R&D Center, Mitsui Chemicals, Inc.*

(Received August 2, 2022; Accepted August 12, 2022)

**ABSTRACT** — In vitro culture of primary hepatocytes for drug screening purposes remains a challenge as cells rapidly lose their function in conventional culture conditions. Thin oxygen permeable membranes have shown, through direct oxygenation, beneficial effects for long-term culture and cellular function with freshly isolated hepatocytes. However, culture of cryopreserved hepatocytes, a standard for the industry, has shown limits due to high cellular damage, leading to low cellular function. In addition, high sorption of drug screening compounds on PDMS oxygen permeable membranes has rendered evaluation of different molecules, aimed at the improvement of the culture of those cells difficult. Here, culture of cryopreserved hepatocytes was performed on PMP membranes, known to exhibit exceptionally low sorption characteristics. A mixture of anti-apoptotic and anti-inflammatory compounds to improve cell viability during adhesion was tested and evaluation in terms of cellular damage and drug metabolism was performed after 24 hr and 72 hr. Components of the mixture were shown to have beneficial effect on Reactive Oxygen Species production after 6 hr of adhesion as well as on mitochondrial activity and LDH release after 24 hr. Effects in improving recovery of albumin and drug metabolism, could be efficiently measured after 72 hr as a result of the use of PMP. The presented results demonstrate the compatibility of PMP oxygen-permeable membrane-based culture with cryopreserved hepatocytes for efficient drug screening.

**Key words:** Cryopreserved hepatocytes, PMP, Oxygen permeable membrane, Drug development

## INTRODUCTION

The liver is known to be a central part of metabolism within the body and has been for years, the target of researchers' investigations for the development of new drug therapies. For analog reasons, the industry is also interested in reliable models which would give more efficient processing to clinical trials. Especially, hepatocytes are known to be the metabolic overachievers of the body. *In vivo* models have been widely investigated for drug screening purposes (Ejima *et al.*, 2016; Ergen *et al.*, 2019; Tokunaga *et al.*, 2017) but there are issues caused by interspecies differences and the ethical problem is becoming more and more preponderant (Liedtke *et al.*, 2013; Liguori *et al.*, 2017). In response to those, *in vitro* culture of hepatocytes has been developed but it was found that those cells would quickly lose their phenotype and thus, would not be compatible with a precise physiological or pathological drug screening. To solve this issue, different methods, including coculture with liver non-parenchymal cells such as Liver Sinusoidal Endothelial Cells (LSECs, Bale *et al.*, 2015; Ware *et al.*, 2018) or Hepatic Stellate Cells (HSCs, Choi *et al.*, 2020; Krause *et al.*, 2009) have been presented. Other methods have included the use of microfluidic devices (Prodanov *et al.*, 2016; Jellali *et al.*, 2016), which allows the inclusion of physiological factors such as flow and shear stress. While being compatible with those solutions, culture on oxygen-permeable membranes such as polydimethylsiloxane (PDMS) was shown to induce *in vivo*-like phenotypes via a biomimetic oxygen supply (Scheidecker *et al.*, 2020). The use of these membranes, linked to direct oxygenation, was also shown to be beneficial to the long-term culture of hepatocytes (Nishikawa *et al.*, 2008) as well as to the establishment of thick coculture tissues (Danoy *et al.*, 2017).

Nonetheless, efficient use of cryopreserved hepatocytes, which are a common standard for the industry, remains limited due to the stress induced by the thawing process in terms of ion balance, osmotic swelling, shift in pH, and oxidative stress (Fuller *et al.*, 2016). Especially, those conditions have been found as favorable to the formation of supra-physiological levels of Reactive Oxygen Species (ROS) which are linked to function and survival impairment (Bhagal *et al.*, 2010; Len *et al.*, 2019). These effects are expected to be further amplified in the culture of cryopreserved hepatocytes on oxygen permeable membranes such as PDMS and have led to difficulties in the establishment of culture conditions relevant for drug screening. Coincidentally, several molecules such as antioxidants and pan-caspase inhibitors have been shown to have a significant effect on the reduction of cellular

damage during the thawing of hepatocytes (Fujita *et al.*, 2005). Especially, treatment with Val-Ala-Asp(Ome)-fluoro-methylketone (Z-VAD-FMK) or with butylated hydroxytoluene (BHT) and ascorbic acid for 6 and 24 hr after thawing were found to significantly enhance cell viability and to promote the recovery of the cell function in hepatocytes after thawing. A combination of those molecules was also found to enhance cell viability (Fujita *et al.*, 2005). While the use of these compounds is expected to be beneficial to the heavily ROS impacted cultures on PDMS oxygen permeable membranes, the evaluation of the cellular function in those conditions is strongly biased by the high sorption of compounds used for drug screening onto PDMS (Nishikawa *et al.*, 2022). In that regard, polymethylpentene (PMP) membranes have been recently proposed as an alternative to PDMS with similar properties in terms of cell culture but with lower sorption characteristics (Nishikawa *et al.*, 2022). It was concluded that culture on PMP membranes allowed for a more accurate evaluation of the metabolism of hepatocytes by measuring the metabolism of different drug compounds through CYP activity.

Based on these results, the present study was focused on efficiently measuring the impact of molecules known to reduce cellular damage on the retrieval of the drug metabolism functions of cryopreserved hepatocytes cultured on oxygen-permeable membranes after thawing. In particular, the influence of Z-VAD-FMK and BHT was evaluated, acting on both the production of reactive oxygen species and on mitochondrial damage as previously observed (Fujita *et al.*, 2005). Other molecules such as Hepatocytes Growth Factor (HGF) and Y-27632 (ROCK inhibitor) were tested as the former is known to be involved in tissue repair, liver homeostasis, and hepatoprotection from apoptosis (Nakamura and Mizuno, 2010; Fausto *et al.*, 2006) and the latter is a potent anti-apoptotic used in post-thawing situations (Bissoyi *et al.*, 2014). The effect of these compounds was measured in terms of ROS and Lactate Dehydrogenase (LDH) production, mitochondrial activity, and albumin production but also in terms of drug metabolism thanks to the use of PMP membranes. In all these measurements, it was confirmed that Z-VAD-FMK was the compound with the most effect on both cellular damage and on the recovery of cellular function, especially after 72 hr of culture. Additionally, measurements of the CYP activity could be performed thanks to the use of PMP membranes and revealed different patterns depending on the substrate. Altogether, the presented results confirm the efficiency of different molecules toward the recovery of the drug metabolism capacity of cryopreserved hepatocytes which could be measured

due to the low sorption characteristics of the PMP membranes. Finally, those results suggest that, upon further evaluations of the material, notably in the field of microfluidics, PMP makes a serious alternative to PDMS which is currently the standard.

## MATERIALS AND METHODS

### Cell culture

PMP plates were obtained from Mitsui Chemicals and coated with 10% Collagen Type I-P (Nitta Gelatin, Osaka, Japan) in MilliQ for 1 hr at 37°C. Cryopreserved Primary Human Hepatocytes (PHHs) were purchased from Sekisui Xenotech (KS, USA) and seeded at a  $2.0 \times 10^5$  cells/cm<sup>2</sup> density after thawing in Hepatocytes Culture Medium (HCM, Williams' E Medium, and Primary Hepatocyte Maintenance Supplements, Gibco, CA, USA). The medium was then changed at 6 hr, 24 hr, and 48 hr, and the culture ended at 72 hr. For the first 24 hr of culture, the medium was supplemented with different combinations of 20  $\mu$ M Z-VAD-FMK (MBL, Tokyo, Japan), 20  $\mu$ M BHT (Sigma, Victoria, Australia), 10  $\mu$ M Y-27632 (Wako, Hiroshima, Japan), and 20 ng/mL HGF (Peprotech, NJ, USA). Cells were maintained in a multi-gas incubator with 10% O<sub>2</sub> at 37°C to provide a biomimetic oxygen supply as previously discussed (Scheidecker *et al.*, 2020). Controls were performed by culturing cells in a multi-gas incubator with 20% O<sub>2</sub> on PMP membranes sealed at the bottom with a polyester film to reproduce conventional culture conditions in Tissue Culture Polystyrene (TCPS) dishes adapted to the mechanical properties of PMP membranes.

### Reactive oxygen species production

The production of Reactive Oxygen Species (ROS) was measured by using a DCFDA Cellular ROS Detection Assay Kit (abcam, Cambridge, UK). Briefly, cells were subsequently washed with the attached buffer, stained with DCFDA for 45 min at 37°C, washed, and kept in culture medium during imaging. All images were taken with a confocal microscope (PowerIX70, Olympus, Tokyo, Japan) immediately after staining. All wells were discarded after the assay to avoid conflict with other measurements and assays.

### LDH production

LDH production was measured using an LDH Cytotoxicity Detection Kit (Roche, Switzerland). Briefly, medium samples were mixed 1:1 after collection with the components of the kit and incubated for 30 min at room temperature in an untreated polystyrene microplate as rec-

ommended by the manufacturer. The resulting conditions were then measured using an iMark Microplate reader (Bio-Rad, CA, USA) with an absorbance set at 490 nm.

### Mitochondrial activity

The mitochondrial activity was measured via a Mitochondrial Viability Assay (abcam). Briefly, cells were incubated with the solution supplied with the kit for 4 hr at 37°C. Fluorescence was then measured at 590 nm with an excitation of 550 nm using a Wallac Arvo Sx 1420 multilabel counter (PerkinElmer, MA, USA).

### RT-qPCR

Total RNA was isolated and purified from samples after 24 hr of culture with Trizol™ Reagent (Life Technologies, CA, USA) following the manufacturer's instructions. The concentration and quality of the samples were assessed via RNA measurement with a BioSpec-nano (Shimadzu Scientific Instruments, Kyoto, Japan). Reverse transcription into cDNA was performed with 1  $\mu$ g of total RNA with a ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). Real-time quantitative PCR was performed using a THUNDERBIRD SYBR qPCR Mix (TOYOBO) according to the manufacturer's recommendations and a StepOnePlus Real-Time PCR system (Applied Biosystems, MA, USA). The primer sequences used are given in Supplementary File 1. ACTB ( $\beta$ -Actin) was used as the reference gene and freshly thawed hepatocytes as the reference sample for the normalization of gene expression data.

### Albumin production

Albumin was quantified via sandwich ELISA using an anti-Human Albumin IgG (Bethyl, TX, USA, capture antibody) and an anti-Human Albumin IgG coupled with peroxidase (Bethyl, detection antibody). The revelation of peroxidase was done via an H<sub>2</sub>O<sub>2</sub>/OPD mixture, and the plate was read at 490 nm using an iMark Microplate reader (Bio-Rad) with an absorbance set at 490 nm.

### Drug screening

Drug metabolism in each condition was evaluated via a cocktail-substrate approach (Kozakai *et al.*, 2013). 500  $\mu$ L of the drug cocktail (Components in Table 1) were incubated for 4 hr in normal culture conditions before samples were taken and stored at -80°C until analysis. 20  $\mu$ L of culture medium was then mixed with 40  $\mu$ L of water and 140  $\mu$ L of methanol containing 100 nM of imipramine for internal control. The mixture was then vortexed and centrifuged at 21,500 g at 4°C for 5 min before use for the LC-MS/MS analysis. For the measurement, an LCMS8050 triple quadrupole mass spectrometer

**Table 1.** Component of the cytochrome P450 substrates cocktail and related metabolites detected by LC-MSMS.

Enzymes	Probe substrates	Concentration in reaction mixture ( $\mu$ M)	Metabolites monitored	Ion mode	m/z monitored
CYP1A2	phenacetin	20	acetaminophen	positive	152.0 > 110.0
CYP2A6	coumarin	2	7-hydroxycoumarin	positive	163.0 > 107.0
CYP2B6	bupropion	5	hydroxybupropion	positive	256.0 > 238.0
CYP2C8	amodiaquine	0,1	N-desethylamodiaquine	positive	328.0 > 283.0
CYP2C9	diclofenac	1	4'-hydroxydiclofenac	positive	312.0 > 230.0
CYP2C19	(S)-mephenytoin	40	4'-hydroxymephenytoin	positive	235.1 > 150.1
CYP2D6	bufuralol	5	1'-hydroxybufuralol	positive	278.0 > 186.0
CYP3A4/5	midazolam	2	1'-hydroxymidazolam	positive	342.0 > 203.0
IS <sup>a)</sup>	imipramine	-	-	positive	281.0 > 86.0

a) Internal standard

(Shimadzu, Kyoto, Japan) coupled with an LC-30A system (Shimadzu) was used.

A CAPCELL PAK C18 MG III (ID 2.0 mm  $\times$  50 mm; Osaka Soda Co. Ltd., Osaka, Japan) was used to perform the chromatography. The step-gradient elution was performed at 50C as follows: 0 to 0.5 min, 95% A/ 5% B; 0.5 to 3.0 min, 95% A/ 5% B to 20% A/ 80% B; 3.0 to 4.0 min, 20% A/ 80% B; 4.0 to 4.1 min, 20% A/ 80% B to 95% A/ 5% B; 4.1 to 5.5 min, 95% A/ 5% B; (A, water containing 0.1% formic acid; B, acetonitrile containing 0.1% formic acid, flow rate at 0.4 mL/min). For each compound, the detected mass number has been listed in Table 1 and the analytical standard curve for each metabolite was made using commercially available compounds. Data analysis was performed using the Lab solutions software (version 5.89, Shimadzu).

### Statistical analysis

Statistical analysis was done by ANOVA to evaluate differences between the groups. When the null hypothesis was rejected, a posthoc Tukey HSD test was performed and differences with  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*), were highlighted and considered statistically significant. All experiments were repeated 3 times independently and at least 3 technical replicates were performed for each independent run. If necessary, exclusion of outliers according to the method detailed by Iglewicz and Hoaglin (Iglewicz and Hoaglin., 1993).

## RESULTS

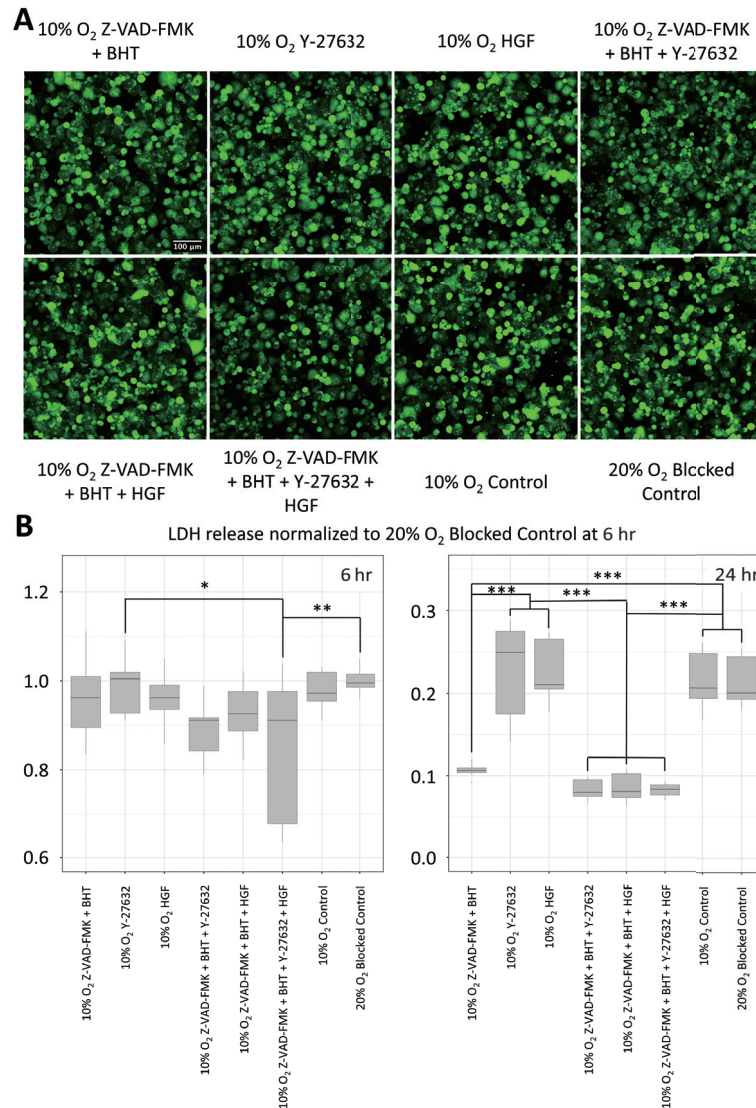
### Reduction of cellular damage during seeding

The efficiency of the different compounds tested was evaluated after 6 hr and 24 hr of incubation after seeding. Qualitatively, the production of ROS was found to be

the lowest in conditions that included Z-VAD-FMK/BHT or Y-27632. By itself, HGF did not appear to have a notable effect on the production of ROS. Interestingly, production of ROS appeared much higher in several cells in the 10% O<sub>2</sub> control on PMP but not in the 20% O<sub>2</sub> control on blocked membranes which further confirms the negative effect of the direct oxygenation on ROS production after cryopreservation (Fig. 1A). Simultaneously, no clear tendency could be extracted from the LDH production at 6 hr, probably due to the high number of dead cells present before seeding as cell viability after thawing was found to be around 80% (Fig. 1B). After 24 hr of culture, significantly lower production of LDH could be observed in conditions which included Z-VAD-FMK/BHT (Fig. 1B), as dead cells were partially washed (Supplementary Fig. 1). No specific difference could be observed between the control conditions and the conditions supplemented with either only Y-27632 or HGF. The effect of the compound was further confirmed as mitochondrial activity, which is known to be significantly altered by cryopreservation of hepatocytes, was also seen to be significantly higher in conditions that included Z-VAD-FMK/BHT (Fig. 2A). Similarly to the release of LDH, no significant difference was observed between the control conditions and the conditions supplemented with either only Y-27632 or HGF. Interestingly, while Z-VAD-FMK is a pan-caspase inhibitor, known to affect caspase-1 to 10, except for caspase-2 (Chauvier *et al.*, 2007), no effect was observed in the gene expression of CASP9 after 24 hr (Fig. 2B). However, the gene expression of CASP3 after 24 hr was found to be significantly regulated among several conditions. Especially, expression in the condition supplemented with Z-VAD-FMK/BHT was found to be significantly lower than in the 20% O<sub>2</sub> blocked control. In addition, the expression in the condition supplemented with both



## Culture optimization of cryopreserved hepatocytes on PMP



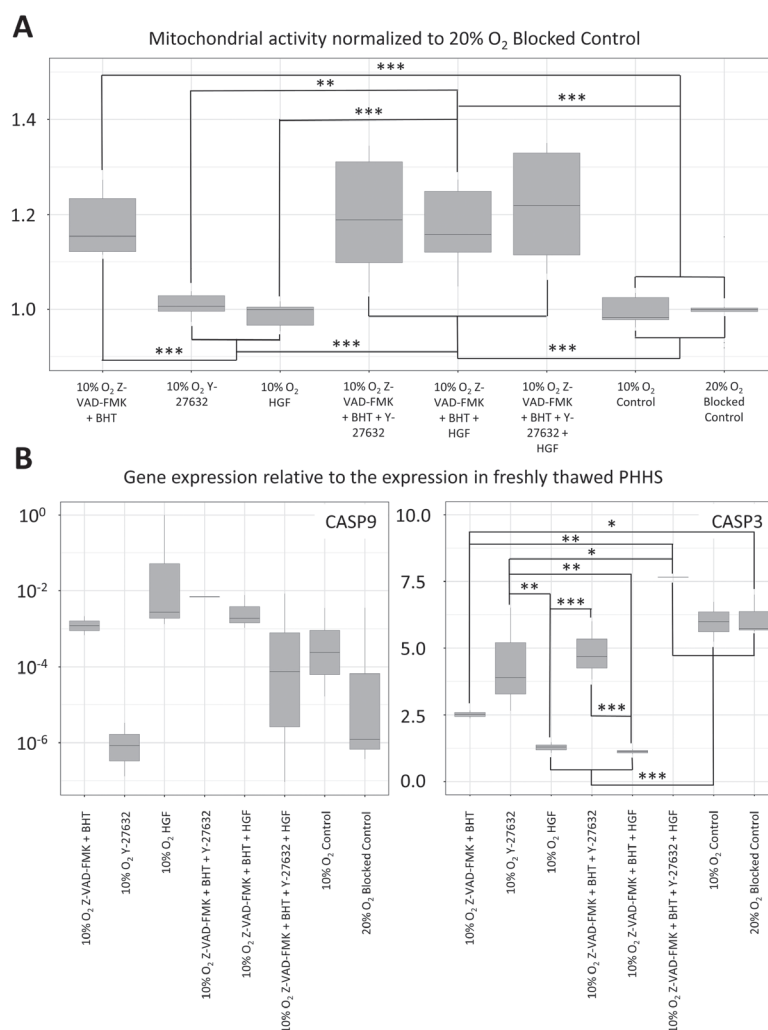
**Fig. 1.** Production of Reactive Oxygen Species (ROS) in the different tested conditions, measured 6 hr after seeding, and imaged by confocal microscopy (A). Production of Lactate Dehydrogenase (LDH) measured in the culture medium 6 hr and 24 hr after seeding, normalized by the production in the 20% O<sub>2</sub> control at 6 hr (B).

Z-VAD-FMK/BHT and HGF was found to be significantly lower than in both controls, but no significant difference was found with the condition supplemented with only HGF. Finally, the expression in the condition supplemented with Z-VAD-FMK/BHT, Y-27632, and HGF was found to be significantly lower than in conditions including either only Z-VAD-FMK/BHT, Y-27632, or HGF.

### Recovery of the hepatic function

While no specific issue was observed in the continuous culture of the cells for 48 hr and 72 hr (Supplementary

Fig. 2, Supplementary Fig. 3), the tissue appeared denser in conditions that included Z-VAD-FMK/BHT, especially as compared to both controls. Nonetheless, the recovery of the hepatic function regarding albumin production and drug metabolism was also monitored over this period. It was found that both Z-VAD-FMK/BHT and HGF allowed for a significant recovery of the albumin production after 72 hr of culture when compared to the levels observed after 24 hr of culture. In the case of the combination of both Z-VAD-FMK/BHT and HGF, the difference was found to be significant from 48 hr and to remain



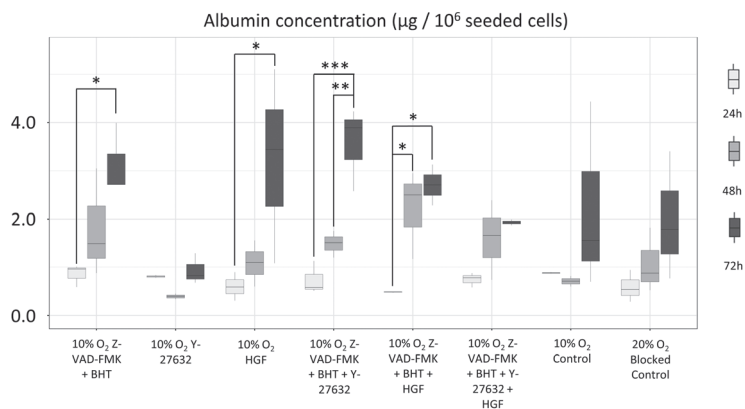
**Fig. 2.** Mitochondrial activity measured in the different culture conditions, measured 6 hr after seeding, and normalized by the activity in the 20% O<sub>2</sub> control at 6 hr (A). mRNA levels of CASP9 and CASP3 measured by rt-qPCR in the different culture conditions, 24 hr after seeding and normalized by the levels measured in freshly thawed primary human hepatocytes (B).

at comparable levels at 72 hr. In the case of the combination of both Z-VAD-FMK/BHT and Y-27632, the levels observed at 72 hr were found to be significantly higher than at 24 hr and 48 hr (Fig. 3). It is also worthy to note that the levels of albumin observed remained between 2 and 4  $\mu\text{g}/10^6$  cells seeded which are in the lower range of what is observed in the literature for fresh PHHs (Ehrlich *et al.*, 2019) but in the higher range of what is observed for unselected cryopreserved PHHs in monoculture (Fitzpatrick *et al.*, 2015).

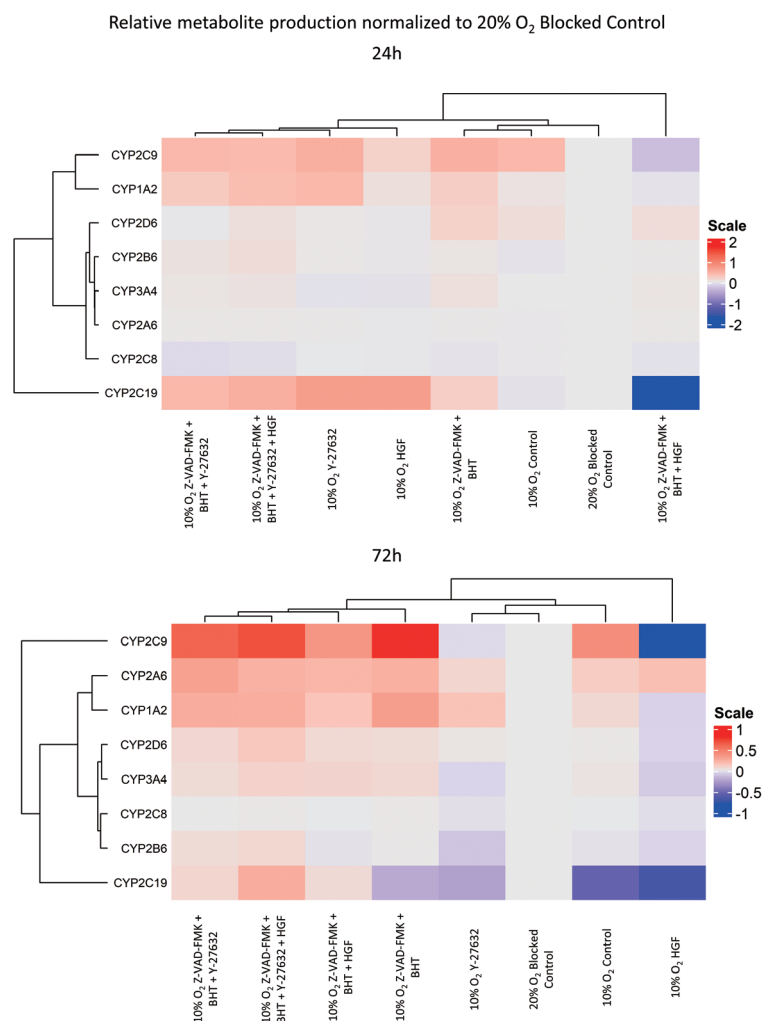
Regarding drug metabolism, differences in each condition could be detected at both 24 hr and 72 hr (Heatmaps in Fig.4, Statistical analysis in Supplementary File 2).

Especially, hierarchical clustering on the dataset obtained at 72 hr allowed for a clear separation of conditions in which Z-VAD-FMK/BHT was added from the others, further confirming the effectiveness of this combination on not only cell viability but also on drug metabolism. Among the differences that were deemed as statistically significant, it was notably found that the condition supplemented with the combination of Z-VAD-FMK/BHT, Y-27632, and HGF exhibited an improved CYP2B6 metabolism over the control at 10% O<sub>2</sub> after 24 hr. After 72 hr, more significant differences in the proposed combinations could be observed. Particularly, the addition of Z-VAD-FMK/BHT and HGF to Y-27632 was found to

## Culture optimization of cryopreserved hepatocytes on PMP



**Fig. 3.** Production of albumin measured by ELISA in the different culture conditions.



**Fig. 4.** Heatmap of the production of the metabolites via CYP450 activity measured 24 hr and 72 hr after seeding, by the cocktail-based approach in the different culture conditions and normalized by the production of each metabolite in the 20% O<sub>2</sub> control. Related statistical analysis is provided in Supplementary File 2.

significantly improve CYP2B6 metabolism after 72 hr. In addition, the combination of Z-VAD-FMK/BHT, Y-27632, and HGF also exhibited an improved CYP2C8 metabolism after 72 hr over conditions that included either only HGF or Y-27632. Moreover, the addition of Z-VAD-FMK/BHT to either HGF or Y-27632 was also found to significantly improve CYP2C8 metabolism after 72 hr. Finally, supplementation with HGF was found to significantly decrease CYP2C8 metabolism when compared to both the 10% O<sub>2</sub> control and the 20% O<sub>2</sub> blocked control.

## DISCUSSION

Cryopreservation of primary hepatocytes remains a largely inefficient method as far as cellular damage inflicted during isolation is further amplified by the freezing-thawing process (Stéphenne *et al.*, 2010). Especially, damages to cryopreserved hepatocytes are known to affect certain drug metabolic pathways in non-optimal conditions (Fuller *et al.*, 2016) which further limits the systematic application of those *in vitro* models by the industry. While advances such as the use of PDMS oxygen-permeable membranes for direct oxygenation (Scheidecker *et al.*, 2020) and cocktails of compounds targeted at limiting cellular damage after thawing (Fujita *et al.*, 2005) could give interesting prospects, evaluation of the cellular function remains biased due to significant sorption in cultures on PDMS (Nishikawa *et al.*, 2022). In this work, the effect of those compounds in culture on oxygen-permeable membranes was successfully assessed through the use of PMP membranes which have been shown to exhibit similar characteristics to PDMS but with improved sorption characteristics (Nishikawa *et al.*, 2022).

Particularly, the use of PMP has allowed for the distinction of patterns not only in terms of cellular damage but also in terms of drug metabolism (Fig. 4). While it is generally accepted that optimal cryopreservation and thawing do not significantly affect drug metabolism in cryopreserved hepatocytes (Li *et al.*, 1999; Steinberg *et al.*, 1999; Smith *et al.*, 2012), oxidative stress has been shown to have a significant effect in the repression of certain drug metabolic pathways (Barouki and Morel, 2001). Especially, as no particular pattern was observed at 24 hr, it could be supposed that there was indeed no specific impact of cryopreservation and thawing on the monitored CYP metabolism as previously observed and suggesting that cellular damage did not have a significant influence at this stage. However, significant differences between conditions were observed for the metabolism via CYP2C8, CYP2C9, CYP3A4, CYP2B6, and CYP2C19 at 72 hr. More interestingly, data clustering on the dataset at 72 hr

has allowed us to isolate conditions in which Z-VAD-FMK/BHT was added which corresponds to conditions in which the production of LDH (Fig. 1B) and the mitochondrial activity (Fig. 2A) suggested lower cellular damage. This result could probably be linked to a higher cell survival at 72 hr due to limited damage in those conditions and which led to the formation of a denser tissue in conditions that included Z-VAD-FMK/BHT (Supplementary Fig. 3). These results are in good adequation with comparable studies within the literature in more conventional culture vessels, and for which an initial exposition to Z-VAD-FMK was confirmed to have a significant influence on drug metabolism in the long term (Ölander *et al.*, 2019).

While additional cellular damage could have been expected due to the quick change from hypoxia to high, direct oxygenation, there was no significant difference between the 10% O<sub>2</sub> control and the 20% O<sub>2</sub> blocked control concerning LDH production (Fig. 1B) and mitochondrial activity (Fig. 2A). Interestingly, intense production of ROS was found in certain cells in the 10% O<sub>2</sub> control but not in the 20% O<sub>2</sub> blocked control (Fig. 1A). As oxidative stress is known to be an important trigger of apoptosis through the mitochondrial pathway (Ölander *et al.*, 2019; Bissoyi *et al.*, 2014), higher cellular damage would have been expected in the former condition than in the latter, but no such observation could be made. This can possibly be linked to the fact that apoptosis was found to be immediately induced in hepatocytes after thawing (Ölander *et al.*, 2019) and thus, before further stimulation by the culture environment. Regarding cellular damage and the post-thawing initiation of apoptosis, caspase-3, 8, and 9 have been found to be mostly involved (Bissoyi *et al.*, 2014). Especially, caspase-3, which was reported to be the downstream effector of apoptosis initiated by ROS (Redza-Dutorzior and Averill-Bates, 2016), was found to be immediately activated at the protein level after thawing (Ölander *et al.*, 2019). In the current experimental setup, mRNA levels of caspase-3 were found to decrease in conditions including either or both Z-VAD-FMK/BHT and HGF (Fig. 2B). As the increase of the caspase-3 mRNA levels was found to be the precursor of cleavage and activation of caspase-3 (Sabbagh *et al.*, 2005), these results can be reasonably related to the fact that the first is a pan-caspase inhibitor (Fujita *et al.*, 2005) while the latter was found to inhibit caspase-3 via Met (Ma *et al.*, 2014). Interestingly, the combination of Z-VAD-FMK/BHT and Y-27632 or the combination of Z-VAD-FMK/BHT, HGF, and Y-27632 did not cause any significant difference in the mRNA levels of caspase-3 when compared to controls. In that regard, Y-27632 was previously



reported to increase mRNA levels of caspase-3 in cancer cells (Liu and Bi, 2016) which might explain the adverse effect observed in the conditions including the compound and be related to the low recovery of albumin production in said condition (Fig. 4).

In the present study, the culture of cryopreserved hepatocytes on oxygen permeable membranes was optimized via the use of PMP plates which allowed evaluation of the effect of the different compounds added. Because of the low sorption characteristics of PMP, not only the influence on cellular damage but also the retrieval of the function could be evaluated via a cocktail-based drug assay. Measurement of the drug metabolism has allowed confirming the major impact of the Z-VAD-FMK/BHT combination in terms of retrieval of the cytochrome P450 metabolism. This was further confirmed by all data regarding cellular damage as far as conditions containing Z-VAD-FMK/BHT were found to present less damage. While further addition of either HGF or Y-27632 did not influence the retrieval of drug metabolism, Y-27632 induced activation of caspase-3, an important downstream effector of apoptosis, suggesting that the compounds may not be recommended in the present configuration. On the other hand, HGF presented interesting perspectives in terms of albumin production retrieval as well as in the reduction of caspase-3 activation. The present dataset suggests that the combination of Z-VAD-FMK/BHT with HGF might be an interesting solution to the retrieval of cellular function on oxygen permeable membranes by limiting cellular damage. Further investigations at the protein level would further confirm those results and studies on the long-term impact of the mixture of the compounds, combined with long-term culture techniques such as collagen sandwich culture on PMP plates, would allow for the development of better *in vitro* models for drug screening purposes.

## ACKNOWLEDGMENTS

The authors would like to thank Tomoaki Matsugi from Mitsui Chemicals, and Toru Horie from D Three Laboratory for the management of the collaboration with Mitsui Chemicals, Inc.

## Fundings

This work was supported by Mitsui Chemicals, Inc. as well as by the Japanese Agency for Medical Research and Development as part of the Project Focused on Developing Key Evaluation Technology: Development of Platform Technology for Drug Discovery through Application of Regenerative Medicine.

**Conflict of interest----** This work was made in collaboration with Mitsui Chemicals, Inc. which has the exclusive right for the manufacturing of PMP.

## REFERENCES

- Bale, S.S., Golberg, I., Jindal, R., McCarty, W.J., Luitje, M., Hegde, M., Bhushan, A., Usta, O.B. and Yarmush, M.L. (2015): Long-term coculture strategies for primary hepatocytes and liver sinusoidal endothelial cells. *Tissue Eng. Part C Methods*, **21**, 413-422.
- Barouki, R. and Morel, Y. (2001): Repression of cytochrome P450 1A1 gene expression by oxidative stress: mechanisms and biological implications. *Biochem. Pharmacol.*, **61**, 511-516.
- Bhagal, R.H., Curbishley, S.M., Weston, C.J., Adams, D.H. and Afford, S.C. (2010): Reactive oxygen species mediate human hepatocyte injury during hypoxia/reoxygenation. *Liver Transpl.*, **16**, 1303-1313.
- Bissoyi, A., Nayak, B., Pramanik, K. and Sarangi, S.K. (2014): Targeting cryopreservation-induced cell death: a review. *Biopreservation. Biobank.*, **12**, 23-34.
- Chauvier, D., Ankri, S., Charriaut-Marlangue, C., Casimir, R. and Jacotot, E. (2007): Broad-spectrum caspase inhibitors: from myth to reality? *Cell Death Differ.*, **14**, 387-391.
- Choi, Y.Y., Seok, J.I. and Kim, D.S. (2020): Flow-based three-dimensional co-culture model for long-term hepatotoxicity prediction. *Micromachines (Basel)*, **11**, 36.
- Danoy, M., Shinohara, M., Rizki-Safitri, A., Collard, D., Senez, V. and Sakai, Y. (2017): Alteration of pancreatic carcinoma and promyeloblastic cell adhesion in liver microvasculature by coculture of hepatocytes, hepatic stellate cells and endothelial cells in a physiologically-relevant model. *Integr. Biol.*, **9**, 350-361.
- Ehrlich, A., Duche, D., Ouedraogo, G. and Nahmias, Y. (2019): Challenges and opportunities in the design of liver-on-chip microdevices. *Annu. Rev. Biomed. Eng.*, **21**, 219-239.
- Ejima, C., Kuroda, H. and Ishizaki, S. (2016): A novel diet-induced murine model of steatohepatitis with fibrosis for screening and evaluation of drug candidates for nonalcoholic steatohepatitis. *Physiol. Rep.*, **4**, e13016.
- Ergen, C., Niemietz, P.M., Heymann, F., Baues, M., Gremse, F., Pola, R., Bloois, L., Storm, G., Kiessling, F., Trautwein, C., Luedde, T., Lammers, T. and Tacke, F. (2019): Liver fibrosis affects the targeting properties of drug delivery systems to macrophage subsets *in vivo*. *Biomaterials*, **206**, 49-60.
- Fausto, N., Campbell, J.S. and Riehle, K.J. (2006): Liver regeneration. *Hepatology*, **43** (S1), S45-S53.
- Fitzpatrick, E., Wu, Y., Dhadda, P., Hughes, R.D., Mitry, R.R., Qin, H., Lehec, S.C., Heaton, N.D. and Dhawan, A. (2015): Coculture with mesenchymal stem cells results in improved viability and function of human hepatocytes. *Cell Transplant.*, **24**, 73-83.
- Fujita, R., Hui, T., Chelly, M. and Demetriou, A.A. (2005): The effect of antioxidants and a caspase inhibitor on cryopreserved rat hepatocytes. *Cell Transplant.*, **14**, 391-396.
- Fuller, B.J., Petrenko, A.Y., Rodriguez, J.V., Somov, A.Y., Balaban, C.L. and Guibert, E.E. (2016): Biopreservation of hepatocytes: current concepts on hypothermic preservation, cryopreservation, and vitrification. *Cryo Lett.*, **37**, 432-452.
- Iglewicz, B. and Hoaglin, D.C. (1993). *How to detect and handle outliers* (Vol. 16). *Asq Press*.
- Jellali, R., Bricks, T., Jacques, S., Fleury, M.J., Paullier, P., Merlier, F. and Leclerc, E. (2016): Long-term human primary hepatocyte

- cultures in a microfluidic liver biochip show maintenance of mRNA levels and higher drug metabolism compared with Petri cultures. *Biopharm. Drug Dispos.*, **37**, 264-275.
- Kozakai, K., Yamada, Y., Oshikata, M., Kawase, T., Suzuki, E., Haramaki, Y., and Taniguchi, H. (2013). Cocktail-substrate approach-based high-throughput assay for evaluation of direct and time-dependent inhibition of multiple cytochrome P450 isoforms. *Drug metabolism and pharmacokinetics*, DMPK-13.
- Krause, P., Saghatolislam, F., Koenig, S., Unthan-Fechner, K. and Probst, I. (2009): Maintaining hepatocyte differentiation *in vitro* through co-culture with hepatic stellate cells. *In Vitro Cell. Dev. Biol. Anim.*, **45**, 205-212.
- Len, J.S., Koh, W.S. and Tan, S.X. (2019): The roles of reactive oxygen species and antioxidants in cryopreservation. *Biosci. Rep.*, **39**.
- Li, A.P., Lu, C., Brent, J.A., Pham, C., Fackett, A., Ruegg, C.E. and Silber, P.M. (1999): Cryopreserved human hepatocytes: characterization of drug-metabolizing activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chem. Biol. Interact.*, **121**, 17-35.
- Liu, X. and Bi, Y. (2016): Y-27632 Increases Sensitivity of PANC-1 Cells to Epigallocatechin Gallate (EGCG) in Regulating Cell Proliferation and Migration. *Med. Sci. Monit.*, **22**, 3529.
- Liedtke, C., Luedde, T., Sauerbruch, T., Scholten, D., Streetz, K., Tacke, F., Tolba, R., Trautwein, C., Trebicka, J. and Weiskirchen, R. (2013): Experimental liver fibrosis research: update on animal models, legal issues and translational aspects. *Fibrogenesis Tissue Repair*, **6**, 1-25.
- Liguori, G.R., Jeronimus, B.F., de Aquinas Liguori, T.T., Moreira, L.F. and Harmsen, M.C. (2017): Ethical issues in the use of animal models for tissue engineering: reflections on legal aspects, moral theory, three rs strategies, and harm-benefit analysis. *Tissue Eng. Part C Methods*, **23**, 850-862.
- Ma, J., Zou, C., Guo, L., Seneviratne, D.S., Tan, X., Kwon, Y.-K., An, J., Bowser, R., DeFrances, M.C. and Zarnegar R. (2014): Novel Death Defying Domain in met entraps the active site of caspase-3 and blocks apoptosis in hepatocytes. *Hepatology*, **59**, 2010-2021.
- Nakamura, T. and Mizuno, S. (2010): The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci.*, **86**, 588-610.
- Nishikawa, M., Kojima, N., Komori, K., Yamamoto, T., Fujii, T. and Sakai, Y. (2008): Enhanced maintenance and functions of rat hepatocytes induced by combination of on-site oxygenation and coculture with fibroblasts. *J. Biotechnol.*, **133**, 253-260.
- Nishikawa, M., Ito, H., Tokito, F., Hirono, K., Inamura, K., Scheidecker, B., Danoy, M., Kawanishi, T., Arakawa, H., Kato, Y., Esashika, K., Miyasako, H. and Sakai Y. (2022). Accurate Evaluation of Hepatocyte Metabolisms on a Noble Oxygen-Permeable Material With Low Sorption Characteristics. *Frontiers in Toxicology*, 56.
- Ölander, M., Wiśniewski, J.R., Flörkemeier, I., Handin, N., Urdzik, J. and Artursson, P. (2019): A simple approach for restoration of differentiation and function in cryopreserved human hepatocytes. *Arch. Toxicol.*, **93**, 819-829.
- Prodanov, L., Jindal, R., Bale, S.S., Hegde, M., McCarty, W.J., Golberg, I., Bhushan, A., Yarmush, M.L. and Usta O.B. (2016): Long-term maintenance of a microfluidic 3D human liver sinusoid. *Biotechnol. Bioeng.*, **113**, 241-246.
- Redza-Dutordoir, M. and Averill-Bates, D.A. (2016): Activation of apoptosis signalling pathways by reactive oxygen species. *Biochimica et Biophysica Acta (BBA)*-. *Molecular Cell Research*, **1863**, 2977-2992.
- Sabbagh, L., Bourbonnière, M., Sékaly, R.P. and Cohen, L.Y. (2005): Selective up-regulation of caspase-3 gene expression following TCR engagement. *Mol. Immunol.*, **42**, 1345-1354.
- Scheidecker, B., Shinohara, M., Sugimoto, M., Danoy, M., Nishikawa, M. and Sakai, Y. (2020): Induction of *in vitro* Metabolic Zonation in Primary Hepatocytes Requires Both Near-Physiological Oxygen Concentration and Flux. *Front. Bioeng. Biotechnol.*, **8**, 524.
- Smith, C.M., Nolan, C.K., Edwards, M.A., Hatfield, J.B., Stewart, T.W., Ferguson, S.S., Lecluyse, E.L. and Sahi, J. (2012): A comprehensive evaluation of metabolic activity and intrinsic clearance in suspensions and monolayer cultures of cryopreserved primary human hepatocytes. *J. Pharm. Sci.*, **101**, 3989-4002.
- Steinberg, P., Fischer, T., Kiulies, S., Biefang, K., Platt, K.-L., Oesch, F., Böttger, T., Bulitta, C., Kempf P. and Hengstler J., (1999): Drug metabolizing capacity of cryopreserved human, rat, and mouse liver parenchymal cells in suspension. *Drug Metab. Dispos.*, **27**, 1415-1422.
- Stéphenne, X., Najimi, M. and Sokal, E.M. (2010): Hepatocyte cryopreservation: is it time to change the strategy? *World journal of gastroenterology*. *WJG*, **16**, 1.
- Tokunaga, Y., Osawa, Y., Ohtsuki, T., Hayashi, Y., Yamaji, K., Yamane, D., Hara, M., Munekata, K., Tsukiyama-Kohara, K., Hishima, T., Kojima, S., Kimura K., and Kohara M., (2017): Selective inhibitor of Wnt/ $\beta$ -catenin/CBP signaling ameliorates hepatitis C virus-induced liver fibrosis in mouse model. *Sci. Rep.*, **7**, 1-11.
- Ware, B.R., Durham, M.J., Monckton, C.P. and Khetani, S.R. (2018): A cell culture platform to maintain long-term phenotype of primary human hepatocytes and endothelial cells. *Cell. Mol. Gastroenterol. Hepatol.*, **5**, 187-207.