An Algorithm that Predicts the Viability and the Yield of Human Hepatocytes Isolated from Remnant Liver Pieces Obtained from Liver Resections

Serene M. L. Lee¹, Celine Schelcher²,³, Rüdiger P. Laubender³, Natalja Fröse¹, Reinhard M. K. Thasler², Tobias S. Schiergens¹, Ulrich Mansmann³, Wolfgang E. Thasler¹ *

¹ Department of General, Visceral, Transplantation, Vascular and Thoracic Surgery, Grosshadern Hospital, Ludwig Maximilians University, Munich, Germany, ² Tissue Bank, Department of General, Visceral, Transplantation, Vascular and Thoracic Surgery, Grosshadern Hospital, Ludwig Maximilians University, Munich, Germany, ³ Institute for Medical Information Processing, Biometry and Epidemiology, Grosshadern Hospital, Ludwig Maximilians University, Munich, Germany

Abstract

Isolated human primary hepatocytes are an essential in vitro model for basic and clinical research. For successful application as a model, isolated hepatocytes need to have a good viability and be available in sufficient yield. Therefore, this study aims to identify donor characteristics, intra-operative factors, tissue processing and cell isolation parameters that affect the viability and yield of human hepatocytes. Remnant liver pieces from tissue designated as surgical waste were collected from 1034 donors with informed consent. Human hepatocytes were isolated by a two-step collagenase perfusion technique with modifications and hepatocyte yield and viability were subsequently determined. The accompanying patient data was collected and entered into a database. Univariate analyses found that the viability and the yield of hepatocytes were affected by many of the variables examined. Multivariate analyses were then carried out to confirm the factors that have a significant relationship with the viability and the yield. It was found that the viability of hepatocytes was significantly decreased by the presence of fibrosis, liver fat and with increasing gamma-glutamyltranspeptidase activity and bilirubin content. Yield was significantly decreased by the presence of liver fat, septal fibrosis, with increasing aspartate aminotransferase activity, cold ischemia times and weight of perfused liver. However, yield was significantly increased by chemotherapy treatment. In conclusion, this study determined the variables that have a significant effect on the viability and the yield of isolated human hepatocytes. These variables have been used to generate an algorithm that can calculate projected viability and yield of isolated human hepatocytes. In this way, projected viability can be determined even before isolation of hepatocytes, so that donors that result in high viability and yield can be identified. Further, if the viability and yield of the isolated hepatocytes is lower than expected, this will highlight a methodological problem that can be addressed.

Introduction

The liver carries out a diverse range of necessary functions, such as homeostasis, metabolism and detoxification. As much of the research on the liver is human-centric, whether for the elucidation of mechanisms, translational research or cell-based therapy, isolated human liver cells remain an important in vitro model for basic and translational research.

One of the main uses of a human in vitro hepatocyte model is for the validation of studies done using animal models due to species differences. Olson et al., [1] showed that when 150 drugs that cause human toxicity are tested, the concordance between toxicity found in animal studies and that observed in clinical practice is 70%. Similarly, Brambilla and Martelli found that when 42 compounds from various chemical families were tested for their toxicity in rat or human hepatocytes, 28 had similar toxicities, 10 were more toxic for rats, 3 were moderately more toxic for human hepatocytes and 1 was lethal for rat hepatocytes at a concentration 30-fold lower than that equally toxic for human hepatocytes [2]. In addition, animal models could also have less genetic variation than humans; Brambilla and Martelli [2] found
Table 1. Summary of factors affecting the viability of isolated hepatocytes.

| Variables     | Decreased viability                                                                 | No change in viability                                                                 | Increased viability                                                                 |
|---------------|-------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| Age           | Increased age [12,38]                                                               | Different ages [15,39]                                                                | Decreased age* [16]                                                               |
| Gender        | Male or female [12,13,39]                                                           | D.                                     |                                                                                |
| Fibrosis      | Fibrotic organs [39]                                                               | Organs that are not steatotic, fibrotic or cirrhotic [39]                            |                                                                                |
| Cirrhosis     | Cirrhotic organs [39]                                                             | Organs that are not steatotic, fibrotic or cirrhotic [39]                             |                                                                                |
| Steatosis     | Severe steatotic organs or organs that are not steatotic, fibrotic or cirrhotic [39] | D.                                     | Visually steatotic liver [12]                                                    |
| Disease       | Malignant disease [15]                                                             | Colonic secondary, cholangiocarcinoma, carcinoid, hepatocellular tumour, unknown primary, hydatid cyst, lung secondary or multi-organ donors [12], primary biliary cirrhosis or primary sclerosing cholangitis [9] | Benign disease [15]                                                             |
| Chemotherapy  | Treated or untreated [14]                                                           | D.                                     |                                                                                |
| Serum enzymes | Increased pre-operative GGT levels [15]                                             | D.                                     |                                                                                |
| Operation type| Right hepatectomy, segmental resection, left hepatectomy, extended right, local excision or multi-organ donor [12] | D.                                     |                                                                                |
| Warm ischemia | Increased time* [16]                                                               | Varying time [11], no pringle or varying Pringle times [12]                          |                                                                                |
| Cold ischemia | >20 h [39]                                                                          | Up to 4 h [12], varying times [38,39]                                               | <10 h [39]                                                                      |

*Statistics done with multiple regression analysis. All other variables were analysed using univariate analyses.
doi:10.1371/journal.pone.0107567.t001

Table 2. Summary of factors affecting the yield of isolated hepatocytes.

| Variables     | Decreased yield                                                                 | No change in yield                                                                 | Increased yield                                                                 |
|---------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Age           | >50 years old [15]                                                              | Different ages [10,13,39,40]                                                      |                                                                                |
| Gender        | Male or female [12,13,39]                                                        | D.                                                                                 |                                                                                |
| Fibrosis      | Fibrotic organs [39]                                                            | Organs that are not steatotic, fibrotic or cirrhotic [39]                        |                                                                                |
| Cirrhosis     | Cirrhotic organs [39]                                                           | Organs that are not steatotic, fibrotic or cirrhotic [39]                        |                                                                                |
| Steatosis     | Severe steatotic organs [39]                                                     | No steatosis, <10% steatosis or >10% steatosis [10], no steatosis or >10% steatosis [13] | Organs that are not steatotic, fibrotic or cirrhotic [39] |
| Disease       | Malignant disease [15]                                                          | Benign hepatic diseases, metastases from colorectal cancer, hepatic primitive malignant tumours or metastases from non-colorectal cancer [10], colonic secondary, cholangiocarcinoma, carcinoid, hepatocellular tumour, unknown primary, hydatid cyst, lung secondary or multi-organ donors [12], benign hepatic disease, metastases from colorectal cancer, hepatic primitive malignant tumours or metastases from non-colorectal cancer [13], primary biliary cirrhosis or primary sclerosing cholangitis [9] | Benign disease [15] |
| Chemotherapy  | Treated or untreated [10,14]                                                      | D.                                                                                 |                                                                                |
| Serum enzymes | Increased pre-operative GGT levels [10]                                          | Increased pre-operative ALT or AST levels [10]                                    |                                                                                |
| Operation type| Right hepatectomy, segmental resection, left hepatectomy, extended right, local excision or multi-organ donor [12] | D.                                                                                 |                                                                                |
| Warm ischemia | Intermittent clamping [13]                                                       | Varying time [11], no pringle or varying Pringle times [12], no clamping, continuous or intermittent clamping [13] | No clamping [13]                                                             |
| Cold ischemia | Up to 4 h [12], up to 5 h [13], varying times [39]                             | D.                                                                                 |                                                                                |
| Perfused liver| 100-200 g liver pieces [12], increased liver weight [16]                        | Varying weights [10,13]                                                          |                                                                                |

doi:10.1371/journal.pone.0107567.t002
that the inter-individual variability in hepatocyte responses to chemicals that cause cytotoxicity is greater for humans than for rats. This would make it harder to detect idiosyncratic drug-induced liver injury.

The usage of human hepatocytes comes with the additional advantage of following the 3R ethical framework [3] to replace the use of research animals when possible. This is as the liver tissue used in this study was obtained from human elective liver resections. After resection, the tissue was immediately brought to a pathologist, who would take what was required for histopathological evaluation. The rest of the tissue, which is not needed, was designated as surgical waste. If a patient had signed an informed

### Table 3. Variables considered for statistical analyses.

| Variables                          | Categories                                  | Abbreviation | Unit |
|------------------------------------|---------------------------------------------|--------------|------|
| **Donor characteristics**          |                                             |              |      |
| Age                                |                                             |              |      |
| Gender                             | Male or female                              |              |      |
| Body mass index                    | BMI                                         |              |      |
| Fibrosis                           | Yes or no                                   |              |      |
| Cirrhosis                          | Yes or no                                   |              |      |
| Diabetes                           | Yes or no                                   |              |      |
| Obesity                            | Yes or no                                   |              |      |
| Hypertension                       | Yes or no                                   |              |      |
| Hypercholesterolemia               | Yes or no                                   |              |      |
| Hyperuricemia                      | Yes or no                                   |              |      |
| Smoking                            | Yes, no or ex-smoker                        |              |      |
| Cigarettes per day                 |                                             |              |      |
| Liver fat                          | Yes or no                                   |              |      |
| Liver fat                          |                                             | %            |      |
| Tumour type                        | Benign or malignant                         |              |      |
| Surgical indication                | Hepatocarcinoma (HCC), metastasis, focalnodular hyperplasia (FNH), klatskin, adenoma, cholangiocarcinoma (CCC) or others |              |      |
| Chemotherapy                       | Treated or untreated                        |              |      |
| ASA physical status classification system | 1, 2, 3 or 6 ASA |            |      |
| Ludwig score                       | No or minimal fibrotic changes, periportalfibrosis, septal fibrosis or cirrhosis |              |      |
| **Clinical chemistry results before operation** |                                             |              |      |
| Alkaline phosphatase activity      | AP                                          | U/L          |      |
| Aspartate aminotransferase activity| GOT                                         | U/L          |      |
| Gamma-glutamyltranspeptidase activity| GGT                                         | U/L          |      |
| Alanine aminotransferase activity  | GPT                                         | U/L          |      |
| Cholinesterase activity            | CHE                                         | U/L          |      |
| Bilirubin                          |                                             | mg/dL        |      |
| Partial thromboplastin time        | PTT                                         | s            |      |
| Quick value                        |                                             | %            |      |
| **Operation parameters**           |                                             |              |      |
| Operation type                     | Hemihepatectomy right (HR), Hemihepatectomy left (HL), segment resection (SR), atypical resection (AR) extended hepatectomy (EH), liver transplantation (LT) or lobectomy (L) |              |      |
| Warm ischemia in vivo              |                                             | min          |      |
| Warm ischemia ex vivo              |                                             | min          |      |
| Weight of resected liver           |                                             | g            |      |
| **Tissue processing and cell isolation parameters** |                                             |              |      |
| Cold ischemia                      |                                             | min          |      |
| Weight of perfused liver           |                                             | g            |      |

doi:10.1371/journal.pone.0107567.t003
consent [4], this discarded tissue could then be collected for hepatocyte isolation.

In order to successfully use human hepatocytes as an in vitro model or for cell-based therapy, hepatocytes must be obtained with good viability and hence quality. Further, as the sources of human hepatocytes are limited and the cost of the entire process from informed consent to successfully obtaining hepatocytes is high, it is also important to understand the factors that can result in a compromised yield. Thus far, the literature on factors that affect viability and yield is contradictory (Tables 1 and 2). Further, the previously done studies had a small sample size ranging from 10 to 149 donors. Therefore, this study aimed to determine, with a large number of donors and hepatocyte isolations carried out over 10 years, the donor characteristics, medical histories and clinical chemistry results before operation.

### Table 4. The number of replicates (N) and the P values obtained after linear regression of the individual variables listed below to viability (%) or yield (million hepatocytes/g liver) of isolated human hepatocytes.

| Variables                              | Viability | Yield |
|----------------------------------------|-----------|-------|
|                                        | N         | P value | N         | P value |
| **Donor characteristics**              |           |        |           |         |
| Age                                    | 1030      | 0.027* | 1026      | 0.00067*|
| Gender                                 | 1032      | 6.3×10⁻⁷* | 1028 | 1.6×10⁻⁸* |
| Body mass index³                       | 1006      | 0.022* | 1002      | 0.026*  |
| Fibrosis                               | 910       | 0.0016* | 905       | 0.074   |
| Cirrhosis                              | 907       | 0.76    | 902       | 1.1×10⁻⁵*|
| Diabetes                               | 1014      | 0.23    | 1009      | 0.022*  |
| Obesity                                | 1015      | 0.89    | 1010      | 0.44    |
| Hypertension                           | 1014      | 0.21    | 1009      | 0.052   |
| Hypercholesterolemia                   | 1012      | 0.32    | 1007      | 0.87    |
| Hyperuricemia                          | 1010      | 0.48    | 1005      | 0.037*  |
| Smoking                                | 573       | 0.93    | 569       | 0.091   |
| Liver fat                              | 886       | 0.00052* | 881 | 0.021*   |
| Liver fat (%)                          | 522       | 0.47    | 517       | 3.6×10⁻⁸*|
| Tumour type                            | 995       | 0.51    | 991       | 0.063   |
| Surgical indication                    | 1017      | 0.053   | 1013      | 1.6×10⁻⁵*|
| Chemotherapy                           | 1027      | 0.31    | 1023      | 3.5×10⁻⁵*|
| ASA physical status classification system | 990  | 0.90    | 986       | 0.054   |
| Ludwig score                           | 813       | 0.0010* | 809       | 7.5×10⁻⁸*|
| **Clinical chemistry results before operation** | | | | |
| Alkaline phosphatase activity (U/L)³  | 797       | 0.067   | 792       | 7.3×10⁻⁷*|
| Aspartate aminotransferase activity (U/L)³ | 712  | 0.00012* | 709 | 0.00016* |
| Gamma-glutamyltranspeptidase activity (U/L)³ | 690  | 0.0015* | 687       | 4.2×10⁻⁷*|
| Alanine aminotransferase activity (U/L)³ | 812  | 2.9×10⁻⁴* | 808 | 2.7×10⁻⁹*|
| Cholinesterase activity (U/L)³         | 714       | 0.97    | 713       | 0.74    |
| Bilirubin (mg/dL)³                     | 810       | 0.0022* | 805       | 0.00087*|
| Partial thromboplastin time (s)³      | 799       | 0.88    | 794       | 0.035*  |
| Quick value (%)                        | 803       | 0.027*  | 798       | 0.015*  |
| **Operation parameters**               |           |        |           |         |
| Operation type                         | 992       | 0.16    | 989       | 3.3×10⁻⁸*|
| Warm ischemia in vivo (min)³           | 602       | 0.47    | 602       | 0.00068*|
| Warm ischemia ex vivo (min)³           | 888       | 3.9×10⁻⁸* | 887 | 0.00054* |
| Weight of resected liver (g)³         | 839       | 0.092   | 836       | 0.00066*|
| **Tissue processing and cell isolation parameters** | | | | |
| Cold ischemia (min)³                   | 913       | 0.027*  | 914       | 0.19    |
| Weight of perfused liver (g)³         | 1030      | 0.68    | 1027      | 1.2×10⁻¹¹*|

*Significant at P≤0.05. Data are transformed to follow a normal distribution by logit¹, fourth root² or natural logarithm³ transformation.
doi:10.1371/journal.pone.0107567.t004
Table 5. The regression coefficients (β), P values and $R^2$ numbers of variables after multivariate analyses for the dependent variable of viability (%) of isolated human hepatocytes.

| Variables                              | Viability (%) | β         | P value  |
|----------------------------------------|---------------|-----------|----------|
| Donor characteristics                  |               |           |          |
| Fibrosis                               |               | −0.18     | 0.040*   |
| Liver fat                              |               | −0.22     | 0.0065*  |
| Clinical chemistry results before operation |           |           |          |
| Gamma-glutamyltranspeptidase activity (U/L) |   | −0.088   | 0.017*   |
| Bilirubin                              |               | −0.17     | 0.0095*  |
| $R^2 = 0.12$, Intercept = 1.81         |               |           |          |

Variables presented are chosen by backward elimination.

$^*$Significant at $P<0.05$, with $N=218$. Data are transformed to follow a normal distribution by logit1 or natural logarithm2 transformation.

doi:10.1371/journal.pone.0107567.t005

operation, tissue processing and cell isolation parameters that affect the viability and the yield of isolated human hepatocytes.

Material and Methods

Ethics Statement

The liver pieces used for hepatocyte isolation were collected from resected liver specimens designated as surgical waste after examination by a pathologist. In particular, the tissue used was dissected from the resection margin of tumours containing morphologically healthy tissue. All liver pieces were collected with their associated clinical data by the Tissue Bank under the Administration of the Human Tissue and Cell Research (HTCR) Foundation (http://www.htcr.de/english/contacts.html) [4]. The HTCR-process included written informed consent, was approved by the Ethics Committee of the Medical Faculty of Regensburg University Hospital (approval number 99/46) and Ethics Committee of the Medical Faculty of Ludwig Maximilians University (approval number 025-12) and complied with the Bavarian Data Protection Act.

Patients

In total, hepatocytes were isolated from remnant liver pieces from 1034 patients from Regensburg University Hospital (December 1997 to December 2002 and July 2010 to December 2011) and Grosshadern Hospital located in Munich (January 2003 to December 2013).

Corresponding data on the donor characteristics, medical histories and operation, tissue processing and cell isolation parameters were collected and entered into a database. The variables of interest for this study are listed on Table 3.

Isolation of Human Hepatocytes

Primary human hepatocytes were isolated using a two-step collagenase perfusion technique [5,6] with modifications [7]. In short, the larger blood vessels on a liver piece with one cut face were cannulated with irrigation cannulae with olive tips. The liver piece was then perfused first with 1 L of Solution 1, which contains 154 mM sodium chloride, 20 mM HEPES, 5.6 mM potassium chloride, 5 mM glucose and 25 mM sodium hydrogen carbonate. Next, it was perfused for 10 min with Solution 2 (152.5 mM sodium chloride, 19.8 mM HEPES, 5.5 mM potassium chloride, 5 mM glucose and 24.8 mM sodium hydrogen carbonate and 0.1 mM EGTA) followed by Solution 3 (152.5 mM sodium chloride, 19.8 mM HEPES, 5.5 mM potassium chloride, 5 mM glucose and 24.8 mM sodium hydrogen carbonate and 0.5 mM calcium chloride dihydrate) for 0.5 L. Finally, it was perfused with Solution 4 (120 mM sodium chloride, 10 mM HEPES, 0.9 mM calcium chloride dehydrate, 6.2 mM potassium chloride and 0.1% w/v albumin), which contains 0.1 to 0.15% w/v collagenase for 9 to 12 minutes or until the liver is sufficiently digested. The liver piece was then placed carefully in a crystallising dish for removal of the Glisson’s capsule before gently shaking the cells loose. The cell suspension was then filtered through a 210 μm nylon mesh followed by a 70 μm nylon mesh before centrifuging at 72 g for 5 min at 4°C to pellet the hepatocytes. Hepatocytes were then washed 3 times before resuspending the cells in Cold Storage Solution (Hepacult GmbH, Germany).

A hemocytometer-based trypan blue dye exclusion assay was done to quantify the viability and total cells yielded by this isolation procedure.

Statistical Analyses

The data were summarised by adequate measures of location and spread. For modelling the outcomes of “viability” and “yield”, linear regression modelling was used when the variables in Table 3 were considered.

To account for the possibility of non-linear relationships between the considered outcome and the continuous covariates (Table 3), fractional polynomials of first and second degree were applied. For this purpose, the multivariable fractional polynomials (MFP) algorithm [8] was used. This algorithm combines the selection of the functional forms of each continuous covariate using fractional polynomials with the selection of all continuous and non-continuous covariates via backward elimination. For the multiple regression models, only donors with a complete set of information for the variables of interest were used. Therefore, 218 donors were considered for viability and 128 donors were considered for yield. The selection level for potential predictors was set to 0.05.

In order to satisfy the assumption of normality, viabilities were transformed by applying the logit and yields by applying the fourth root. Graphical procedures were used to assess the fit of the model. All tests were performed two-sided and a p-value lower than 0.05.
Results

A total of 1034 hepatocyte isolations were done with an average viability of 78±10% and average yield of 13±11 million viable hepatocytes per gram liver with the values represented in means ± standard deviation.

Univariate analyses to determine relationships of variables to viability and yield of hepatocytes

After linear regression analyses were carried out, variables with or without a significant relationship to the viability and the yield of hepatocytes were listed in Tables 4 and 5 respectively. In addition, figures were generated for the variables with significant relationships to the viability (Figures 1 to 4) and the yield (Figures 5 to 9) of hepatocytes.

It was found that the viability of hepatocytes was decreased by increases in age, body mass index (BMI), aspartate aminotransferase (GOT) activity, gamma-glutamyltranspeptidase (GGT) activity, alanine aminotransferase (GPT) activity, bilirubin content in the blood, quick value, warm ischemia time \textit{in vivo} and cold ischemia time (Table S1). In addition, the viability of hepatocytes was also decreased for males and for donors with fibrosis, liver fat or Ludwig scores indicating periportal fibrosis or septal fibrosis (Table S1).

In the case of the yield of hepatocytes, it was found that the yield was decreased by increases in age, BMI, liver fat, alkaline phosphatase (AP) activity, GOT activity, GGT activity, GPT activity, bilirubin content in the blood, partial thromboplastin time (PTT), warm ischemia time \textit{in vivo} and weight of resected or perfused liver (Table S2). Further, the yield of hepatocytes was also decreased for males and for donors with cirrhosis, diabetes, hyperuricemia or certain surgical indications, operation types or Ludwig scores (Table S2). However, the yield of hepatocytes can be increased by increases in warm ischemia time \textit{ex vivo}, Quick value and in donors treated with chemotherapy (Table S2).

Multivariate analyses to determine the variables that affect the viability and the yield of hepatocytes

After multivariate analysis, the number of variables that have a significant effect on the viability of hepatocytes was reduced to 4 variables. It was found that the viability of hepatocytes was significantly decreased by the presence of fibrosis, liver fat and with increasing GGT activity and bilirubin content (Table 5).

For the yield of hepatocytes, it was found that yield was significantly decreased by the presence of liver fat and a Ludwig score indicating septal fibrosis. In addition, the yield of hepatocytes was decreased by increasing GOT activity, cold ischemia time and weight of perfused liver. However, the yield of hepatocytes was increased with chemotherapy treatment (Table 6).
Figure 2. Variables measured in the blood or serum that have significant relationships with the viability (%) of hepatocytes after linear regression analyses. Figures show relationships between viability and (A) aspartate aminotransferase activity (GOT; U/L), (B) gamma-glutamyltranspeptidase activity (GGT; U/L), (C) alanine aminotransferase activity (GPT; U/L), (D) bilirubin (mg/dL) or (E) quick value (%). Values were deemed significant when $P < 0.05$.

doi:10.1371/journal.pone.0107567.g002
Generation of a model that allows for the calculation of projected viability and yield of hepatocytes

The information obtained from the multivariate analyses allowed the generation of formulae for the calculation of projected viability and the yield of isolated human hepatocytes as shown below.

For the calculation of projected viability (% of hepatocytes)

**Formula 1.** Linear predictor of viability

\[
\text{Linear predictor of viability} = b_0 + b_1 \times \log_e(\text{bilirubin}) + b_2 \times \text{fibrosis} + b_3 \times \text{liver fat} + b_4 \times \log_e(\text{GGT} + 1)
\]

The values of the various constants are as follows; \(b_0 = 1.809\), \(b_1 = -0.169\), \(b_2 = -0.178\), \(b_3 = -0.216\) and \(b_4 = -0.088\). While the continuous variables can be directly substituted with the absolute values recorded for a patient, categorical variables have to be substituted with “0” or “1”. For “fibrosis” – “0” for donors with no fibrosis and “1” for donors with fibrosis; for “liver fat” – “0” for donors with no liver fat and “1” for donors with liver fat.

**Formula 2.** Viability (%) = \(e^{(\text{linear predictor of viability})} \times 100\)

For the calculation of projected yield (million hepatocytes/g liver)

**Formula 3.** Linear predictor of yield

\[
\text{Linear predictor of yield} = b_0 + b_1 \times \text{Chemotherapy} + b_2 \times \log_e(\text{Cold ischemia} + 1) + b_3 \times \log_e(\text{Cirrhosis} + b_4 \times \text{Liver fat} + b_5 \times (\text{Ludwig score}) + b_6 \times (\text{Periportal fibrosis} + b_7 \times (\text{Ludwig score}) + b_8 \times \log_e(\text{GOT activity} + 1)
\]

The values of the various constants are as follows; \(b_0 = 3.137\), \(b_1 = 0.19\), \(b_2 = -0.099\), \(b_3 = -0.159\), \(b_4 = -0.007\), \(b_5 = -0.108\), \(b_6 = 0.042\), \(b_7 = -0.211\) and \(b_8 = -0.114\). While the continuous variables can be directly substituted with the absolute values recorded for a patient, categorical variables have to be substituted with “0” or “1”. For “chemotherapy” – “0” for donors with no chemotherapy and “1” for donors treated with chemotherapy; for the variable “Ludwig score”, the Ludwig score category of the donor should be set to “1” while all other not applicable Ludwig score categories should be set to “0” for calculation. For example, if the donor has “cirrhosis”, this variable should be set to 1 at the same time as setting the variables “periportal fibrosis” and “septal fibrosis” to 0.

**Formula 4.** Yield (million hepatocytes/g liver) = \(e^{(\text{linear predictor of yield})} \times 100\)

Validation of the models for calculating projected viability and yield of isolated hepatocytes

The appropriateness of the models for calculating the projected viability and yield of isolated hepatocytes are very similar and can be seen in Figures 10 and 11 respectively. Firstly, the residuals versus fitted plots (Figures 10A and 11A) show that there is no systematic relationship between the residuals and the predicted (or so-called fitted) values. These two figure panels (Figures 10A and 11A) show that there is no systematic relationship between the residuals and the predicted (or so-called fitted) values.
11A) also show that there are no systematic tendencies in the errors, such as heteroscedasticity etc.

Secondly, the normal quintile plots (Figures 10B and 11B) show that the residuals are approximately normally distributed. This distribution is necessary in order to obtain valid test statistics and $P$ values of the regression coefficient.

Thirdly, the square root of the standardised residuals versus fitted plots (Figures 10C and 11C) show that there is no systematic relationship between the residuals and the predicted (or fitted) values. As before, these graphs also do not show systematic tendencies in the errors such as heteroscedasticity. However, in contrast to the residuals versus fitted plot, the standardised residuals were normalised once again, so that the residuals to have unit variance, using an overall measure of the error variance.

Finally, the standardised residuals versus leverage plots (Figures 10D and 11D) show the influence of regression results when leaving out a single observation from the dataset. Leverage can be used to detect multivariate outliers in the data, but in this case, the leverage is so small that no limits indicating big leverage of a single or several observations appear in our data.

Discussion

This study aimed to determine donor characteristics, medical histories and operation, tissue processing and cell isolation parameters that affect the viability and the yield of isolated human hepatocytes. In order to do this, univariate analyses were first run to determine the variables that had a significant relationship to the viability or the yield of isolated human hepatocytes. Next, multiple regression analyses were run to determine the relative contributions of the various variables on the outcomes of viability and yield. From the results of the multiple regression analyses, a model was built to predict the viability and the yield of isolated hepatocytes (see Formulae 1–4 in results).

Residual analyses were then done to check the regression assumptions in order to ensure that the model was appropriate.

This study has to the authors’ knowledge, the largest number of donors examined for univariate analyses with 1034 donors and a sample size between 517 and 1032 for the individual variables. In contrast, other studies that carried out univariate analyses had sample sizes between 10 and 149 [9–15]. As a result, this study detects statistical significance in more variables than in the other studies (Tables 4, S1 and S2), probably due to an increase in statistical power. However, when multiple regression analyses were done, a reduced number of variables were found to be statistically significant. Typically, this is the case when multiple regression analyses are carried out, but it is important to consider that there is a loss of statistical power as the sample sizes were 218 for viability and 128 for yield of isolated hepatocytes as only cases with completed data on all the variables of interest were considered. However, as the other study that has conducted multiple regression analyses to the authors’ knowledge has a sample size of 90 [16], this study still contributes useful information that will be discussed below.

When the absolute viability and yield numbers are considered, the hepatocytes isolated according to the authors’ protocol [7] have a good balance of high viability (77 ± 0.3) and yield (13.4 ± 0.4) comparable to other groups with good results (Table 7). The comparison of variables found to have significant effects on the viability and yield of hepatocytes to what is known in the literature is challenging, as the results obtained by other groups are often contradictory (Tables 1 and 2). As such, the following paragraphs will instead focus on discussing the results of the multiple regression analyses.

After multiple regression analyses, this investigation has found that the viability of isolated human hepatocytes was significantly decreased by the presence of fibrosis, liver fat and with increasing GGT activity and bilirubin content (Formula 1). In the case of the
Figure 5. Donor characteristics that have significant relationships with the yield (million hepatocytes/gram liver) after linear regression analyses. Figures show relationships between yield and (A) age, (B) gender, (C) body mass index (BMI), (D) diabetes, (E) hyperuricemia or (F) chemotherapy. Values were deemed significant when $P < 0.05$.

doi:10.1371/journal.pone.0107567.g005
yield of isolated human hepatocytes, it was found that the yield was significantly decreased by the presence of liver fat, a Ludwig score indicating septal fibrosis and by increasing GOT activity, cold ischemia time and weight of perfused liver. Further, the yield of hepatocytes was increased with chemotherapy treatment (Formula 3).

Increased cold ischemia time has been found to decrease the yield of isolated hepatocytes. In this study, the liver piece used for hepatocyte isolation was not perfused to remove the blood in the tissue before transport on ice to the laboratory. As a result, the formation of blood clots can occur in the tissue in the cases with longer transportation times even though the clotting process is slowed by low temperatures [17]. This could then affect the perfusion of the liver during the hepatocyte isolation process and decrease the yield. Yield is also decreased when the weight of the perfused liver is increased. Alexandre et al. [10] showed that the percentage of undigested tissue left after the isolation process is significantly increased in larger pieces of liver above 101 g. Also,

![Figure 6. Variables measured in the blood or serum that have significant relationships with the yield (million hepatocytes/gram liver) after linear regression analyses. Figures show relationships between yield and (A) alkaline phosphatase activity (AP; U/L), (B) aspartate aminotransferase activity (GOT; U/L), (C) gamma-glutamyltranspeptidase activity (GGT; U/L), (D) alanine aminotransferase activity (GPT; U/L), (E) bilirubin (mg/dL), (F) partial thromboplastin time (PTT; s) or (G) quick value (%). Values were deemed significant when P<0.05. doi:10.1371/journal.pone.0107567.g006](image)

![Figure 7. Liver variables that have significant relationships with the yield (million hepatocytes/gram liver) of hepatocytes after linear regression analyses. Figures show relationships between yield and (A) cirrhosis, (B) liver fat, (C) liver fat (%) or (D) Ludwig score. Values were deemed significant when P<0.05. For the variables of Ludwig score, operation type and surgical indication, variables not sharing the same alphabet are significantly different, P<0.05. doi:10.1371/journal.pone.0107567.g007](image)
the isolation set-up used in this study can provide a maximum of 8 cannulae for perfusing the liver piece [7]. Although the rate of perfusion per cannula is kept at similar levels independent of the size of the liver [7], it may be that portions of a larger liver are not perfused due to additionally available open blood vessels not being cannulated.

Fibrosis develops in response to many types of chronic liver diseases [18]. In such diseases, apoptosis plays a critical role both in liver injury and the subsequent fibrosis [18]. During the process of apoptosis, hepatocytes form apoptotic bodies that are phagocytosed by hepatic stellate cells resulting in an up-regulation of Transforming Growth Factor β (TGF-β) and procollagen α1, leading to subsequent inflammation and fibrogenesis [19–21]. Since fibrosis is closely linked with inflammation and cell death, it is not surprising that hepatocytes isolated from fibrotic livers have a significantly lower viability. In particular, this study found that septal fibrosis resulted in a significantly decreased yield of viable hepatocytes. The process of septal fibrosis begins with the extension of the septa between central veins through interhepatic cellular space and the space of Disse on the sides of the sinusoids [22]. As fibrosis proceeds, capillarization and then venularization occurs [22,23]. During this process, the fenestrations in some hepatic sinusoids are lost and the development of basal laminae occur alongside the collagenization of the extravascular spaces of

Figure 8. Operation variables that have significant relationships with the yield (millions hepatocytes/gram liver) of hepatocytes after linear regression analyses. Figures show relationships between yield and (A) surgical indication, (B) operation type, (C) warm ischemia in vivo (min) or (D) weight of resected liver (g). Values were deemed significant when P<0.05. Abbreviations; hepatocarcinoma (HCC), focal nodular hyperplasia (FNH), cholangiocarcinoma (CCC), hemihepatectomy right (HR), hemihepatectomy left (HL), segment resection (SR), atypical resection (AR), extended heptectomy (EH), lobectomy (L) and liver transplantation (LT).

doi:10.1371/journal.pone.0107567.g008
Disse [22,24]. This capillarization and fibrosis has been postulated to impair the leakage of macromolecules by creating a new barrier between the sinusoids and the hepatocytes [24]. This assertion has been supported by an approach utilising MRI, which shows that the extravascular distribution of high molecular weight contrast agents (6 or 52 kDa) is limited in a model of sinusoidal fibrosis [24]. These observations could explain the decreased yield obtained in this study due to septal fibrosis as collagenase that is 110 kDa will have limited access to the extracellular matrix in the direct vicinity of hepatocytes. Together with an increased amount of collagen surrounding capillaries that have to be digested, it is likely that the release of hepatocytes into a cell suspension is impaired and hence the reduced yield.

The presence of liver fat results in a significant decrease in the viability of hepatocytes. Hepatic steatosis, which is commonly found in obese or heavy alcohol drinkers, has been postulated to

![Image](image-url)

**Figure 9. Tissue processing and cell isolation variables that have significant relationships with the yield (million hepatocytes/gram liver) of hepatocytes after linear regression analyses.** Figures show relationships between yield and (A) warm ischemia *ex vivo* (min) or (B) weight of perfused liver (g). Values were deemed significant when P<0.05.

doi:10.1371/journal.pone.0107567.g009

### Table 6

The regression coefficients (β), P values and R² numbers of variables after multivariate analyses for the dependent variable of yield (million hepatocytes/g liver) of isolated human hepatocytes.

| Variables                                      | Yield¹ | β     | P value |
|------------------------------------------------|--------|-------|---------|
| **Donor characteristics**                     |        |       |         |
| Liver fat (%)                                  |        | −0.0069 | 0.0056* |
| Chemotherapy                                   |        | 0.19  | 0.0036* |
| Ludwig score                                   |        |       |         |
| -No or minimal fibrotic changes (reference)    |        | -     | -       |
| -Periportal fibrosis                           |        | 0.042 | 0.56    |
| -Septal fibrosis                               |        | −0.21 | 0.047*  |
| -Cirrhosis                                     |        | −0.11 | 0.52    |
| **Clinical chemistry results before operation**|        |       |         |
| Aspartate aminotransferase activity (U/L)²     |        | −0.11 | 0.016*  |
| **Tissue processing and cell isolation parameters** |        |       |         |
| Cold ischemia (min)²                           |        | −0.099 | 0.042*  |
| Weight of perfused liver (g)                   |        | −0.16 | 0.0018* |

R² = 0.32, Intercept = 3.14

*Significant at P<0.05, with N=128. Data are transformed to follow a normal distribution by fourth root¹ or natural logarithm² transformation.

doi:10.1371/journal.pone.0107567.t006

Variables presented are chosen by backward elimination.
be a “first hit” that increases sensitivity to a “second hit” that could then trigger a cascade leading to steatohepatitis [25]. Steatohepatitis is characterised by inflammation and liver cell damage and could therefore lead to lower hepatocyte viability. Further, steatotic hepatocytes have been found to have increased sensitivity to hypoxic injury i.e. lower viability, due to the attenuation of Hypoxia Inducible Factor 1α expression and protein accumulation [26]. The presence of liver fat also results in a significant decrease in the yield of hepatocytes. It has been found that fat accumulation in hepatocytes results in microvascular alterations [27]. Various studies have demonstrated that lipid accumulation results in enlargement of hepatocytes, which widen the parenchymal cell plates, narrow and distort the lumens of the sinusoids and hence reduce the intrasinusoidal volume [27]. As a result, the sinusoids have impaired tissue perfusion and become poor conduits for conducting the collagenase-containing buffer used in the cell isolation process and possibly leading to a lower yield of hepatocytes. In addition, during centrifugation steps in the isolation process, steatotic hepatocytes tend to form a pellet less effectively, making it more likely that hepatocytes are lost when the supernatant is aspirated off (Authors’ observation).

Levels of bilirubin and activities of GGT and GOT in the serum are standard assay parameters in liver function tests. Elevated levels of bilirubin and GGT, known indicators of hepatocyte injury [28, 29], have been found here to lower viability of isolated hepatocytes. Increased activity of GOT significantly decreased the yield of hepatocytes. This could be because a high level of GOT has been found to be a diagnostic marker for a number of diseases that can affect microcirculation or have increased collagen deposition, such as advanced alcoholic disease [30] or non-alcoholic chronic liver diseases with significant fibrosis [31, 32],

Figure 10. The model for calculating projected viability is appropriate. (A) Residuals versus fitted plot. (B) Normal quantile plot. (C) Square root of the standardised residuals versus fitted plot. (D) Standardised residuals versus leverage plot.

doi:10.1371/journal.pone.0107567.g010
Hewes et al. [14] found that previous chemotherapy does not affect the median yield of isolated human hepatocytes (4.6 million viable cells per gram of liver). In contrast, this study found a significantly increased yield of hepatocytes from donors pre-treated with chemotherapy with median yields of 17 compared to 10 million viable cells per gram of liver from donors without chemotherapy. It could be possible that the analysis done here was able to pick up a significance due to an increased statistical power, as this study has 128 replicates compared to 47 replicates done in Hewes et al.’s study [14]. An additional support for this reasoning is that the univariate analysis done with 1027 replicates also indicated a statistical difference ($P = 3.5 \times 10^{-5}$). It is possible that chemotherapy has reduced extracellular matrix proteins, such as collagen, allowing for a more complete digestion of the liver piece with collagenase, which resulted in an increased yield of isolated hepatocytes. This is supported by the study of Drozdz and Kucharz [33], which found that a cytostatic drug, azathioprine caused a decrease in total collagen content in the liver. Further, Sorafenib, a drug that is approved for the treatment of hepatocarcinoma has been found to act as an antifibrotic agent that reduced collagen deposition in fibrosis models such as bile duct ligation, thioacetamide or dimethyl nitrosamine administration in rats or carbon tetrachloride administration in mice [34–36]. Doxorubicin, another commonly used chemotherapeutic drug, also reduces collagen content in bile duct ligated rats by strongly inhibiting hepatic stellate cell proliferation [37].

In conclusion, this study has determined the variables that affect the viability and yield of isolated human hepatocytes. Further, this study has generated algorithms (Formulae 1–4) for the prediction of the viability or yield. A publicly accessible webpage (http://www.

Figure 11. The model for calculating projected yield is appropriate. (A) Residuals versus fitted plot. (B) Normal quantile plot. (C) Square root of the standardised residuals versus fitted plot. (D) Standardised residuals versus leverage plot.

doi:10.1371/journal.pone.0107567.g011
Table 7. Viabilities (%) and yield (million hepatocytes per gram liver) of isolated human hepatocytes obtained by various groups.

| Viability | Mean   | Median | N    | Reference | Yield   | Mean   | Median |
|-----------|--------|--------|------|-----------|---------|--------|--------|
|           |        |        |      |           |         |        |        |
| 91 ± 2    | -      | 14     | [11] |           | -       | 125    | 30     |
|           | 89     | 90     | [16] |           | 18.7 ± 1.7 | -     | 50     |
| 83 ± 1    | -      | 67     | [12] |           | 13.4 ± 0.4 | 10.3  | 1028   |
| 83 ± 1    | -      | 72     | [13] |           | 10.6 ± 7.8 | -     | 41     |
| 80 ± 8    | -      | 41     | [42] |           | 8.2 ± 5.7  | -     | 42     |
| 78 ± 0.3  | -      | 10     | [9]  |           | 7.9 ± 1.2  | -     | 14     |
| 77 ± 0.3  | 79     | 1032   | Authors’ own |           | 7.7 ± 1.8  | -     | 58     |
| 77 ± 9    | -      | 42     | [42] |           | 7.1 ± 1.0  | -     | 10     |
| 70 ± 2    | -      | 50     | [15] |           | -        | 6.0    | 90     |
| 64 ± 3    | 74     | 58     | [39] |           | 5.8 ± 0.8  | -     | 72     |
|           | 71     | 47     | [14] |           | 5.2 ± 0.5  | -     | 67     |
| 60 ± 4    | -      | 58     | [13] |           | -        | 4.6    | 47     |
|           | 56     | 20     | [38] |           | 4.0 ± 0.7  | -     | 149    |
|           | 25     | 30     | [41] |           | 2.6 ± 0.5  | 1.5    | 58     |

Values were expressed as means ± standard error of the mean.

doi:10.1371/journal.pone.0107567.t007
to hepatocyte viability, hepatocytes. P variables not sharing the same superscript alphabet are significantly different, P<0.05. For the variables of Ludwig score, operation type and surgical indication, variables not sharing the same superscript alphabet are significantly different, P<0.05. Yield values were transformed to follow a normal distribution by the logarithm.

(DOC)

**References**

1. Olson H, Betton G, Robinson D, Thomas K, Monro A, et al. (2000) Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol 32: 56–67.

2. Brambilla G, Martelli A (1993) Human hepatocyte primary cultures in toxicity assessment. Cytotechnology 11 Suppl 1: 86–8.

3. Russell WMS, Burch RL (1959) The principles of humane experimental technique. London: Methuen.

4. Thasler WE, Weiss TS, Schillhorn K, Stoll PT, Irrgang B, et al. (2003) Prognostic variables of right liver lobectomy for colorectal liver metastases: the effects of prior chemotherapy. Liver Int 24: 371–378.

5. Berry MN, Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J Cell Biol 43: 506–520.

6. Rhee JH, Gores GJ (2002) Death receptor-mediated apoptosis and the liver. Am Fam Physician 59: 2223–2230.

7. Lee SML SC UM TSS RMKT. Wrote the paper: SMLL CS WET RMKT

8. Conceived and designed the experiments: WET SMLL CS. Performed the experiments: SMLL CS NF WET. Analyzed the data: SMLL CS RPL UM

9. Acknowledgments

This work was made possible by the Human Tissue and Cell Research Foundation, which makes human tissues available for research. Our thanks also go to the technicians from the Groshadern Hospital and the Regensburg University Hospital Tissue Banks for the collection of the liver samples and from the Cell Isolation Core Facility in Munich and Hepacult GmbH for carrying out the liver perfusion, hepatocyte isolation and data entry. Finally, special thanks go to Luke Bax for his help in creating the webpage, where investigators can enter their donor information to get a prediction of viability and yield of hepatocytes.

**Author Contributions**

Conceived and designed the experiments: WET SMLL CS. Performed the experiments: SMLL CS NF WET. Analyzed the data: SMLL CS RPL UM

**Table S1**

| The number of replicates (N), P values, multiple R² values (R²), intercepts, regression coefficients (β) obtained after linear regression of the individual variables to viability (%) of isolated human hepatocytes. | *Significant relationship of the indicated variable to hepatocyte viability, P<0.05. For the variables of Ludwig score, operation type and surgical indication, variables not sharing the same superscript alphabet are significantly different, P<0.05. Yield values were transformed to follow a normal distribution by the log*1. |

**Table S2**

| The number of replicates (N), P values, regression coefficients (β), intercepts and multiple R² values (R²) obtained after linear regression of the individual variables to the yield (million/g liver) of isolated human hepatocytes. | *Significant relationship of the indicated variable to hepatocyte yield, P<0.05. For the variables of Ludwig score, operation type and surgical indication, variables not sharing the same superscript alphabet are significantly different, P<0.05. Yield values were transformed to follow a normal distribution by the fourth root.* |

**Supporting Information**

15. Kavathara T, Teo C, Douglas DN, Norabakhsh M, Lewis JT, et al. (2010) Factors affecting hepatocyte isolation, engraftment, and replication in an in vivo model. Liver Transpl 16: 974–982.

16. Shionoya T (1927) Studies in Experimental Extracorporeal Thrombosis: IV. Effects of Certain Physical and Mechanical Factors on Extracorporeal Thrombosis with and without the Use of Anticoagulants. J Exp Med 46: 945–948.

17. Youn JH, Gores GJ (2002) Death receptor-mediated apoptosis and the liver. J Hepatol 37: 400–410.

18. Zhan SS, Jiang JX, Wu J, Halsted C, Friedman SL, et al. (2006) Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. Hepatology 43: 845–848.

19. Brenner C, Galluzzi K, Kepp O, Kroemer G (2013) Decoding cell death signals in liver inflammation. J Hepatol 59: 583–594.

20. Bhunchet E, Fujieda K (1993) Capillarization of the sinusoids in liver fibrosis: noninvasive assessment with contrast-enhanced MRI in the rabbit. Magn Reson Med 28: 961.

21. Maria De Souza M, Tolentino M Jr, Assis BC, Cristina De Oliveira Gonzalez A, Maria Correia Silva T, et al. (2006) Pathogenesis of septal fibrosis of the liver. (An experimental study with a new model). Pathol Res Pract 202: 883–889.

22. Van Beers BE, Mateme R, Amri L, Hesney J, Tempel P, et al. (2003) Increased HIF1alpha activation: a novel mechanism for increased vulnerability of steatotic hepatocytes to hypoxic stress. Free Radic Biol Med 32: 1531–1542.

23. Cao Y, Liao Y, London E (2001) Hepatocyte isolation from waste liver surgical resections. A multilaboratory study. Liver Int 24: 371–378.

24. Van Beers BE, Mateme R, Amri L, Hesney J, Tempel P, et al. (2003) Decreased HIF1 alpha activation: a novel mechanism for decreased vulnerability of steatotic hepatocytes to hypoxic stress. Free Radic Biol Med 32: 1531–1542.

25. Farrell GC, Teoh NC, McCuskey RS (2008) Hepatic microcirculation in fatty liver disease. Liver Int 24: 371–378.

26. Anavi S, Harmelin NB, Madar Z, Tirosh O (2012) Oxidative stress impairs HIF1alpha activation: a novel mechanism for increased vulnerability of steatotic hepatocytes to hypoxic stress. Free Radic Biol Med 32: 1531–1542.

27. Day CP, James OF (1998) Special considerations in interpreting liver function tests. Am Fam Physician 59: 2223–2230.

28. Maniatis T, Craig NL, Davidson BR, et al. (2000) A formal test of the method of moments. Cytotechnology 11 Suppl 1: S6–8.

29. Iqbal S, Elcombe CR, Elias E (1991) Maintenance of mixed-function oxidase activity in primary cultures of human hepatocytes. Cell Tissue Bank 4: 49–56.

30. Berry MN, Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J Cell Biol 43: 506–520.

31. Seglen PO (1975) Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion. Exp Cell Res 82: 391–398.

32. Lee SML SC UM TSS RMKT. Wrote the paper: SMLL CS WET RMKT

33. Cryer PE, Cushman SW (1989) Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. Hepatology 13: 843–848.

34. Brenner C, Galluzzi K, Kepp O, Kroemer G (2013) Decoding cell death signals in liver inflammation. J Hepatol 59: 583–594.

35. Bhunchet E, Fujieda K (1993) Capillarization of the sinusoids in liver fibrosis: noninvasive assessment with contrast-enhanced MRI in the rabbit. Magn Reson Med 28: 961.

36. Zhan SS, Jiang JX, Wu J, Halsted C, Friedman SL, et al. (2006) Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. Hepatology 43: 845–848.

37. Van Beers BE, Mateme R, Amri L, Hesney J, Tempel P, et al. (2003) Capillarization of the sinusoids in liver fibrosis: noninvasive assessment with contrast-enhanced MRI in the rabbit. Mag Reson Med 49: 692–699.

38. Day CP, James OF (1998) Special considerations in interpreting liver function tests. Am Fam Physician 59: 2223–2230.

39. Maniatis T, Craig NL, Davidson BR, et al. (2000) A formal test of the method of moments. Cytotechnology 11 Suppl 1: S6–8.

40. Iqbal S, Elcombe CR, Elias E (1991) Maintenance of mixed-function oxidase activity in primary cultures of human hepatocytes. Cell Tissue Bank 4: 49–56.

41. Cryer PE, Cushman SW (1989) Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. Hepatology 13: 843–848.

42. Brenner C, Galluzzi K, Kepp O, Kroemer G (2013) Decoding cell death signals in liver inflammation. J Hepatol 59: 583–594.

43. Bhunchet E, Fujieda K (1993) Capillarization of the sinusoids in liver fibrosis: noninvasive assessment with contrast-enhanced MRI in the rabbit. Magn Reson Med 28: 961.

44. Zhan SS, Jiang JX, Wu J, Halsted C, Friedman SL, et al. (2006) Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. Hepatology 43: 845–848.

45. Van Beers BE, Mateme R, Amri L, Hesney J, Tempel P, et al. (2003) Capillarization of the sinusoids in liver fibrosis: noninvasive assessment with contrast-enhanced MRI in the rabbit. Mag Reson Med 49: 692–699.
32. Fotiadu A, Gagalis A, Akriviadis E, Kotoula V, Sinakos E, et al. (2010) Clinicopathological correlations in a series of adult patients with non-alcoholic fatty liver disease. Pathology International 60: 87–92.
33. Drozdz M, Kucharz E (1977) The effect of cytostatic drugs on collagen metabolism in guinea pigs. Arch Immunol Ther Exp (Warsz) 25: 773–778.
34. Wang Y, Gao J, Zhang D, Zhang J, Ma J, et al. (2010) New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis. J Hepatol 53: 132–144.
35. Deng YR, Ma HD, Tsuneyama K, Yang W, Wang YH, et al. (2013) STAT3-mediated attenuation of CCl4-induced mouse liver fibrosis by the protein kinase inhibitor sorafenib. J Autoimmun.
36. Hong F, Chou H, Fried M, Friedman SL (2013) Antifibrotic activity of sorafenib in experimental hepatic fibrosis: refinement of inhibitory targets, dosing, and window of efficacy in vivo. Dig Dis Sci 58: 257–264.
37. Greupink R, Bakker HI, Bouma W, Reker-Smit C, Meijer DK, et al. (2006) The antiproliferative drug doxorubicin inhibits liver fibrosis in bile duct-ligated rats and can be selectively delivered to hepatic stellate cells in vivo. J Pharmacol Exp Ther 317: 514–521.
38. Mitry RR, Hughes RD, Aw MM, Terry C, Misli-Vergani G, et al. (2003) Human hepatocyte isolation and relationship of cell viability to early graft function. Cell Transplant 12: 69–74.
39. Alexandrova K, Griesel C, Barthold M, Heuff HG, Ott M, et al. (2005) Large-scale isolation of human hepatocytes for therapeutic application. Cell Transplant 14: 845–853.
40. Gramignoli R, Tahan V, Dorko K, Skvorak KJ, Hanel MC, et al. (2013) New potential cell source for hepatocyte transplantation: discarded livers from metabolic disease liver transplants. Stem Cell Res 11: 563–573.
41. Bartlett DC, Hodson J, Bhogal RH, Youster J, Newsome PN (2014) Combined use of N-acetylcysteine and Liberase improves the viability and metabolic function of human hepatocytes isolated from human liver. Cytotherapy 16: 800–809.
42. Gramignoli R, Geen ML, Tahan V, Dorko K, Skvorak KJ, et al. (2012) Development and application of purified tissue dissociation enzyme mixtures for human hepatocyte isolation. Cell Transplant 21: 1245–1260.