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急性肝衰竭和肝細胞移植的研究

Acute liver failure & hepatocyte transplantation

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國立臺灣大學博士學位論文
口試委員會審定書

急性肝衰竭和肝細胞移植的研究

Acute liver failure & hepatocyte transplantation

本論文係何承懋君（D94421101）在國立臺灣大學臨床醫學研究所完成之博士學位論文，於民國 105 年 03 月 02 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

（指導教授）	（指導教授）

系主任、所長 （簽名）

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To those who let me experience the world

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中文摘要

背景及目的

急性肝衰竭是一個不常見但卻有高致命風險的症候群。目前的處理大多是根據專家經驗。肝移植是一個黃金標準治療。相較於肝器官移植，肝細胞移植是有發展潛力的另一個選擇。本研究擬針對急性肝衰竭及肝細胞移植的相關題材進行研究，從群體世代的流行病學，個體急性肝衰竭微觀病生理觀察，肝細胞體外功能評估，到最後肝細胞移植臨床前期動物實驗。目前對於體外肝細胞在移植前功能快速評估的工具很少，從肝門靜脈進行肝細胞移植時的輸注速度對移植細胞早期的 engraftment 及 repopulation 的影響也不清楚。因此，吾人欲研究 1) 台灣族群急性肝衰竭的發生率、病因、預後及相關因子探討。2) 急性肝衰竭肝再生及細胞分化的病生理機轉。3) 肝細胞對靛氰綠 (indocyanine green, ICG) 特異性吸收(經由 OATP1B1 受體)及釋放(面向小膽管的細胞膜)是否可以當作一個快速體外肝細胞功能評估工具。4) 急性肝衰竭大鼠模式下，不同細胞輸注速度對移植肝細胞殖入(engraftment)及繁盛(repopulation)的影響，並試圖以這種臨床可應用的方式改善細胞移植成效 。本論文從四大面向(族群流行病學，橫斷面病生理學，體外細胞學研究，臨床前動物模式轉譯學) 探討相關議題。

研究對象與方法

1)族群研究

從台灣健保資料庫中篩選出在 2005 年 1 月至 2007 年 9 月因急性肝衰竭相關診斷入院的病人。急性肝衰竭則進一步藉由相關檢驗醫囑、相關處方、住院天數、及沒有先前肝病就醫史等方式確保疾病嚴重度及排除其他可能干擾診斷因子。預後因子再作 Cox 迴歸分析。

2)橫斷面病生理學

吾人從急性肝衰竭的病人病肝中，以免疫組織染色法觀察組織中的前驅細胞 (用 CK19, EpCAM 當標記)，肝細胞(CPS-1, HNF4α)，膽道細胞(HNF1β)，細胞分化(NUMB)及增生(Ki-67)的變化。

3)體外研究

從肝臟移植剩餘肝組織分離出來的人類肝細胞(1 x 10^6 cells) 在 37°C 下、分別置於細

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胞懸浮液和培養基狀態下，加入不同濃度 ICG (0-2 mg/ml)浸泡 30 分鐘。之後將細胞移至不含 ICG 的培養液中 3 小時，分別於 1, 2, 3 小時後收集上清液，測量 ICG 濃度。細胞存活率用 trypan blue 排除法、MTT (mitochondrial dehydrogenase activity) 及 SRB (cell attachment) assays 測定。HepG2 細胞用來當作 control 比較。

4)動物實驗

以 D-galactosamine 誘發 Sprague-Dawley 大鼠急性肝衰竭後，從肝門靜脈分別以 30, 70 或 100 秒將 1 x 10^7/1 ml 新鮮分離的肝細胞打入肝臟，並於術後 2 天及 7 天觀察 early engraftment (2 天) and repopulation (7 天)。

結果

1)族群研究方面

從 28,078 位潛在急性肝衰竭病人中， 篩選出 218 位條件符合者。發生率為每百萬人年 80.2 位，而且隨年齡增加而上升。平均年齡是 57.9±17.1 歲，中位存活時間為 171 天。最常見的病因是病毒性(45.4%，B 型肝炎為主)和酒精/毒藥物(33.0%)。獨立預後因子包括酗酒，惡性腫瘤，每周檢查 total bilirubin 頻率，敗血症，使用血液透析，以及使用氫離子幫浦阻斷劑 (proton pump inhibitor)。他們的風險比值(HR)及 95%信賴區間分別為 1.67 (1.01-2.77), 2.90 (1.92-4.37), 1.57 (1.40-1.76), 1.85 (1.20-2.85), 2.12 (1.15-3.9), 0.94 (0.90-0.98)。在存活超過三個月的 130 病人中，66 人(50.8%)後來追蹤發現有肝硬化的傾向。8 人(3.7%)曾經接受肝移植評估， 只有 1 位接受移植且存活。

2)橫斷面病生理方面

組織中可觀察到大量肝細胞死亡及顯著的小膽管反應 (ductular reaction)。利用免疫組織染色，可以觀察到不同分化階段的小膽管反應，從早期原始前驅細胞(CK19 濃染, 細胞核大細胞小)往外圍成螺旋狀逐漸分化進入肝細胞前期(CK19 淡染或消失, 細胞核小細胞大)。 隨著細胞分化進行，這群中間肝細胞(intermediate hepatocyte)也會有 NUMB、EpCAM 的表現，部分細胞甚至已經出現成熟肝細胞特有與尿素代謝相關的 CPS-1 表現，但是很少觀察到分裂增生的細胞 (無 Ki67 表現)。

3)體外研究方面

體外有功能的肝細胞數分鐘內可納入 ICG，並可在 1~2 小時內排除細胞外。排出的 ICG 在 1 小時後很快達到一個飽和濃度。ICG 濃度超過 1.0 mg/ml 對肝細胞有毒性。

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相較於 0.5 mg/ml 或對照組，在 1.0 mg/ml ICG 濃度下，肝細胞有較高粒線體去氫酶活性(0.025 ± 0.0004 v.s 0.019 ± 0.0008 or 0.020 ± 0.002, P < 0.05)。當 HepG2 細胞浸置在不同 ICG 濃度(control, 0.5 mg/ml, 1.0 mg/ml)中，上清液白蛋白量呈遞減趨勢(98.9 ± 0.02, 66.6 ± 0.05, 39.1 ± 0.4 ng/ml)，偵測細胞增生的[^{3}H]-thymidine incorporation 也呈現相同趨勢。

4)動物實驗方面

不同輸注速度會影響移植肝細胞的 engraftment (P = 0.018) 及 repopulation (P = 0.037)，而且有統計上的顯著差異。其中以 70 秒輸注速度成效較好，移植的肝細胞能夠立即穿越過肝竇內皮血管層，很少累積在門靜脈小管中，也有較顯著的肝功能改善。三組的平均首次門靜脈壓高峰分別是 14.8 ± 6.5, 17.7 ± 3.7, 13.6 ± 3.0 mmHg，之間並無統計差異。

結論

台灣族群的急性肝衰竭主要跟病毒感染有關，病人有惡性腫瘤及酗酒者預後較差，使用 proton pump inhibitor 則有較佳的預後。半數的存活者有肝硬化。急性肝衰竭病肝有明顯的 ductular reaction，雖然新生的前期肝細胞已經表現出成熟肝細胞特有的功能，仍不足以成功完成肝臟再生的救援功能，所以肝細胞移植仍能提供急性肝衰竭治療上的實際需求。體外有功能的肝細胞數分鐘內可納入 ICG，並可在 1~2 小時內排除細胞外。再進一步改善後，ICG 可發展成快速體外評估肝細胞功能的檢測工具。在急性肝衰竭大鼠模式中，不同肝細胞輸注速度會導致不同早期殖入和繁盛的結果。這些經由動物實驗得到的觀念驗證具有實質臨床意義，可以容易進行臨床的轉譯應用。總體而言，台灣在急性肝衰竭的細胞治療仍有相當多的研究及進步空間。

關鍵字: 急性肝衰竭，預後，族群，肝細胞，前驅幹細胞，靛氰綠 (ICG)，肝細胞移植，殖入 (engraftment)，繁盛 (repopulation)

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英文摘要

Background and objective

Acute liver failure (ALF) is uncommon but fatal. Current management is based mostly on clinical experience. Hepatocyte transplantation is a promising alternative to liver transplantation in patients with acute liver failure. The study is to investigate ALF from longitudinal population-scale epidemiological analysis, through individual cross-sectional histopathophysiological observation, ex vivo functional evaluation of hepatocytes, to preclinical animal experiment of hepatocyte transplantation. To this end, we investigated 1) the incidence, etiology, outcomes, and prognostic factors of ALF in Taiwan. 2) pathophysiological expression of regeneration and differentiation in acute failure liver. 3) whether the uptake and release of indocyanine green (ICG) by hepatocytes could be used as a rapid in vitro assay for hepatocyte functional assessment. 4) the impact of the rate of intraportal hepatocyte transplantation on early engraftment and repopulation and to improve the engraftment and repopulation efficiencies of hepatocyte transplantation for treatment of a rat model of acute liver failure in a clinically useful way without preconditioning.

Materials and methods

1) For population study, patients with the admission diagnosis of ALF between January 2005 and September 2007 were identified from the Longitudinal Health Insurance Database of Taiwan. ALF was further confirmed by disease severity based on laboratory orders, prescriptions, and duration of hospital stay, and acute onset without prior liver disease. Prognostic factors were identified using Cox regression analysis.

2) For microscopic cross-sectional observational study, a human explant liver from acute HBV infection was examined for immunohistochemical expression of progenitors [marker: CK19, epithelial cell adhesion molecule (EpCAM)], differentiation [NUMB (an inhibitor of the Notch pathway), carbamoyl phosphate synthetase 1 (CPS-1, urea cycle enzyme), HNF4α, and HNF1β], and proliferation (Ki-67).

3) For in vitro study, human hepatocytes (1 x 10^6 cells) isolated from unused donor livers were incubated at 37°C for 30 min with ICG (0-2 mg/ml) in both cell suspension and on collagen-coated culture plates. Cells were then incubated in medium without ICG for 3 h with supernatants collected at 1, 2, and 3 h for measurement of ICG release. Viability of cells

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was determined by trypan blue exclusion, MTT (mitochondrial dehydrogenase activity) and SRB (cell attachment) assays. HepG2 cells were also used as comparison.
4) For animal study, acute hepatic injury was induced in Sprague-Dawley rats with D-galactosamine. Hepatocytes (1 x 10^7/ml) were infused intraportally over 30, 70, or 100 seconds to study early engraftment (2 days) and repopulation (7 days).

Results

1) For population study, during the study period, 218 eligible cases were identified from 28,078 potential eligible ALF patients. The incidence was 80.2 per million person-years in average and increased with age. The mean age was 57.9±17.1 years and median survival was 171 days. The most common etiologies were viral (45.4%, mainly hepatitis B virus) and alcohol/toxin (33.0%). Independent prognostic factors included alcohol consumption (HR 1.67 [1.01-2.77]), malignancy (HR 2.90 [1.92-4.37]), frequency of check-ups per week for total bilirubin (HR 1.57 [1.40-1.76]), sepsis (HR 1.85 [1.20-2.85]), and use of hemodialysis/hemofiltration (HR 2.12 [1.15-3.9]) and proton pump inhibitor (HR 0.94 [0.90-0.98]). Among the 130 patients who survived ≥90 days, 66 (50.8%) were complicated by liver cirrhosis. Eight (3.7%) were referred for liver transplantation evaluation, but only one received transplantation and survived.
2) For cross-sectional study, histological examination of the explant liver showed submassive necrosis and prominent ductular reaction. The road of hepatocyte differentiation was nicely shown from the bipotential progenitor cells (thick stained, small cell size, high nuclear-cytoplasm ratio) and gradually spirally spreading outward to form daughter intermediate hepatocytes (light stained, larger cell size, lower nuclear-cytoplasm ratio). These differentiating cells did not proliferate actively, and express EpCAM and transition of NUMB and CPS-1.
3) For in vitro study, ICG was taken up and secreted by hepatocytes with the release reaching a plateau level soon after 1 hour. Concentrations of ICG above 1.0 mg/ml, had toxic effects on hepatocytes. Hepatocytes incubated with 1.0 mg/ml ICG had higher mitochondrial dehydrogenase activity compared to 0.5 mg/ml ICG or control (0.025 ± 0.0004 v.s 0.019 ± 0.0008 or 0.020 ± 0.002, $P < 0.05$). Incubation of HepG2 cells with ICG reduced albumin production (98.9 ± 0.02, 66.6 ± 0.05, 39.1 ± 0.4 ng/ml for control, 0.5 mg/ml, and 1.0 mg/ml ICG respectively) and also decreased [^{3}H]-thymidine incorporation in a dose-response manner.

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4) For animal study, three groups had significant difference in hepatocyte engraftment (P = 0.018) and repopulation efficiencies (P = 0.037) and infusion over 70 seconds produced superior outcomes. After the 70-second infusion, the transplanted cells immediately transmigrated the sinusoidal endothelial layer and rarely accumulated in the portal venules, with improved liver function significantly. The mean first peak pressures, without significant difference, were 14.8 ± 6.5, 17.7 ± 3.7, and 13.6 ± 3.0 mmHg in the 30, 70, and 100-second groups, respectively.

Conclusion

ALF in Taiwan is mainly due to viral infection. Patients with malignancy and alcohol exposure have worst prognosis. The use of proton pump inhibitor is associated with improved survival. Half of the ALF survivors have liver cirrhosis. Prominent ductular reaction with at-least partially functional hepatocyte differentiation did not guarantee successful regeneration in acute liver failure and there is demand left for hepatocyte transplantation. With further refinement of ICG could be used to develop a rapid assay for assessment of the function of isolated human hepatocytes. Differential hepatocyte transfusion rate contribute to accelerated early engraftment and repopulation in rats with acute liver injury. These proof-of-concept findings are of clinical significance because they are easy to translate into practice. Further studies are needed for improvement of hepatocyte transplantation for ALF in Taiwan, albeit some problems solved.

Key words: acute liver failure, prognosis, population, hepatocyte, progenitor cell, indocyanine green (ICG), hepatocyte transplantation, engraftment, repopulation

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Abbreviation

ACLF	acute-on-chronic liver failure
ADSC	adipose-derived stem cell
AFP	Alpha-fetaprotein
ALF	acute liver failure
ALT	alanine aminotransferase
ANOVA	analysis of variance
Arg-1	arginase 1
AST	aspartate aminotransferase
ATP	adenosine triphosphate
CBD	common bile duct
CK19	cytokeratin 19
CPS-1	carbamoyl phosphate synthetase 1
CT	computed tomography
D-gal	D-galactosamine
DMNA	dimethylnitrosamine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPIV	dipeptidyl peptidase IV
ECM	extracellular matrix
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
EpCAM	epithelial cell adhesion molecule
EtBr	ethidium bromide
FDA	fluorescein diacetate
GGT	gamma-glutamyltranspeptidase
GMP	good manufacturing practice
GTP	good tissue practice
HBSS	Hanks' Balanced Salt Solution
HBV	hepatitis B virus
HCV	hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	hepatitis E virus
HGF	hepatocyte growth factor
HNF1β	hepatocyte nuclear factor 4 beta
HNF4α	hepatocyte nuclear factor 4 alpha
HR	hazard ratio
ICU	intensive care unit
IL	interleukin
IMV	inferior mesenteric vein
INR	international normalized ratio
LHID	Longitudinal Health Insurance Database
LT	liver transplantation
MRP2	Multidrug resistance-associated protein 2
MSC	Mesenchymal stem cell
MTT	mitochondrial dehydrogenase activity
NHI	National Health Insurance
NHIRD	National Health Insurance Research Database
NOTCH	neurogenic locus notch homologue protein
PPI	proton pump inhibitor
RNA	ribonucleic acid
SD	Sprague-Dawley
SRB	sulphorhodamine
SVF	stromal vascular fraction
Tbx3	T-box 3
TWEAK	TNF-like weak inducer of apoptosis
UW	University of Wisconsin
WME	Williams’medium E

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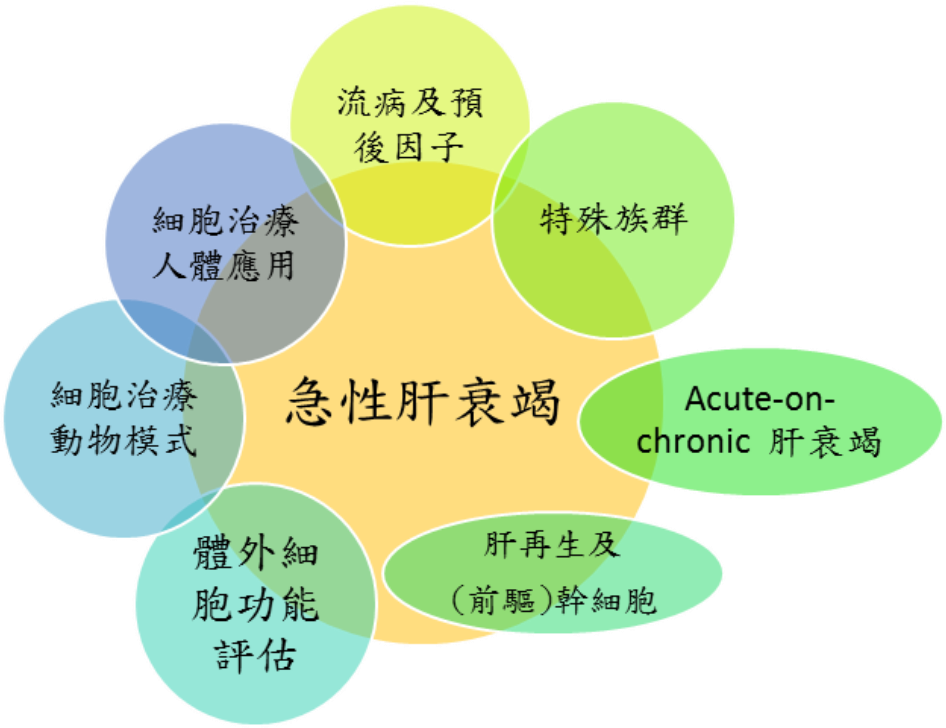
Chapter 1

Overview 緒論



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臨床上的急性肝衰竭

急性肝衰竭是一個肝細胞短期大量壞死，使肝功能快速不足且進展到衰竭，常合併多重器官一起變差，死亡率甚高的疾病症候群。致病原因可以是病毒感染(如 A 型、B 型或 E 型肝炎病毒)、毒藥物 (如 acetaminophen)、急性缺氧性肝損傷 (acute ischemic injury)、腫瘤浸潤、急性 Budd-Chiari syndrome、中暑、吃到毒菇、Wilson's disease 急性發作等等 (Bernal et al. 2013)。很多情況下是原因不明的。即便今日醫學大幅進展，死亡率仍高約四成至五成 (Bernal et al. 2013, Ho et al. 2014)。

在西洋醫學發展史上是相對落後且發展緩慢的。西元 1946 年 Lucke 和 Mallory 曾描述到致命的急性肝炎 (Lucke et al. 1946)，之後到 1970 年猛爆性肝衰竭(fulminant hepatic failure) 一詞在美國才被具體的提出 (Trey et al. 1970)，描述一個先前沒有肝臟疾病的人發生嚴重但可能回復的肝損傷，並於發生肝損傷徵狀後 8 周之內產生肝腦病變。1993 年，King's College Hospital 的 O'Grady 進一步根據病人病程時序細分出超急、急、亞急性肝衰竭 (O'Grady et al. 1993)。直到 2005 年美國肝病學會 (American Association for the Study of Liver Diseases) 統合出一個操作型定義：在沒慢性肝病存在下，26 周內出現凝血功能障礙 (INR>1.5)及肝腦病變 (Polson et al. 2005)。至此之後，凝血功能異常及肝昏迷就被普遍公認當作診斷急性肝衰竭的 biomarkers。另外，美國肝病學會對 Wilson's disease、B 型肝炎垂直感染健康帶原者及自體免疫肝炎發生急性肝衰竭是否也包含在內採取較寬容的態度 (Polson et al. 2005)。因此像 B 型肝炎健康帶原者接受免疫調節劑而產生 B 型肝炎病毒活化引發急性肝衰竭的情況，可發生在換心人(Yang et al. 2014)或自體免疫患者接受治療時 (Hsu et al. 2014)，甚至像換肝者(一個新的沒病肝)發生單純皰疹引發的急性肝衰竭 (Ho et al. 2008)，都可算廣義的急性肝衰竭。

急性肝衰竭在已開發國家並不常見，推估每年每百萬人少於十例新個案 (Bernal et al. 2013)。美國一年將近 2800 例 (Khashab et al. 2007)。主要的發病原因在不同地域國家也有所不同：如英美兩國以 acetaminophen 過量為主，南亞印度以 HEV 為主，日本以 HBV 為主 (Bernal et al. 2013)。吾人根據台灣健保資料庫樣本推估每年每百萬人約有 80 例，發病年齡層也較歐美高，發病因則以 HBV 為多 (Ho et al., 2014)。在第二章

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(Chapter 2) 中，吾人進一步利用健保資料庫貼近對台灣的急性肝衰竭的流行病學、病因、預後及危險因子加以分析整理，得到第一手的大樣本資料(如一年的存活率 49.3%)，可作為後續治療追蹤時預估的基礎。

急性肝衰竭與肝再生

急性肝衰竭時組織學上可見大片範圍的肝細胞壞死或凋亡現象 (Rutherford et al. 2008)，在此時通常會啟動肝細胞增生並促進肝再生 (Ding et al. 2010)，但後者(增生)的速度常趕不上前者(傷亡) (Rutherford et al. 2008)。肝再生的研究很早就開始了。典型的肝再生研究模式是以研究動物大塊肝切除後的變化 (Higgins et al. 1931)。根據早期的研究，肝再生時有多種肝內細胞的 DNA 在不同時序進行複製，早期肝再生研究多集中在肝細胞及細胞生長激素的研究上(Michalopoulos et al. 1997)。近期的研究更發現肝細胞不是都具有一樣的增生功能。在平常維持 homeostasis 時，中央靜脈附近的肝細胞具有早期前驅肝幹細胞的 biomarker (Tbx3) 會負責向門靜脈端補充需替換的肝細胞(不表現 Tbx3) (Wang et al. 2015)。門靜脈附近的肝細胞則是在肝受損傷後肝細胞增生補充的來源 (Font-Burgada et al. 2015)。此外，肝再生研究更聚焦在肝內非肝細胞之間的交互影響，如強調肝竇血管內皮細胞再生 (Ding et al. 2010)，膽道細胞再生 (Chen et al. 2015) 、肝細胞之間藉 exosome 傳遞細胞生長激素的方式促進肝再生(Nojima et al. 2015) 及血小板藉 RNA transfer 促進肝細胞增生 (Kirschbaum et al. 2015)。

對應到臨床上，活體捐肝者在肝切除後的 mRNA 早期一致的變化 (Figure 1) 則可一窺人體的肝再生奧秘 (Ho et al. 2007)。大範圍肝細胞壞死也可由肝毒化物所引發，常用做動物模式研究的毒物有 D-galatosamine (Keppler et al. 1968)、acetaminophen、carbon tetrachloride、thioacetamide (Rahman et al. 2000) 等等，當中以第一個最常使用也較穩定、有再現性 (reproducible)。因此，D-galatosamine 被吾人拿來當作細胞治療的急性肝衰竭的動物模式 (第五章 Chapter 5)。

急性肝衰竭與前驅幹細胞

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急性肝衰竭後的肝復原往往不如捐肝者切除後的健康肝再生完美 (Ho et al. 2014)，甚至無足夠的肝細胞供作增生來源或/且執行肝臟功能，此時臨床上可觀察到肝前驅幹細胞的活化增生 (詳見第三章, Chapter 3, Figure 11, Ho et al. 2015)。這些肝前驅幹細胞最早是由 Farber 發現的 (Farber et al. 1956)，當時稱這些形狀呈卵圓形的小細胞為卵圓細胞。後來被證明可分化成肝細胞，所以又稱卵圓幹細胞 (Evarts et al. 1987)。它們會表現的標記含括膽道細胞 (CK-7, CK-8, CK-9, OV-6, glutathione-S-transferase, connexin 43, mouse A6 antigen)、肝母細胞 (AFP, gamma-glutamyltranspeptidase (GGT), muscle pyruvate kinase)及造血幹細胞 (Thy-1 (CD90), c-kit, CD34, sca-1) (Yu 2009)。 這些前驅幹細胞從 canals of Hering 增生出來，可分化成肝細胞或膽道細胞 (Spee et al. 2010) (圖詳見第三章, Chapter 3, Figure 18, Ho et al. 2015)。現在更清楚的知道這種前驅幹細胞先天被設定要分化膽道細胞，當訊息分子 wnt 3 被因肝細胞受傷而激活的巨噬細胞分泌出來時，原設定模式 (藉 Notch 訊息傳遞軸維持) 被中介因子 NUMB 解除設定，因而走向分化成肝細胞的路途 (Boulter et al. 2012)。臨床上，我們也觀察到這些前驅幹細胞在急性肝衰竭大量肝細胞死亡時會大量出現且朝不同方向分化：一方走向肝細胞且表現 NUMB；另一方走向膽管細胞而不表現 NUMB (圖詳見第三章, Chapter 3, Figure 14 and 15, Ho et al. 2015)。此外，巨噬細胞也可藉分泌細胞激素 TWEAK (TNF-like weak inducer of apoptosis) 引發 ductular reaction (在正常小鼠模式下) (Bird et al. 2013)。Elsegood 等人進一步在慢性肝損傷的肝組織中驗證巨噬細胞和誘發 ductular reaction 的關聯性 (Elsegood et al. 2015)。而且巨噬細胞在肝切除後的小鼠肝再生模式中的重要角色也被提出 (Nishiyama et al. 2015)。這種肝前驅幹細胞協助肝再生的替代方案看似一個完美的備案，臨床上肝再生失敗的例子仍比比皆是。Roskams's group 曾發表過在急性肝衰竭病人病理組織中，肝細胞減少一半之後，肝細胞增生能力大減。肝細胞剩下越少，前軀幹細胞活化越多，但病人卻大多是以死亡或接受肝移植收場 (即救援失敗) (Katoonizadeh et al. 2006)。

對應到 D-galactosamine 引發的急性肝衰竭大鼠模式，原生自體肝細胞會在肝受傷後快速增生，在七至十天完成肝臟的修補 (Yu 2009)。若加上 retrorsine 抑制原生肝細胞增生，卵圓幹細胞 (大鼠的前驅幹細胞) 會快速被啟動活化、增生並分化成肝細胞 (Yu 2009)。若於卵圓幹細胞活化增生的高峰期(retrosine + D-galactosamine 藥物處

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理後的第四天)施以肝細胞移植，這些卵圓幹細胞將停止分化成肝細胞，轉而分化成膽道細胞 (Yu 2009)。

急性肝衰竭的微觀免疫分子機轉

急性肝衰竭時大量肝細胞壞死會造成 innate 免疫反應過度活化，大量的發炎物質 (如 HMGB1, TNFα, IL-1) 從肝臟溢出至全身，引起個體的無菌 systemic inflammatory response syndrome (Possamai et al. 2014)。隨後抑制發炎的細胞激素 (主要是 IL-10, secretory leukocyte protease inhibitor) (原本是想讓發炎過程趕快緩解並促進修復的代償反應) 跟著溢出肝臟外，造成單核球對入侵體內細菌無感的免疫癱瘓 (immuneparesis) 及敗血症 (Antoniades et al. 2014)。這在病毒性及自體免疫性病因 (非短暫一次性病因，如 acetaminophen 中毒) 的急性肝衰竭時細胞死亡持續進行，導致發炎與抑制發炎細胞激素的調節混亂，形成對個體極為不利的局面 (Possamai et al. 2014)。肝內的巨噬細胞在這裡扮演重要的致病機轉角色。現在逐漸了解到巨噬細胞會因微環境不同有促進 (M1) 及抑制發炎 (M2) 兩極的功能，然而完全處在兩極的某一端並非常態 (Possamai et al. 2014)。如何利用這個陰陽兩極的概念於臨床治療急性肝衰竭的免疫調節，則是另一個極待解決的問題。

急性肝衰竭的治療及策略

急性肝衰竭的治療有的跟致病因有關：如 B 肝病毒引起的可用抗 B 肝病毒用藥; acetaminophen 過量或缺血性引起的可用 n-acetylcysteine；大多是跟致病因無關、跟全身受影響的器官系統有關：如腎臟替代療法，升壓強心劑，減腦壓處置，降氨療法，抗生素/抗黴菌療法，血漿置換療法等等 (Mark et al. 2015)。在急性肝衰竭病程進展期，可預期的是生命個體急需的是成熟足量的肝細胞以維持生命，行有餘力勉強提供一個合適的環境供幹細胞分化。肝細胞移植在這種情況下便具有 ”救急” 的功能。另一種假說是幹細胞 (自身或外加) 的分泌物 (如 exosome) 可緩解急性肝衰竭的發炎反應，減輕傷害，甚而促進肝再生 (van Poll et al. 2008, Xagorari et al. 2013, Tan et al.

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2014)。不過，這就是從這個疾病的另一個面向來解決問題。在第六章 (Chapter 6) 會著墨在肝細胞和間質幹細胞同時移植的探討。

肝臟移植是急性肝衰竭的黃金標準治療，臨床病程不可逆的病人可由國際上通用的標準篩選出來，如 King's College Criteria (O'Grady et al. 1993)、Clinchy criteria (Bernuau et al. 1986)、Japanese criteria (Mochida et al. 2008)。但是，急性肝衰竭病人常極度衰弱，即便接受移植，也預期會有較高的手術風險及手術併發症。Germani 就報告過這類病人的存活率比其他肝移植病人硬是低了一成 (Germani et al. 2012)。有時，全肝壞死會引起 toxic liver syndrome (心血管性休克、腎衰竭 ± 呼吸衰竭) (Ringe et al. 1993)。緊急時刻可先採取全肝切除及 portocaval shunt 手術，暫時穩定病人的代謝性酸中毒及 hemodynamics；再等待肝移植做的二階段的治療 (Ringe et al. 1988, Henderson et al. 1994)。甚至也有藉此手術緩解腦水腫的報告 (Rozga et al. 1993)。另外對急性肝衰竭的肝可能還是有回復的機會時也有做輔助性部分肝移植手術，等自身肝再生成功後免去終身服用免疫抑制劑的不便 (van Hoek et al. 1999, Weiner et al. 2015)。不論如何，這些病人常處於迫切等肝移植的狀態，可是在台灣大部分的病人無法如期等到換肝 (Ho et al. 2014)。因此，發展替代療法 (如細胞移植)便成為一個迫切需要且非常重要的課題。

肝細胞移植的發展

肝細胞移植的發想在四十年前就有了，初期是發現在 Crigler-Najjar syndrome 的模式動物 Gunn 大鼠 (缺少 uridine diphosphate glucuronyltransferase) 在進行了經門靜脈肝細胞移植後可降低膽紅素 (Matas et al. 1976)。Mito 也發現把肝細胞打入大鼠脾臟中移植細胞也可存活下來 (Mito et al, 1979)。另外也發現在 D-galactosamin 或 dimethylnitrosamine (DMNA) 所引起的急性肝衰竭模式大鼠上進行肝細胞移植可以改善存活率 (Sutherland et al. 1977, Sommer et al. 1979, Makowka et al. 1980)。隨後的 1980-90 年代更造成相關研究非常流行 (Makowka et al. 1981, Braun et al. 2000, Kobayashi et al. 2000)。不過，大多數的動物實驗告訴我們移植肝細胞只有二成至三成存活的下來，其實只佔全部肝臟細胞的 0.5-1% (Yu 2009)。進行多次的移植或可增加移

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植肝細胞存活的機會 (Rajvanshi et al. 1996)。以往臨床前動物模式上使用 retrorsine 用來增加移植肝細胞的 engraftment 效率的做法臨床上並不適用，促使肝細胞移植界廣泛尋找臨床上可能可應用的新策略，例如門靜脈部分栓塞、移植前肝放射處置 (Dhawan 2015)、或部分肝切除後再行細胞移植 (Jorn et al. 2015)。移植前先給藥物的策略也有進展，例如改變肝血管反應的 endothelin-1 receptor blockade (bosentan, darusentan), nitroglycerin, prostacyclin；阻斷發炎或細胞激素的 etanercept, thalidomide；破壞肝竇內皮細胞邊界的 cyclophosphamide, doxorubicin, rifampicin/phenytoin；活化 stellate cells 以促進 extracellular matrix remodeling 的 naproxen, celecoxib 等 (Forbes et al 2015)。吾人利用輸注移植肝細胞速度的差異可產生不同的 engraftment 效率 (Ho et al 2015) 則提供另一個臨床上可應用的治療策略。

肝細胞移植的微觀機轉

肝細胞大小約為 20-30μm, 遠端肝門靜脈和肝竇微血管大小約為 6μm (Yu 2009)，而正常肝竇微血管上的孔洞大小約為 100 nm (Gandillet et al. 2003)。分離出的肝細胞失去細胞間的 tight junction 牽制而成圓球狀，經門靜脈注入的肝細胞若無變形擠壓，是無法離開遠端肝門靜脈和肝竇微血管系統，進入 parenchyma 中的。因此大部分移植的肝細胞若沒在一天之內脫離循環系統，就會被 Kupffer cells 所消滅殆盡 (Yu 2009)。甚至移植的肝細胞是趁微循環因移植細胞卡塞住引發缺血再灌流 (ischemia-reperfusion) 效應，刺激 Kupffer cells 活化並釋出 tumor necrosis factorα，造成肝竇微血管通透性改變；同時也有因發炎引起血管內皮完整性被破壞的當下，伺機逃離循環系統，常駐肝臟 (Gupta et al. 1999)。這也成了肝細胞移植 engraftment 最廣為接受的機制假說。這種情形會造成暫時性(約三小時)的肝門靜脈栓塞及門脈高壓，在嚴重急性肝衰竭的大鼠會產生持續的門脈高壓，而加速死亡 (Yu et al. 2004)。不過，弔詭的是，大鼠上使用抑制發炎的 tumor necrosis factorα 抑制劑 (etanercept) 反可增加移植細胞的存活及 engraftment (Viswanathan et al. 2014)。稍後在第四章，我們將提到經由門靜脈輸注肝細胞的動物實驗中，可發現移植肝細胞當下短時間內即可擠出肝竇微血管，甚至可觀察到正在擠出的過程 (詳見第五章, Chapter 5, Figure 36)。肝內血管堵塞的情形反而在較慢注射速度時較常發生 (Ho et al. 2015)。門脈壓力的變化大多在半小時內回到移植前

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狀態。雖稱不上推翻先前假說，吾人的實驗發現的確提供移植肝細胞 engraftment 另一個創新的機轉。

移植肝細胞突破血流血管障壁後的下一關是要融入 liver parenchyma. 這通常要花 1 至 5 天的時間讓移植肝細胞和原生肝細胞的細胞膜結構重整(包含 gap junction 及 bile canalicular network) (Gupta et al. 1995, Slehria et al. 2002, Forbes et al. 2015)。活化的 stellate cells 會藉由釋放 matrix metalloproteinases 及 tissue inhibitors of metalloproteinases 幫助融入的過程中細胞外間質 (extracellular matrix)所需要的破壞與重建 (Benten et al. 2005)。理論上，移植成功的肝細胞具有增生的能力，會依肝受損情況而增生 (Forbes et al. 2015)。往往實驗觀察到的現象並非如此。若不先壓抑原生肝細胞的增生，移植的肝細胞並不會有明顯的增生現象。Font-Burgada 等人則暗示門靜脈周圍的肝細胞表達一些膽管相關基因 (如 Sox-9)，增生能力較強，又沒致癌能力，可為較佳的肝細胞來源 (Font-Burgada et al. 2015)。

臨床肝細胞移植的發展

在臨床文獻上最早是日本 Mito 於 1992 年成功發表 (Mito et al. 1992)。肝細胞移植是將成熟肝細胞自捐贈者肝臟分離純化出來後，經肝門靜脈或脾動脈輸注入受贈者體內，使所捐肝細胞融入肝實質中，以改善肝功能的新技術。全球至少已有超過八十例臨床經驗，受贈對象可為罹患先天性肝疾病，如 Crigler-Najar Syndrome type I，urea cycle defects (ornithine transcarbarmylase deficiency、argininosuccinate lyase deficiency、carbamoylphosphate synthase type 1 deficiency、citrullinemia)，factor VII deficiency，hemophilia A，glycogen storage disease type I，argininosuccinate lyase deficiency，Refsum disease，α1 antitrypsin deficiency，familial hypercholesterolemia，maple syrup disease，neonatal hemochromatosis，progressive familial intrahepatic cholestasis type 2，急性肝衰竭，懷孕脂肪肝及 acute-on-chronic 肝衰竭等等。相關的動物模式也被測試 (Weber et al. 2009)，如 spf-ash mice (ornithine transcarbamylase deficiency) (Michel et al. 1993)，fumarylacetoacetate hydrolase knockout mice (tyrosinemia) (Overturf et al. 1997)，mdr2 knockout mice (progressive familial intrahepatic cholestasis) (De Vree et al. 2000)，

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hypercholesterolemic Watanabe rabbit (Wiederkehr et al 1990)。 其角色定位在至少作肝臟移植的橋接或急性肝衰竭的自我康復，進而追求所捐肝細胞的長期存活及個體存活。這項手術侵入性較器官移植小，病人術後的恢復期也比較短。缺點是得多次輸注細胞 (因個體一次能承受的移植細胞量有限且大多數所輸注的細胞未融入肝實質前即被免疫細胞所消滅)、細胞存活期不確定並且追蹤困難等等。臨床上甚至觀察到經由肝細胞移植後，受贈者身上會產生對 donor hepatocytes 的新生抗體 (de novo donor-specific antibody) (Jorns et al. 2015)，產生體液免疫反應 (humeral immune response) 及巨噬細胞藉抗體輔助吞噬外來細胞的反應 (alloantibody-dependent, complement-independent cell mediated cytotoxicity) (Horne et al. 2008, Zimmerer et al. 2015)。即使如此，臨床實證告訴我們：接受妥善執行的肝細胞移植手術後，病人是安全無虞的，並且因原發的肝疾病不同有不同程度的病情改善。筆者曾於 2008 年 2 月至 King's College Hospital 進修臨床肝細胞移植相關課題 (Figure 2)。英文文獻中近 1/3 兒童肝細胞移植個案報告都出自此處 (Dhawan 2015)。筆者就臨床肝細胞移植手術，做一個基本介紹。

肝細胞分離術及臨床級無菌細胞分離製備實驗室

臨床級細胞分離的實驗室可供各種細胞分離純化及製備。其環境維護以及微生物監控都有一定的標準作業流程規範 (Figure 3A)。舉例來說，細胞處理室的菌落量得遠小於準備室的菌落量、hood 的落塵量要達到 100 級區的標準。用於臨床的細胞一定要在 GTP (good tissue practice)/GMP (good manufacturing practice) 合格的環境下分離、純化、冷凍儲存、解凍、製備，才能用於人體。這幾項步驟以及未來可加入的程序和肝細胞移植手術的關係可以流程圖 (Figure 2) 表示。

一般肝臟來源主要是未用於肝移植手術的屍肝，或僅使用部份屍肝的剩餘肝組織。另外，也有少數肝切除手術的肝組織。這些肝組織經器官保存液低溫灌流後，在無菌塑膠袋包覆下，被送往無菌細胞分離室等候處理。各種灌洗液、保存液如 HBSS (Hanks' Balanced Salt Solution), EMEM (Eagle's Minimum Essential Medium) , UW solution(University of Wisconsin solution)、試藥如 EGTA (ethylene glycol tetraacetic acid), collagenase, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), DMSO

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(dimethyl sulfoxide), 50% glucose, acetylcysteine)、無菌水、agar plates 及血液培養瓶等等也一併攜帶入室(Figure 3B) 。進入最終細胞分離室前要經過兩層隔離衣，兩階段準備房間更衣，依序增加無菌層級。

進入無菌分離室後隨即進行準備工作，將溫水浴準備好，配製各種灌洗液 (依灌流順序先 pure HBSS 洗出留在組織中的器官保存液，再 HBSS + EGTA，HBSS + $CaCl_2$ 打斷細胞間的 tight junction，EMEM + HEPES + Collagenase 讓細胞脫離 basement membrane)；其次，開始處理肝組織，包括秤重、清除血管內剩餘血栓、大血管 cannulation。未 cannulated 的血管斷端儘量予以關閉，以利稍後酵素作用完全。無菌操作是很重要的，同時隨時要保持管線的無菌狀態。管線連接完成即可利用機器 pump 排氣，灌流 (Figure 3C)。此時視肝組織大小決定每階段的灌流量；一般每 200-300 克肝組織約一瓶 500 ml HBSS；Collagenase 的濃度約 0.5g/500 ml EMEM (視不同廠牌建議而定)。全程監控灌流過程，避免空氣跑入及可能的染污。當酵素作用佳時，肝臟呈現透明沙袋狀。此時即可終止酵素灌流，取出已分解的肝組織，另置無菌塑膠袋中，置於冰上，等待下一步處理。

將縫在肝組織上的管線移除，並移除未分解的白色結締組織。剪破肝膜，切割那些被消化的肝實質，釋放肝細胞於 ice-cold EMEM 中 (Figure 3D)。隨即加以紗布過濾雜質，分裝入離心管中以 50g 低速離心 (Figure 3E,F)。離心完後吸除管內上清液，輕敲散細胞沉積物，新加入 EMEM，搖勻，離心，重覆數次直到上清液澄清為止。此時吸除上清液，計算細胞總沉積量。吸取少量肝細胞沉積，稀釋後加入 trypan blue，置於 hemocytometer，計算活細胞數及比例。依公式細胞數/ml=計算值 x 2 x 10^4 x dilution factor，即可估出總細胞數及產量 (Yield) (總細胞數/肝組織重量)。細胞分離術即告完成。接下來要把細胞冷凍或馬上稀釋作細胞輸注，而有不同的程序。

若無合適受贈者存在，細胞品質也不錯(活細胞佔 60%)，細胞將可冷凍保存，待日後急需時解凍使用。保存液以 UW 為主，最終細胞濃度為 10^7/ml，最終 DMSO 濃度 10%，最終葡萄糖濃度 5%；以此原則加以配置溶液並包裝密封。(Figure 3G)包裝標示完成後，隨即送至 step-rate freezing freezer 加以冷凍至-140°C 保存。

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若從冷凍肝細胞製備，將冷凍細胞取出，快速解凍至近乎完全，以 EMEM 緩慢稀釋至十倍體積，混合均勻，低速離心五分鐘，取出含 UW solution 和 DMSO 的上清液。將細胞沉積物打散，再檢測活細胞比例及數量，並以五倍的 TM-1 (transplant media) + 一倍的 20% Albumin 混合液加以稀釋至濃度 10^7/ml 並保存在 ice-cold 環境下儘快使用。

臨床肝細胞移植手術見習經驗

病人的術前準備和一般手術相同，移植路徑可從 IMV (inferior mesenteric vein)事先裝設的 catheter 至體外方便輸注細胞，或由 umbilical vein catheter 打入。輸注前後須特別注意並紀錄生命徵象及門脈壓力的變化，以 1ml/min 速度緩緩輸入，並以生理食鹽水沖洗管路 (Figure 3H)。每次細胞輸注量約 100 millions/kg，並給予短期類固醇注射以及和肝移植一樣的抗排斥藥物。術後給予靜脈營養，短期禁食，觀察 vital signs 及門脈壓。抽血檢測膽紅素、尿素、氨、及肝酵素等等肝相關指數的變化。必要時間隔一日重覆手術數次，直到臨床症狀及症候改善。

Stéphenne 等人曾證明肝細胞移植後可在一個 3 歲患有 argininosuccinate lyase deficiency 病童肝上存活一年並發揮功能(Stéphenne 2006)。但是，植入肝細胞的功能通常在移植 9 個月後漸漸減少(Dhawan 2015)。詳細的原因並不是很清楚，但是應該跟 engraftment 差或受到免疫系統的攻擊很有關係 (Dhawan 2015)。

臨床肝細胞移植的瓶頸

一般來說，完整的人肝至多約可分離出 10^{11} 個肝細胞，這個數目可以分給多人次使用，也可以冷凍起來供將來解凍使用。雖然肝細胞在冷凍及解凍的過程中極易受傷死亡，現有的冷凍保存肝細胞可長達 32 個月，解凍後拿去移植仍可存活並保有肝細胞功能 (Mitry et al. 2002)。理論上，移植肝細胞總量的 5%通常夠用 (Dhawan 2015)。不過，急性肝衰竭的病人可能需要量更多。至少 40 多例的急性肝衰竭病人接受過肝細胞移植 (Strom 1999)，累積的經驗告訴我們治療後氨和膽紅素會下降、肝昏迷會改善；然而個體卻沒有存活夠久 (Dhawan 2015)。2009 年在倫敦開的肝細胞移植國際共識會

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議指出不同 centers 的細胞製程及臨床 protocols 讓肝細胞治療結果很難互相比較 (Puppi et al. 2012)。因此，肝細胞品質的標準化、protocol 一致化及如何增加移植肝細胞的殖盛率 (repopulation)成了將來的重要課題 (Puppi et al. 2012)。

雖然實驗上在模式動物得到大的進展，臨床上實際的治療反應不一，原因大都出在動物實驗的環境設定常不適用於臨床 (Ho et al. 2015)，分離出的肝細胞常取自無法做器官移植的肝 (Dhawan 2015)，所以結果不像動物實驗般穩定，無法適時得到大量優質的肝細胞。因此，體外肝細胞在細胞移植前功能的快速評估是一項重要的課題 (Ho et al. 2012)，第三章將詳細討論。如何將動物實驗的正向結果成功轉譯至臨床應用，也是讓臨床肝細胞治療穩定進步的要點，第四章從臨床可行的角度發想，在實際觀察過臨床肝細胞移植的流程中 (Figure 10)，建立假說並設計動物實驗去驗證 (Ho et al. 2015)。並希望進一步藉由肝細胞臨床製程的簡化改善，減少場地空間的依賴及維護，希望達到和傳統製程一樣甚至更好的肝細胞產品，即時提供臨床急性肝衰竭病人使用。

論文體系

整篇論文關聯及架構體系可參見 Figure 4。從臨床觀察型研究出發，探討台灣急性肝衰竭的現況，利用健保資料庫函蓋的普及性，了解潛在台灣族群的大小及特性。Acute-on-chronic 肝衰竭的表現和急性肝衰竭有類似重疊的地方，也是肝細胞治療的潛在目標疾病 (Dhawan 2015)，綜論中雖未詳細介紹，將於 future perspective 中加以詳述。肝再生及前驅幹細胞的相關基礎研究在第三章(Chapter 3)進行探討，並也將在未來持續進行。體外肝細胞功能的評估佔肝細胞治療療效良窳一個很重要的地位。臨床可行的肝細胞治療動物實驗則是轉譯應用醫學的必要之途。細胞萃取的流程改造工程雖非一蹴可幾，但將可讓臨床細胞治療的成本降低且普及，最終回歸這類族群的臨床治療應用。

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主要研究的問題與重要性

1 探討急性肝衰竭在台灣的整體流行病學現況，了解可能目標族群的大小、特性及存活率，勾勒出可進步的空間。以提供將來設計臨床試驗的參考依據。

2 藉急性肝衰竭動物模式實驗，以臨床可行的策略，提出增加肝細胞移植 engraftment 效率的方案，縮短實驗和臨床經驗的 discrepancy，提早臨床肝細胞移植廣泛應用，挽救更多病人的生命。

研究假說與特定目的

1 在台灣健保普及的狀態下，研究假說為可藉資料庫分析出急性肝衰竭病人的流行病學特徵。設定急性肝衰竭健保資料的操作型定義，逐步將範圍縮小靠近目標族群，並分析其流病資料。

2 在急性肝衰竭大鼠模式實驗中，研究假說為經門靜脈的肝細胞輸注速率會影響移植肝細胞的 engraftment。因此分組設計不同輸注速度，觀察移植後不同時間點 donor 肝細胞的位置及數量，並加以分析比較。

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Acute liver failure: Clinical perspective

Acute liver failure (ALF) is a syndrome characterized of multiple organ functional deterioration rapidly induced by massive hepatocyte necrosis and liver decompensation. The mortality of ALF can be as high up to 50 % and can be caused by viral infection (eg, hepatitis virus A, B or E), toxin or drug (eg, acetaminophen), ischemia, malignancy infiltration, venous obstruction (eg, Budd-Chiari syndrome), Wilson disease, or heat stroke (Bernal et al. 2013). The etiology of ALF varied in geographic regions and indeterminate or unknown etiology occur in many circumstances. The definition is based on the coagulopathy and hepatic encephalopathy in a previously healthy liver (Polson et al. 2005). ALF occurs rarely in the developed countries, estimating about less than 10 cases per million person-years. In our epidemiological nationwide study (Chapter 2), however, it was estimated about 80 cases per million person-years (Ho et al. 2014). We can expect our target population of interest based on this analysis.

Acute liver failure: regenerative perspective

Liver regeneration is triggered when massive hepatocyte necrosis or apoptosis (Rutherford et al. 2008). ALF occurs when the balance tilts toward the injury instead of regeneration. Hepatocytes proliferate fully to recover in the model of liver regeneration of massive physical loss of liver volume or in living donor liver resection (Higgins et al. 1931, Ho et al. 2007). Earlier studies of liver generation pay more attention on the hepatocyte and its related cytokines (Michalopoulos et al. 1997). Recent research found that the hepatocyte near the central vein was actually contributing to the house-keeping cell renewal in daily cell loss (Wang et al. 2015). The hepatocyte near the portal vein, instead, contributes to the replacement of cell loss by injury (Font-Burgada et al. 2015). Interaction between the hepatocyte and the non-parenchymal cells was ever better understood. For example, the regeneration of sinusoidal endothelial cells (Ding et al. 2010) and biliary cells (Chen et al. 2015), and transfer for growth factors by exosome (Nojima et al. 2015) and RNA by platelets (Kirschbaum et al. 2015). In Chapter 5, we used D-galactosamine induced acute liver failure in rats as the animal model of hepatocyte transplantation because D-galactosamine is a common and stable agent that cause reproducible acute liver injury.

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Acute liver failure: perspective of progenitor cells

Recovery from acute liver failure by injury generally is not as complete as the living liver donor (Ho et al. 2014). In many cases, there is not enough hepatocytes to meet the demand of regeneration. Ductular reaction occurs in these circumstances. Liver progenitor cells, or known as oval cells, were first discovered by Farber (Farber et al. 1956). They express markers of CK-7, 8, 9, OV-6, glutathione-S-transferase, connexin 43, mouse A6 antigen, AFP, gamma-glutamyltranspeptidase (GGT), muscle pyruvate kinase, CD90, c-kit, CD34, sca-1 and can differentiate into hepatocytes or cholangiocytes (Yu 2009). They are set to differentiate toward cholangiocytes by default and will shift toward hepatocytes under the action of wnt 3 released by activated macrophages (Boulter et al. 2012, Elsegood et al. 2015). NUMB played a central role in the transition. Clinically, we observed the differentiation of the progenitor cells (marked by CK19) toward hepatocytes with the expression of NUMB in Chapter 3 (Ho et al. 2015). The spared mechanism of rescue clinically usually still end up with host death or liver transplantation (Katoonizadeh et al. 2006).

Acute liver failure: Immunologic perspective

Massive hepatocyte necrosis will over activate the innate immunity, which later induce inflammatory substances (such as HMGB1, TNFα, and IL-1) in whole liver and overflow through systemic circulation around the host. It induce sterile inflammatory response syndrome systematically (Possamai et al. 2014). Anti-inflammatory substances (such as IL-10 and secretory leukocyte protease inhibitor) will be induced locally in the liver and spread out of liver in the later round, which cause immune paralysis of monocytes and susceptible to sepsis (microbial invasion) (Antoniades et al. 2014). It can result in immune chaos when the insulting triggers continues to be existed (Possamai et al. 2014). Macrophage polarization between M1 (pro-inflammation) and M2 (pro-resolution) is important in the perturbation and modulation of the microenvironment of acute liver failure and needs further studies.

Acute liver failure: Therapeutic perspective

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Specific agents for acute liver failure are limited and etiology-dependent. Most treatment strategies are supportive in organ dysfunction due to the systemic inflammatory response (Mark et al. 2015). Liver transplantation is the gold standard of treatment. These patients are usually weak and could not tolerate the procedure. Hepatocyte transplantation plays an alternative critical role in the rescue of acute liver failure when there is acute paucity of enough mature hepatocytes. Hepatocyte transplantation can either bridge to liver transplantation or help native liver regenerate in these circumstances.

Hepatocyte transplantation as the treatment for acute liver failure (see Chapter 4-1 and Chapter 5-1 for more information)

Mechanism of engraftment

The size mismatch between hepatocytes (20-30μm), the lumen of portal venule or sinusoidal endothelium (6μm), and the sinusoidal fenestration (100nm, Gandillet et al. 2003) argues against hepatocyte transfer into parenchyma (Yu 2009). The current hypothesis of engraftment, therefore, describes the breakage of endothelium by Kupffer cells after ischemia-reperfusion injury induced by transplanted cells occupying the vessels (Gupta et al. 1999). Portal hypertension by obstruction can be fetal if it is persisted in the rat model of acute liver failure receiving hepatocyte transplantation (Yu et al. 2004). In Chapter 5, we found that donor hepatocytes can migrate out of sinusoidal endothelium into the parenchyma shortly after transplantation in adequate speed of transfusion. The obstruction was more often observed in slow rate of infusion. Portal pressure was back to pre-transplant status most of the time in the adequate speed of transfusion. It is a novel finding with translational significance and mechanistic revolution. After entering into parenchyma, donor cells need to reorganize the membrane structure around (such as gap junction and bile canalicular network) and stellate cells will reshape the extracellular matrix (Benten et al. 2005).Donor hepatocytes will not proliferate actively unless suppressing the proliferative ability of native hepatocyte in experiment. It is another area of interest that needs to be solved in order to achieve widespread clinical application of hepatocyte transplantation.

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Framework of the study

The overall study framework is illustrated in Figure 4. We will start to investigate the epidemiologic features of acute liver failure in Taiwan by using the nationwide coverage of the National Health Insurance database and realize the size and characteristics of the potential population of interest (Chapter 2). Acute-on-chronic liver failure, shared some common features with acute liver failure, is also among the potential population of interest regarding hepatocyte transplantation and will be described in perspective (Chapter 6). Liver regeneration and progenitor cell biology is going to be explored in Chapter 3 by demonstrating the microscopic real-word appearance of acute liver failure and will be continuing in the near future. Functional evaluation of ex vivo hepatocytes before transplant is another important issue and will be addressed in Chapter 4. Animal model of hepatocyte transplantation with clinical translational impact is of paramount status and is detailed in Chapter 5. Modification of the cell isolation procedure to meet clinical needs is undergoing and briefly described in Chapter 6. We hope that the overall study design is feedback to clinical application and benefit the patients in suffer.

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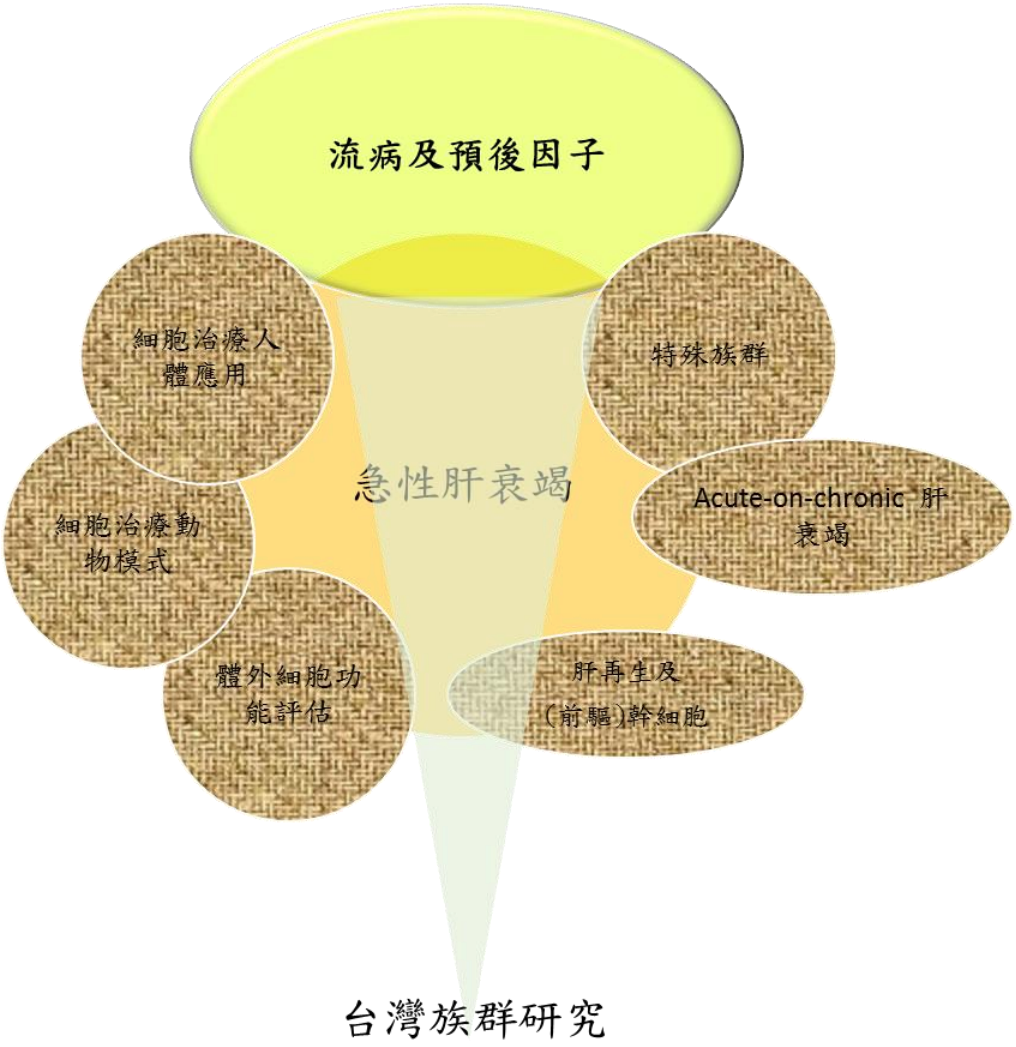
Chapter 2

Acute liver failure in Taiwan

Population study



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(1) Background

Acute liver failure (ALF) is an uncommon clinical syndrome that often has a course associated with rapidly progressive multi-organ failure and devastating complications like coagulopathy and encephalopathy in patients without previous liver disease. Its etiologies include a multitude of infectious, immunologic, infiltrative, or metabolic diseases, and have considerable geographical and ethnic variations (Bernal et al. 2010). In developing countries, viral causes predominate, whereas drugs or toxins are recognized as common causes in the United States and United Kingdom (Bernal et al. 2010). Reports estimate an overall incidence of fewer than 10 cases per million persons per year in developed countries (Bower et al. 2007, Escorsell et al. 2007, Bernal et al. 2013). Because ALF is an orphan disease, large clinical trials are extremely difficult and its management is currently based on clinical experience rather than on solid evidence (Polson et al. 2005, Lee et al. 2012, Bernal et al. 2013). Conclusions are also very difficult to reach even in a systemic review (Wlodzimirow et al. 2012) because of the varying definition of ALF among studies. As such, mortality rate remains high at 60-80% (Shalimar et al. 2013).

The most widely accepted definition of ALF includes evidence of coagulation abnormality and a degree of mental alteration (encephalopathy) in a patient without pre-existing liver disease (Bernal et al. 2010, Lee et al. 2012). No single institute has established considerable case series except Kings College Hospital (Bernal et al. 2013), while most currently available reports are multi-center collaborations (Brandsaeter et al. 2002, Ostapowicz et al. 2002, Larson et al. 2005, Escorsell et al. 2007, Kim et al. 2013, Oketani et al. 2013).

As the mandatory universal health insurance program offering comprehensive medical care coverage, the National Health Insurance of Taiwan has covered up to 99% of residents in Taiwan for several years since 1996 (Bureau of National Health Insurance, 2011). With a longitudinal follow-up of more than twenty-million subjects and validated diagnoses of catastrophic illness (Chen et al. 2011; Wu et al. 2012), the National Health Insurance Research Database (NHIRD) provides a very suitable research material to explore the outcome of a rare disease or clinical entity. The aim of this study is to analyze the incidence, characteristics, hospital course, prognosis, and complications of ALF in Taiwan using the longitudinal cohort information of the NHIRD.

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(2) Methods

The institutional review board of National Taiwan University Hospital, Taipei, Taiwan approved this study (NTUH REC: 201212001W). As a retrospective study using an encrypted database, the institutional review board waived the need for informed consent.

Data source

The Longitudinal Health Insurance Database (LHID) 2005, a subset database of the NHI program, contains the entire original claim data from 1996 to 2007 of 1,000,000 beneficiaries randomly sampled from the year 2005 Registry for Beneficiaries of the NHI program.

Patient selection

From the LHID 2005, patients who were admitted due to ALF for the first time from January 1, 2005 to September 30, 2007 were identified. Patients with possible ALF was identified based on in-patient records with compatible diagnoses, laboratory orders for ammonia and international normalized ratio (INR), and prescription of lactulose (regardless to the route of administration) and stayed in hospital for ≥7 days to ensure severe liver injury. The compatible diagnoses of ALF included ICD-9-CM (International Classification of Diseases, Ninth Revision, Clinical Modification) code for acute liver failure (570.0), hepatic coma (472.2), autoimmune hepatitis (571.42), acute alcoholic hepatitis (571.1), hepatitis unspecified (573.3), jaundice (782.4), viral hepatitis (070.0-070.9), and hepatitis B carrier (V02.61).

To ensure no preexisting liver disease, patients were excluded if they had any of the following diagnoses within 3 years prior to the index admission: [1] chronic hepatitis (ICD-9-CM 571.4); [2] hepatic stone (ICD-9-CM 574.5); [3] hepatocellular carcinoma (ICD-9-CM 155.0); [4] intra-hepatic cholangiocarcinoma (ICD-9-CM 155.1); [5] gall bladder cancer (ICD-9-CM 156.0); [6] extra-hepatic bile duct cancer (ICD-9-CM 156.1); [7] malignant neoplasm of the pancreas or Ampulla of Vater (ICD-9-CM 157.9, 156.2); [8] liver metastasis (ICD-9-CM 197.7); and liver cirrhosis (ICD-9-CM 571.2, 571.5, 571.6). Those admitted after October 1, 2007 were excluded to ensure a minimal follow-up duration of 3 months. Summary list of the inclusion and exclusion criteria were shown below.

Inclusion criteria: either or the following

1. Admission due to ACUTE HEPATIC FAILURE (ICD-9: 570), or HEPATIC COMA (ICD-9: 572.2)

2. Admission for liver-associated diagnosis (defined below) plus either of the following two conditions

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i) any of the parameters, including PT, PT INR, total bilirubin, direct bilirubin, AST, or ALT, been checked >= 4 time in 2 weeks during admission

ii) NH3 ben checked >= 4 times in 2 weeks during admission and receiving lactulose >= 1000 mL in 2 weeks during admission

Liver-associated diagnoses:

(1) SYSTEMIC INFLAMMATORY RESPONSE SYNDROME DUE TO NON-INFECTIOUS PROCESS WITH ACUTE ORGAN DYSFUNCTION (ICD-9: 995.94)

(2) DISSEMINATED INTRAVASCULAR COAGULOPATHY (DIC) SYNDROME (ICD-9: 286.6)

(3) AUTOIMMUNE HEPATITIS (ICD-9: 571.42)

(4) ACUTE ALCOHOLIC HEPATITIS (ICD-9: 571.1)

(5) CONTAMINATED OR INFECTED BLOOD, OTHER FLUID, DRUG, OR BIOLOGICAL SUBSTANCE (ICD-9: E875)

(6) HEPATITIS unspecified (ICD-9: 573.3)

(7) JAUNDICE (ICD-9: 782.4)

(8) VIRAL HEPATITIS (ICD-9: 070.0 – 070.9)

(9) HEPATITIS B CARRIER (ICD-9: V02.61)

Exclusion criteria: previous liver disease (defined below) within previous 3 years

Previous liver disease

(1) liver-associated diagnoses

(2) chronic hepatitis (ICD-9: 571.4)

(3) hepatic stone (ICD-9: 574.5)

(4) HCC (ICD-9: 155.0)

(5) cholangiocarcinoma, intrahepatic (ICD-9: 155.1)

(6) malignant neoplasm of gall bladder (ICD-9: 156.0)

(7) malignant neoplasm of extrahepatic bile ducts (ICD-9: 156.1)

(8) malignant neoplasm of ampulla of vater (ICD-9: 156.2)

(9) malignant neoplasm of pancreas (ICD-9: 157.9)

(10) liver metastasis (ICD-9: 197.7)

For every enrolled patient, the demographic data, laboratory tests, medications, clinical procedures, and outcomes were retrieved from the LHID 2005 and the possible etiology of

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acute hepatic failure was determined.

Demographic data

Demographic information including sex, age, underlying co-morbidity (i.e. diabetes mellitus, chronic obstructive pulmonary lung disease, end-stage renal disease, autoimmune disorder, acquired immune deficiency syndrome, and malignancy), and low income were collected as in a previous report (Lee et al. 2012).

Laboratory tests, medications, and procedures

The frequency of laboratory tests, including INR, total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and ammonia were calculated. Prescriptions of medications, including lactulose, diuretics, vasopressin (glypressin, somatosatin, and sandostatin), and proton pump inhibitors, were converted from the claims data according to the defined daily doses (DDD) and grouped according to their pharmacologic categories (World Health Organization, 2011). The performance of procedures (intubation for ventilator, plasmapheresis, hemodialysis, hemofiltration, upper gastrointestinal panendoscopy, echo-guided fluid tapping, and blood transfusion), transplantation-associated laboratory test (human leukocyte antigen), or liver transplantation procedure were recorded. Transfusion of fresh frozen plasma >30 units in a week or >100 units during the whole course of index admissions were considered as plasmapheresis.

Etiologic contribution

The etiology of ALF was based on the priority of virus infection, alcohol, and metabolic causes, and then hepatotoxin (see descriptions below for potential hepatotoxins), if ever. Malignant infiltration was attributed to the presence of malignancy and the lack of the etiologies mentioned above.

Potential Hepatotoxin

Potential hepatotoxins included paracetamol, anti-tuberculosis drugs (isoniazid, rifampin, rifabutin, and pyrazinamide), non-steroidal anti-inflammatory drugs, antibiotics or anti-viral agents (ketoconazole, terbinafine, tetracycline, amoxicillin, erythromycins, clindamycin, trimethoprime, ritonavir, indinavir, saquinavir, nelfinavir, zidovudine, didanosine, abacavir, nevirapine, stavudine, and efavirenz), lipid-lowering drugs (atorvastatin, lovastatin, simvastatin, pravastatin, gemfibrozil, and ezetimibe), anti-epileptics (carbamazepine, valproate, and phenytoin), anti-psychotics (chlorpromazine, risperidol, quetiapine, olanzapine, clozapine, bupropion, fluoxetine, paroxetine, sertraline, trazodone, mirtazapine,

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imipramine, and amitriptyline), immune modulators (sulfasalazine, azathioprine, methotrexate, adalimumab, and etanercept), anti-hypertensives (lisinopril, enalapril, captopril, losartan, and irbesartan), steroid derivatives (danazol, fluoxymesterone, nandrolone, oxymetholone, stanozolol, and testosterone), methyldopa, nicotinamide, nicotinic, allopurinol, amiodarone, baclofen, clopidogrel, and Chinese herbal therapy.

Follow-up and outcome

The patients were followed-up until death, withdrawal of health insurance, or December 31, 2007. The date of death was obtained from the Cause of Death Data included in the LHID 2005. Based on the discharge diagnosis (ICD-9-CM), intra-hospital complications were noted, including hemorrhage (gastrointestinal [GI] tract: 578.9, 531.4, 532.4, 530.82; brain: 431, 432.0, 432.9, 852.0-4, 767.0, 772.2; unspecified: 459.0), sepsis (995.91, 995.92), pneumonia (481, 482, 484, 486), extra-hepatic organ damage (renal insufficiency: 584.5-9, 572.4; respiratory failure: 518.81, 518.84, 786.0, 799.1), and seizure (345.0-4).

Statistical analysis

Data were expressed as means ± standard deviation, median (inter-quartile range [IQR]), or number (percentage) when appropriate. The Student's *t* test or χ^2 test was used for inter-group comparison. The survival curves of different etiologic groups were generated using the Kaplan-Meier method and compared using the log-rank test. The Cox's proportional hazard model was used to identify independent prognostic factors. The p value in each variable was derived from the Wald test in the Cox's model and was used to predict and identify independent prognostic factors. Sensitivity analyses were further performed in the sub-population who had no concomitant malignancy, since it was difficult to attribute the etiology of ALF accurately in patients with concomitant malignancy. Risk factors for intra-hospital complications were analyzed using logistic regression analysis. A two-sided $p < 0.05$ was considered significant. All analyses were performed with the Statistical Package for Social Sciences (SPSS)® version 18.0 (IBM Corporation, Armonk, NY, USA).

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(3) Results

Demographic Characteristics of the ALF Cohort

A total of 28,078 potentially eligible admissions for acute liver failure were identified from the 2,719,680.2 person-years of follow-up since 2005 in LHID 2005 (Figure 5). Among them, 14,482 admissions before 2005 were excluded, as well as 9,880 with prior history of liver diseases within 3 years, 3,430 without records of INR, ammonia, or lactulose, and 50 with hospital stay less than 7 days. Another 14 admissions were excluded to guarantee an observation time ≥3 months. Four were also excluded due to non-first admissions. The remaining 218 patients were enrolled in this study.

The 218 ALF patients (150 males) had a mean age of 57.9±17.1 years and median age of 57.3 years (range, 45.4-72.5 years). The incidence was 80.2 per million person-years (218 cases in 2,719,680.2 person-years) and this increased with age (Figure 6). The median follow-up duration was 171 days (range, 7-1059 days). Attributable etiologic exposures were viral infection (45.4%, mainly hepatitis B virus), chemicals (alcohol or toxin) (33.0%), infiltrative malignancy (4.6%), miliary tuberculosis (1.4%), and others (metabolic or pregnancy: 2.3%; indeterminate: 13.3%) (Figure 7).

The primary site of concomitant malignancy and etiologic exposure of ALF patients were listed in Table 1. The most common malignancy was hepatocellular carcinoma (63%), followed by colorectal cancer, lung cancer, and head and neck cancer (7% each).

The clinical characteristics were presented in Table 2. Of the 218 patients, 88 (40.4%) died within 90 days after admission, with a median survival of 29 (IQR, 7-93) days. Eighty-one (37%) died during their index admission. Among the 130 patients who survived ≥90 days, the median follow-up duration was 458.5 (IQR, 45-1059) days. The former group was statistically significantly older (60.0 vs. 56.5 years; p=0.018) and had longer hospital stay (p=0.020), higher probability of intensive care unit (ICU) admission (p=0.018), and higher prevalence of concomitant malignancy (47% vs. 14%; p<0.001).

Severity and In-hospital Complications of the ALF Cohort

Compared to those who survived ≥90 days, the patients who died within 90 days after admission received more frequent check-ups of total bilirubin (1.9 vs. 0.4 per week; p<0.001) and ammonia (1.4 vs. 1.0 per week; p=0.008), and were more likely to receive plasmapheresis (15% vs. 8%, p=0.045) (Table 3). There were no differences between the two groups regarding the presence of ascites and esophageal varices, frequency of check-up for AST, ALT, direct bilirubin, and INR levels, and proportion of patients who underwent

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procedures (i.e. panendoscopy, computed tomography, or brain magnetic resonance imaging).

Compared to those who survived ≥90 days, patients who died within 90 days after admission were more likely to be complicated by sepsis (39% vs. 20%; p=0.003), require more frequent transfusion of fresh frozen plasma (8.1 vs. 3.6 units/week; p<0.001), use vasopressin (24% vs. 12 %; p=0.025), and require renal replacement therapy (18% vs. 5 %; p=0.001) and ventilator support (50% vs. 27%; p=0.001) (Table 3).

Logistic regression analysis revealed that peptic ulcer (hazard ratio [HR] [95% confidence interval] 6.96 [2.87-16.91]; p<0.001) and respiratory failure (HR: 3.20 [1.30-7.85]; p=0.011) were independent risk factors of in-hospital hemorrhage complication. For the occurrence of sepsis, renal insufficiency (HR 2.55 [1.15-5.65]; p=0.021), computed tomography (HR 2.61 [1.27-5.34]; p=0.009), and frequency of check-ups per week for total bilirubin (HR 1.35 [1.05-1.72]; p=0.019) were risk factors.

Long-term Sequelae

Among the 130 patients who survived ≥90 days after admission, 66 (51%) were complicated by liver cirrhosis, including 22 with encephalopathy, and 21 with ascites. During follow-up, 20 (15%) required vasopressin in subsequent admissions and 29 (22%) underwent panendoscopy. Sixty-four (49%) patients, including 10 without liver cirrhosis, received lactulose whereas 72 (55.4%), including 19 without liver cirrhosis, received diuretics.

Survival Analysis

The one- and two-year survival probabilities were 49.3% and 45.9%, respectively. Eight were referred for liver transplantation (LT) evaluation. Among them, three survived without LT and one survived with LT. Kaplan-Meier analysis revealed that the survival of 59 patients with concomitant malignancy were significantly worse than that of the 159 without malignancy (p<0.001) (Figure 8, which illustrated the survival curves of ALF patients stratified according to the status of concomitant malignancy). In the latter group, the one- and two-year survival rates were 61.9% and 57.3%, respectively, and were 14.6% and 14.6%, respectively, in the former group. Among patients without malignancy, the Kaplan-Meier survival curves for different etiologic groups were shown in Figure 9.

The results of multivariate Cox regression revealed that in patients with ALF, the independent factors associated with poor survival were alcohol consumption (HR 1.67 [1.01-2.77]; p=0.046), malignancy on index admission (HR 2.90 [1.92-4.37]; p< 0.001), frequency of check-ups per week for total bilirubin (HR 1.57 [1.40-1.76]; p<0.001), sepsis (HR 1.85 [1.20-2.85]; p=0.005), and use of hemodialysis/hemofiltration (HR 2.12 [1.15-3.9]; p=0.015)

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and proton pump inhibitors (HR 0.94 [0.90-0.98]; p=0.005) (Table 4).

Sensitivity analysis focusing on the sub-population without malignancy showed that five variables - alcohol consumption (HR 2.43 [1.31-4.53]; p=0.005), frequency of check-ups per week for total bilirubin (HR 1.91 [1.63-2.23]; p<0.001), sepsis (HR 1.79 [1.03-3.1]; p=0.039), and use of hemodialysis/hemofiltration (HR 2.38 [1.19-4.79]; p=0.015) and proton pump inhibitors (HR 0.95 [0.91-0.99]; p=0.025) - remained significant prognostic factors (Table 4). Among patients without concomitant malignancy, the adjusted survival curves for patients stratified by etiology of ALF (Figure 10) demonstrated that alcoholic patients had the worst survival, while those with hepatitis C virus (HCV) or toxin exposures had more favorable outcomes.

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(4) Discussion

Analyzing the nationwide ALF cohort, the present study has four main findings. First, the incidence of ALF was 80.2 per million person-years, which increased with age. Second, viral infection was the most common attributable etiology (45.4%). Third, the mortality rate was 40.4% within 90 days after admission and liver cirrhosis occurred in about half of the survivors. Lastly, alcohol consumption, malignancy on index admission, frequency of check-ups per week for total bilirubin, sepsis, and use of hemodialysis/hemofiltration and not using proton pump inhibitors were poor prognostic factors for ALF.

The observation that the incidence of ALF increases with age is interesting. The median age of the 218 ALF patients is 57.3, quite older than the 38 years of a previous study with 308 ALF patients (Ostapowicz et al. 2002). In another previous study using the NHIRD (1997-2004) to assess drug-induced liver injury, the age distribution is also skewed towards >60 years (Lee et al. 2012) This may indicate the ageing population of Taiwan. Ageing is accompanied by diminished metabolism and elimination of toxin or alcohol (Meier et al. 2008, Tanemura et al. 2012), decreased water distribution volume (Timchenko et al. 2009), and reduced liver regeneration when the liver is placed under stressful conditions like hepatectomy or acute liver injury (Timchenko et al. 2009, Tanemura et al. 2012, Bernal et al. 2013). Acute liver injury or post-ischemic liver injury is greater in older adult mice than in younger ones (Okaya et al. 2005, Collins et al. 2013). Older donor age is a well-known risk factor of poorer outcome of liver recipients (Hoofnagle et al. 1996, Lake et al. 2005, Tanemura et al. 2012). Ageing may also explain the higher ALF incidence in the study compared to that in literature (Bower et al. 2007, Escorsell et al. 2007, Bernal et al. 2013).

In the nationwide cohort, the major etiological exposure of ALF in Taiwan is HBV infection, followed by toxins and alcohol. HBV infection is a leading cause of ALF in Japan and Spain, while toxins are more common in the United States, United Kingdom, and Korea (Brandsaeter et al. 2002, Polson et al. 2005, Escorsell et al. 2007, Kjashab et al. 2007, Bernal et al. 2010, Bernal et al. 2013, Suk et al. 2012). While studies conducted in Japan exclude patients with alcohol exposure for analysis (Fujiwara et al. 2008, Oketani et al. 2011, Oketani et al. 2013), some in the United States consider alcohol as playing a contributing but unclear role in ALF (Larson et al. 2005, Bower et al. 2007). In the current ALF cohort, about one fourth have multiple etiologic exposures, suggesting that their ALF may be attributed to multiple hits in a short period of time. There have been reports showing that acute hepatitis C in patients with concurrent chronic HBV infection is associated with a substantial risk of

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ALF (Chu et al. 1994, Chu et al. 1999). It is also clear that genetic polymorphisms, or the effects of concomitant drugs, alcohol, or diseases, can alter the threshold for exposure to other toxic metabolites and result in ALF (Kaplowitz et al. 2005).

Despite the well-known high mortality rate of ALF, reports on its long-term sequelae are lacking. This study shows that liver cirrhosis occurs in about half of ALF survivors within less than 3 years. Cases of ALF with features suggestive of an autoimmune pathogenesis have higher incidence of chronic hepatitis in long-term follow-up than those without such features (Stravitz et al. 2011). Acute liver injury, even those caused by single-dose or short-term administration of hepatotoxic agents such as temozolomide, can be followed by prolonged liver damage (Grant et al. 2013). Furthermore, the quality of life is significantly impaired in long-term survivors of ALF (Rangnekar et al. 2013). All of these findings suggest that ALF may have some sustained irreversible impact. As the course of ALF is widely heterogeneous in nature, further long-term clinical observational study is needed to characterize potential late complications of ALF and improve follow-up care of survivors.

The use of proton pump inhibitors is an independent protective factor of survival of ALF patients in multivariate analysis. Despite the lack of firm evidences, acid suppression by a proton pump inhibitor is recommended to prevent upper gastrointestinal bleeding in intubated ALF patients or those in the intensive care unit (Polson et al. 2005). By inference, proton pump inhibitors are likely to contribute to decreased incidence of significant upper gastrointestinal bleeding in patients with ALF (Cook et al. 1994, Lee et al. 2012). Peptic ulcer disease, in current study, is also an independent risk factor for intra-hospital hemorrhage. Critical illness, such as respiratory failure and renal failure requiring renal replacement therapy, may also increase the risk of stress ulcer bleeding (Skillman et al. 1969). To date, this is the first study to show the survival benefit of using proton pump inhibitors in patients with ALF on their index admission. This study provides positive evidence for recommending the use of proton pump inhibitors in ALF patients.

Liver transplantation is considered as a life-saving procedure for patients with ALF, but is not popular as a timely treatment option in this cohort. It may be due to the rapid course of ALF and the limited organ source. Artificial liver support with plasmapheresis and hemodialysis/hemofiltration plays a bridging role while a donor liver or the regeneration of the native liver is being awaited (Fujiwara et al. 2008). Earlier studies report mortality rates near 85% before transplantation (Germani et al. 2012). However, in the post-transplantation era, one-year survival rates are estimated to be 60-80% (Ostapowicz et al. 2002, Liou et al.

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2008, Germani et al. 2012, Bernal et al. 2013).

During the study period, there were 88 liver transplant procedures (69 living donors and 19 deceased donors) in the National Taiwan University Hospital. Among them, two deceased-donor liver transplantations were performed for ALF patients. The study cohort was a random sampling of one out of 23 million population of Taiwan. Thus, it is representative of the general, but not the transplant center, population. However, early recognition, prompt referral, and living donors in areas of low organ donation rates may save more lives of patients with ALF (Escorsell et al. 2007, Khashab et al. 2007, Fujiwara et al. 2008, Bernal et al. 2010, Lee et al. 2012, Bernal et al. 2013).

This study has some limitations. Although the target cohort has been approached by utilizing multiple criteria, including the diagnosis, prescription of medications, and laboratory tests, the insurance reimbursement database has a built-in shortage of no information of laboratory data, radiographic findings, and medications not covered by insurance (i.e. over-the-counter drugs). The frequency of laboratory testing and the statistics about hospital stay and ICU admission could be biased by the judgment of the attending physicians , but generally it is reasonable that more frequent laboratory testing and longer hospital or ICU stay would be expected in more critically ill patients. Nevertheless, it is very difficult to validate the diagnosis of ALF and the cause-effect relationship. Furthermore, patients with chronic liver disease may have been included if the disease has not been established within 3 years prior to the index admission (Lee et al. 2012). Therefore, using the 218 sample cases as the numerator to calculate the incidence of the ALF may have some bias. This may also occur in previous studies and may be a reason for the heterogeneous clinical characteristics (Koretz et al. 1978, Saracco et al. 1988, Chu et al. 1990, Lee et al. 2012). However, it is less likely that a slowly progressed or even stable underlying liver disease which requires no medical help within recent 3 years will rapidly deteriorate and result in liver failure without acute and new hepatic insult.

In summary, the incidence of ALF increases with age in Taiwan. Viral infection is the major etiology. Mortality rate is about 40% within 3 months and half of the survivors have concomitant liver cirrhosis. Patients with malignancy and alcohol exposure have the worst prognosis. Use of proton pump inhibitors has a protective effect. Liver transplantation for ALF is not highly utilized in Taiwan and early referral to a transplant center is recommended.

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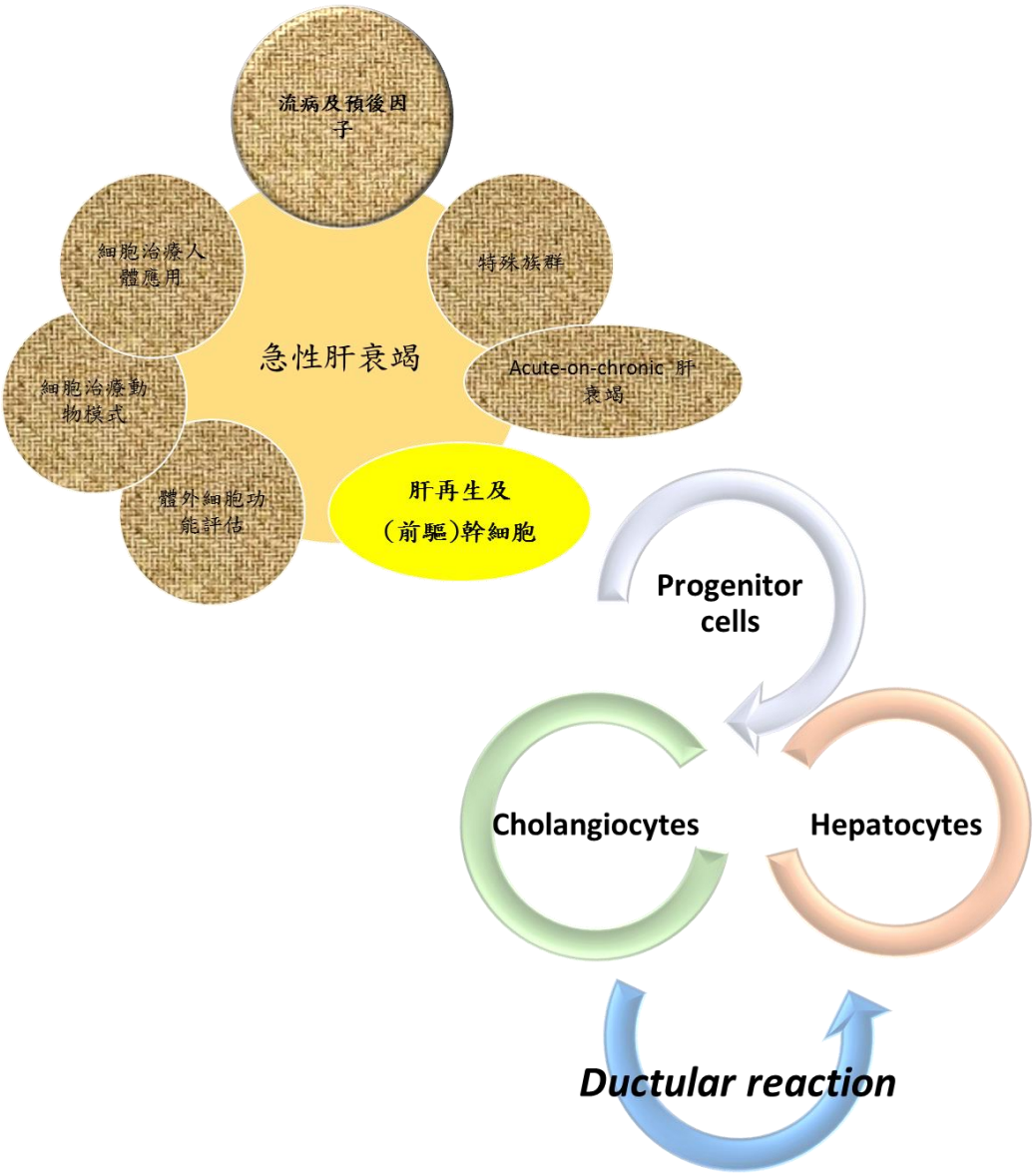
Chapter 3

Acute liver failure *in micro*

Histological study



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(1) Case scenario

A 22-year-old man, who was otherwise healthy before, was referred for acute liver failure complicated with hepatic encephalopathy, renal insufficiency and sepsis with the initial presentation of fever, vomiting, and abdominal pain for 5 days. Laboratory surveys revealed a recent fresh hepatitis B viral infection as evidenced by the high positivity of IgM antibody against hepatitis B core antigen. The liver failure syndrome progressed even though he received specific antiviral agents, broad-spectrum antibiotics and intensive supportive care. The patient underwent urgent deceased donor liver transplantation 2 weeks since admission. The native liver weighted 1.1 kg without cirrhosis and the volume of straw-colored ascites were 2.4 liters, as the operation findings.

(2) Methods

Parrafin-fixed specimen of the explant was examined by immunohistochemical staining for markers of progenitor cells [cytokeratin 19 (CK19), epithelial cell adhesion molecule (EpCAM)], hepatocytes [HNF4α, carbamoyl phosphate synthetase (CPS-1, a urea cycle enzyme)], cholangiocytes (HNF1β), differentiation (NUMB, an inhibitor of the Notch pathway), and proliferation (Ki-67). Serial sections were compared and examined between marker expressions in addition to pathologic morphology.

(3) Results

Histological examination of the explant liver showed submassive necrosis and prominent ductular reaction. Characterized by immunostaining of CK19 (Figure. 11), the road of hepatocyte differentiation was nicely shown from the bipotential progenitor cells (thick stained, small cell size, high nuclear-cytoplasm ratio) and gradually spirally spreading outward to form daughter intermediate hepatocytes (light stained, larger cell size, lower nuclear-cytoplasm ratio) (curved arrow) (Figure. 11). These differentiating cells did not proliferate actively (Ki-67 staining, Figure. 12), and express EpCAM (Figure. 13) and transition of NUMB (Figiure. 14, 15) and CPS-1 (Figure 16). Progenitor cells differentiating toward hepatocytes exhibited HNF4α and CPS-1 (Figure 17B, C, long and thin arrow) and cholangiocytes, HNF1β without CPS-1 (Figure 17B, D, short and thick arrow).

Interestingly, they were negative for HBsAg and HBcAg, suggesting no further HBV infection since differentiation from mother progenitor cells. Canal of Hering can be identified

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by lines of low cuboidal cells which were CK19+ (arrow, Figure. 18, 18A), and CPS1- (arrow, Figure. 19B). Although shown actively as a sparing mechanism for rescuing liver regeneration, ductular reaction occurred in this case failed to achieve overall functional recovery.

(4) Conclusion

Prominent ductular reaction with at-least partially functional hepatocyte differentiation did not guarantee successful regeneration in acute liver failure and there is demand left for hepatocyte transplantation.

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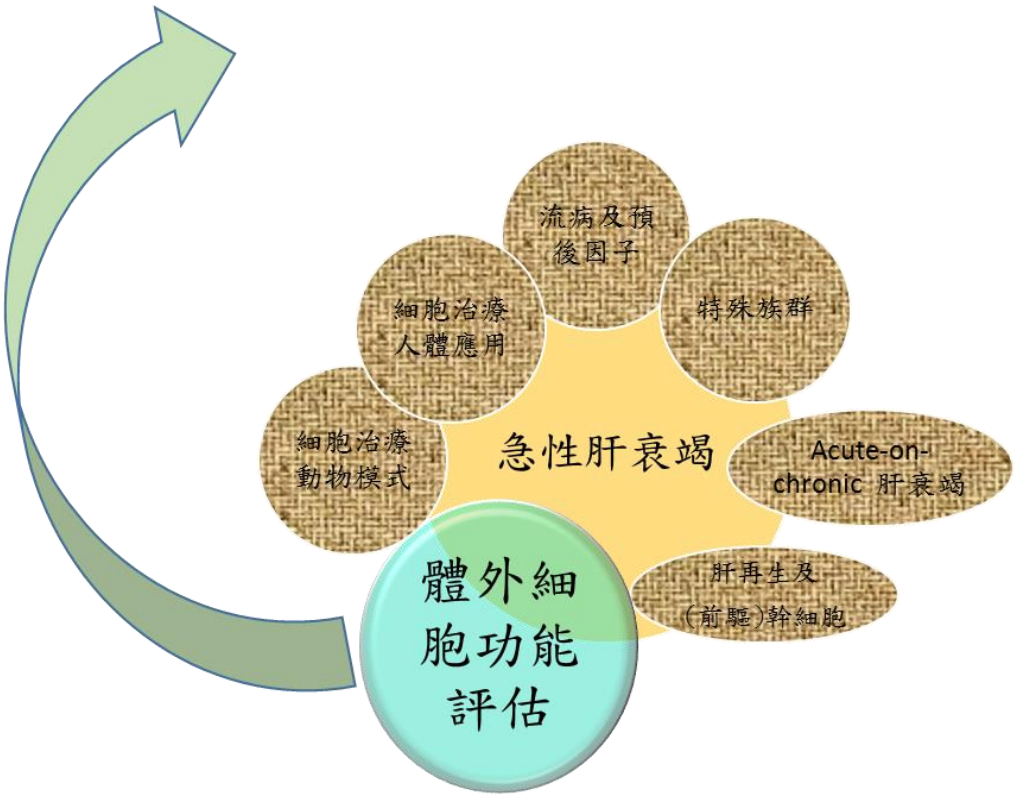
Chapter 4

Hepatocytes *in vitro*

Are they good enough

Investigation of the use of indocyanine green for functional assessment of human hepatocytes for transplantation



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(1) Background

Hepatocyte transplantation was first introduced into clinical practice in 1992 (Mito et al. 1992) as a promising alternative to liver transplantation or as a bridging therapy for patients with metabolic diseases and acute liver failure (Nussler et al. 2006, Puppi, et al. 2009). Hepatocyte transplantation is a safe and less invasive procedure for patients with liver disease than whole organ transplantation. Animal studies have clearly proven the efficacy of hepatocyte transplantation, however, this has not translated into clinical practice where there is often limited benefit (Haridass et al. 2008). One of the major reasons for this is the quality of the hepatocytes that have been infused, which are often isolated from livers that have been rejected for transplantation. Currently trypan blue exclusion is used as a rapid test of cell viability, which determines cell integrity by staining the nuclei of dead cells. This test does not reflect the metabolic function of the hepatocytes, which is important in vivo. Measures of specific synthetic function such as albumin, and clotting factor 7 synthesis require cell culture, and are not applicable to determine suitability of hepatocytes for immediate infusion into a patient.

Current commercial assays use technologies that are either nonspecific to hepatocytes (ATP detection; redox activity, membrane integrity) or not available for routine clinical use (P450 assays by HPLC, mass spectrometry). Indocyanine green (ICG) is an organic anion used in hepatobiliary surgery to assess liver reserve before resection and is specifically eliminated by the liver (Cherrick et al. 1960) ,ICG uptake by hepatocytes assessed by microscopy has been recently used to assess the *in vitro* function of stem cell derived hepatocytes (Agarwal et al. 2008; Yamada et al. 2002). At the cellular level, ICG is taken up by hepatocytes via the transporter OATP1B1 (OATP2 (rat)/OATP-C (human) which is exclusively expressed in the basolateral membrane of hepatocytes (Campbell et al. 2004, Ito et al. 2005, König et al. 2006), as used by bilirubin (Scharschmidt et al. 1978) and then excreted into the bile cannaliculus by MRP2 which requires ATP (Simon et al. 2006, Huang et al. 2001). ICG uptake can also reflect the degree of hepatic triglyceride content in a dose-responsive relationship (Takahashi et al. 2000).

In this study we have investigated whether quantitative measurement of the uptake and release of ICG by human hepatocytes has potential to develop a rapid test of metabolic function prior to hepatocyte transplantation.

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(2) Methods

Isolation and culture of human hepatocytes

Hepatocytes were isolated from unused donor liver tissue using a modified collagenase perfusion technique (Mitry et al. 2003). Donor data and liver characteristics are shown in Table 5. Viability was assessed by 0.4% trypan blue exclusion test. Cell suspensions contained 1.0×10^6 cells. Collagen-coated culture plates were seeded with fresh or defrosted cells, which were then incubated in Williams' medium E (WME) supplemented, as previously described (Mitry et al. 2004). HepG2 (human hepatocellular carcinoma cell line) cells were cultured in RPMI1640/ 10% fetal calf serum overnight before use. Cell suspensions were used for experiments with ICG immediately. Cells were cultured overnight to determine the effects of ICG treatment on hepatocyte functions.

ICG Treatment

ICG dry powder 5 mg (Cardiogreen ® Sigma Aldrich, Gillingham, Dorset, UK) was dissolved in 1 ml solvent (100 μl DMSO (CryoSure-DMSO**,** WAK-Chemie Medical GmbH) and culture media added to obtain a stock of 5 mg/ml. The solution was shaken for 2 minutes to ensure that the powder was completely dissolved. The experimental solutions used were different concentrations of ICG (0.25, 0.5, 1.0, 2.0 mg/ml) and in further experiments at 0.5 mg/ml and 1.0 mg/ml.. Cells were incubated with ICG as suspension or plated for 30 minutes at 37℃ in 95% O2/5% CO2. Cells were washed with PBS and centrifuged to obtain the pellets if in suspension and then re-incubated in media alone to determine ICG release. Supernatants were collected after 1, 2, and 3 hours for measurement of ICG concentration against a standard curve using a DYNEX Technologies MRX microplate reader, (supplied by Prior Laboratory Supplies Ltd. East Sussex, UK), at OD 820 nm. Plates were re-incubated overnight, and cell function analyzed using the following assays.

Cell attachment—sulphorhodamine (SRB) assay

To determine cell numbers on culture plates, the SRB assay was performed as described previously (Mitry et al. 2000). In each well of the 96-well plate, 50 μl of ice-cold 50% trichloroacetic acid solution was gently layered on top of the medium overlaying the cells.

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The plates were then incubated for 60 minutes at 4 ℃. Wells were rinsed 5 times with water and then cells stained with 0.4% SRB solution (100 µl stain/well) for 15 minutes at room temperature. SRB staining solution was poured off, wells were rinsed 5 times with 1% acetic acid to remove unbound dye, and left to air dry. The bound SRB dye was then solubilized by adding unbuffered Tris-base solution (200 µl/well), and plates placed on a plate shaker for 1 hour at room temperature. Plates were then read at OD 564 nm, using a microplate reader.

Mitochondrial activity—modified MTT assay

The assay was as described previously (Mitry et al. 2005). Briefly, 20 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution was added to the 200 µl medium in each well of the 96-well plate, and the plate incubated at 37 ℃, for 4 hours. The medium was then removed by aspiration, 120 µl isopropanol/HCl added per well, the plate shaken for a 15 min and the absorbance at OD 630 nm measured.

[^{3}H]-thymidine incorporation into HepG2 cells

The effect of the ICG (0, 0.5 and 1 mg/ml) on DNA synthesis of HepG2 cells was assessed using [^{3}H]-thymidine incorporation assay. The medium in each well was replaced with an equal volume (200 µl) of fresh medium containing [^{3}H]-thymidine (0.5 µCi/well), (Amersham International plc. Amersham, Bucks. UK). The plates were incubated overnight. The cells were then harvested onto glass fibre membranes using a cell harvester (FilterMate, Packard Instruments, Pangbourne, Berkshire, UK). The filters were dried and the radioactivity counted (MATRIX 9600 Plate Counter, Packard Instruments) to determine the incorporation of radioactivity into the cells.

Albumin synthesis

Concentration of albumin in culture media was determined by enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX) using a sheep anti-human albumin antibody. The assay was done according to the manufacturer's instructions. The absorbance was read at 450nm.

Cell viability - staining with fluorescein diacetate (FDA)/ethidium bromide (EtBr)

A stock solution of FDA (Sigma-Aldrich, Dorset, UK) was prepared by dissolving 5 mg/ml in DMSO. The FDA working solution was freshly prepared by adding 0.01 ml of stock to 5

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ml of EtBr (Sigma-Aldrich) stock solution prepared by dissolving 10 mg/ml in PBS. Cells in supernatants were collected by washing with 0.5 ml PBS at room temperature and centrifugation for 50 x g, 4℃ for 4 minutes. Cell were resuspended with 0.2 ml FDA/EtBr solution and incubated for 6 minutes at room temperature. Cells were then collected again by removing the staining solution, washing twice with PBS and centrifugation. Stained cells were resuspended with 2 drops of antifading regeagent and placed onto a microscope slide with a coverslip. The cells were observed under a fluorescent microscope (filter set 09 ZEISS, ex = 450 - 490nm, em = 520 nm), while nuclei were stained red (ex = 506 nm, em = 610 nm) at 100x to 400x magnification.

Taurine treatment of hepatocytes

Fresh human hepatocytes were cultured overnight with taurine (Sigma-Aldrich) at 20 mM, then cells were incubated with ICG and tested as above.

Statistical analysis

Statistical analysis of the results was carried out using the Student's t-test and Pearson correlation test. $P<0.05$ was considered significant.

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(3) Results

ICG uptake and release by hepatocytes and HepG2 cells

Human hepatocytes and HepG2 cells took up ICG with cells turning green after incubation for 30 minutes. After three hours the ICG was released by the cells (Figure 20) The release of ICG by human hepatocytes (Figure 21) and HepG2 cells (Figure 22) was related to the initial ICG concentration during uptake from both the loss of green color of the cells and appearance of ICG detected microscopically in the culture medium. There was a significant correlation between the ICG release and the viability of human hepatocytes measured by trypan blue uptake ($r=0.85$, $P=0.008$). The pattern of ICG release in human hepatocytes showed a rapid release reaching a plateau level soon after 1 hour. (Figure 23) This effect was seen with cells both in suspension and in culture, but tended to be more rapid in culture.

Effect of incubation with ICG on hepatocyte function

The effects of increasing concentrations of ICG on hepatocyte function were determined. With the MTT assay, hepatocytes incubated with 1.0 mg/ml ICG had higher mitochondrial dehydrogenase activity compared to 0.5 mg/ml ICG or control (0.025 ± 0.0004 v.s 0.019 ± 0.0008 or 0.020 ± 0.002, $P < 0.05$ for hepatocytes; 0.038 ± 0.004 v.s. 0.025 ± 0.003 or 0.025 ± 0.004, $P < 0.05$ for HepG2 cells) (Figure 24).

Incubation of HepG2 cells with ICG reduced albumin production (98.9 ± 0.02, 66.6 ± 0.05, 39.1 ± 0.4 ng/ml for control, 0.5 mg/ml, and 1.0 mg/ml ICG respectively) and also decreased [^{3}H]-thymidine incorporation in a dose-response manner (Figure 25, 26).

Cells had lower attachment when tested 6 hours after incubation with increasing concentrations of ICG (Figure 27). However, if the plates were reincubated overnight the cells reattached. To investigate these further cells in the supernatants were collected after incubation with ICG (0-2 mg/ml) and stained with FDA/EtBr. Greater numbers of viable cells were detached at higher ICG concentrations (0.5 mg/ml and 1 mg/ml) than those at 0.25 mg/ml ICG and control (Figure 28).

Effect of taurine on hepatocyte transport of ICG.

Pretreatment of fresh human hepatocytes with taurine in culture overnight gave greater amounts of ICG release and the pattern of ICG release was maintained with high ICG concentrations (Figure 29). Compared with control, taurine also resulted in a higher degree of

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cell attachment, and enhanced reattachment overnight following ICG incubation (Figure 30). Pretreatment with taurine prevented the stimulatory effect on the MTT assay at 1.0 mg/ml ICG, but not at 0.5 mg/ml (Figure 31).

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(4) Discussion

The results of these experiments show that there is a distinct pattern of uptake and release of ICG by human hepatocytes which can be quantitated using readily available laboratory equipment. Hepatocytes take up ICG in 30 min and then excrete the unchanged dye in 1 to 2h. The specific uptake of ICG by intact hepatocytes is followed by excretion via the ATP-dependent MRP-2 transporter, which is the rate-limiting step (Huang et al. 2001), thus being a dual measure of cell metabolic function. Cells with impaired function will have reduced amounts of ATP which will limit ICG excretion. There was a characteristic ICG release curve for hepatocytes which showed a peak in the second hour of incubation. In this preliminary study only a relatively small number of cell batches were used. Further experiments are needed to define the ICG pattern for cells isolated from fatty livers, which are likely to have impaired function (Green et al. 1998) and those from non-heart-beating donors exposed to warm ischemia (Soric et al. 2007).

There was a correlation of ICG excretion with cell viability by trypan blue exclusion suggesting that trypan blue exclusion is related to cell function in the range of cell viabilities studied. However, ICG is specific to hepatocytes and should reveal more about cellular function than just membrane integrity. Ideally any measure of hepatocyte function should be correlated to engraftment and function of hepatocytes after transplantation.

HepG2 cells, although they are transformed cells, had a similar release pattern of ICG to "normal hepatocytes". They are thus a useful tool for developing the conditions for an ICG test having high viability as "best quality" hepatocytes are not often available. However, HepG2 cells may not predict metabolism in adult human liver cells, because their expression of drug-metabolizing enzymes is different (Wilkening et al. 2003). Incubation of human hepatocytes and HepG2 cells at concentrations above 0.5 mg/ml had effects on cell metabolism including stimulation of mitochondrial dehydrogenase activity and inhibition of albumin synthesis and proliferation of HepG2 cells. Other studies in retinal cells have shown high concentrations of ICG to be toxic with a proapototic effect (Kawahara et al. 2007). The concentration used to test hepatocytes in vitro should be 0.5 mg/ml to avoid these effects

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In the cell attachment experiments using the SRB assay, we found that cells incubated with ICG had lower attachment during ICG release but recovered to give better attachment the next day. It is interesting to speculate whether this is in some way related to location of the ICG uptake OATP transporter on the basolateral membrane, which attaches to the collagen coating on the culture plates (Musat et al. 1993). Whereas the excretion of ICG is via the transporter in the canalicular membrane of the hepatocyte.

Taurine which has been shown to be involved in membrane stabilization (Green et al. 1998) and can act as an antioxidant (Das et al. 2008) was used to modulate cell function to see the effects on ICG transport. With hepatocytes pretreated with taurine they appeared to better tolerate higher concentrations (1.0 mg/ml) of ICG with increased cell attachment. Thus ICG disposition could detect a protective effect on cell function.

In a recent study, Donato et al (Donato et al. 2008) rapidly assessed the cellular P450 enzyme function of human hepatocytes within 1 hour by HPLC-MS/MS. However, these assays require sophisticated equipment and are not available in everyday clinical use. They also determined urea synthesis, which is a good specific marker of hepatocyte metabolic function, though this did not correlate with the cell viability. It is likely that a panel of rapid assays will give the most useful data.

In conclusion, *in vitro* hepatocyte function can be assessed by the ICG release pattern within two hours. Further refinement of this assay, particularly in reducing the time taken, should lead to a test of hepatocyte function to help assess the quality of isolated human hepatocytes for transplantation.

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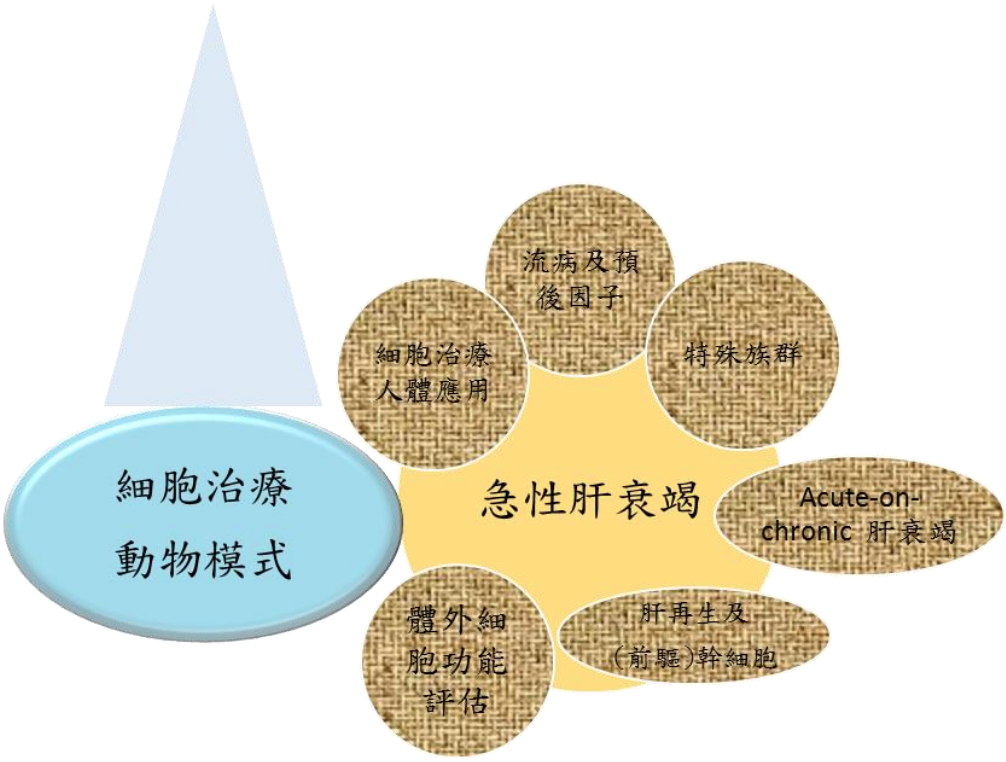
Chapter 5

Hepatocyte transplantation

Strategy for better engraftment

Translational study with clinical implication



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(1) Background

Hepatocyte transplantation is a promising alternative to liver transplantation in cases of acute liver failure (Puppi et al. 2012). Since the first case of clinical human hepatocyte transplantation performed by Mito et al. (Mito et al. 1992) in 1992, hepatocyte transplantation has gained proof-of-principle success more in animal experiments than in clinical use worldwide (Mazaris et al. 2005, Fisher et al. 2006, Dhawan et al. 2010). The key issue has become translating bench success in hepatocyte transplantation into improvements in its clinical application (Dhawan et al. 2010, Puppi et al. 2012). Many of the preconditioning "recipes" used to improve engraftment in animal studies are inappropriate for use in humans; 2 examples are retrorsine, which inhibits native hepatocyte proliferation and thus enhances relative graft cell survival, and monocrotaline, which disrupts sinusoidal endothelial barriers and thus assists graft cell migration. Partial hepatectomy, portal vein embolization, and hepatic irradiation before cell transplantation might be feasible but are surely highly risky in patients with acute liver injury. These difficulties limit the development and use of hepatocyte transplantation (Dagher et al. 2009, Yamanouchi et al. 2009, Soltys et al. 2010, Guha et al. 2011, Koenig et al. 2011, Puppi et al. 2012).

Hepatocytes can be delivered through the portal vein, which is preferred in cases of acute liver failure with normal architecture; the spleen, when chronic liver disease with cirrhosis is encountered; or the hepatic vein, which has recently been identified as an alternative route (Goto et al. 2011). Whatever the route(s), transplanted hepatocytes usually block the terminal portal veins, causing a transient increase in portal pressure that later facilitates the entry of the cells into sinusoidal areas by an ischemic-reperfusion injury-mediated mechanism (Gupta et al. 1999, Koenig et al. 2005, Koenig et al. 2011). For example, in the dipeptidyl peptidase IV (DPPIV) knock-out rat model of Koenig et al. individual hepatocytes were integrated into the parenchyma 24 hours after transplantation, and un-engrafted cells were cleared by Kupffer cells within 48 hours (Koenig et al. 2005). Increasing engraftment in the early post-transplantation period, before Kupffer cell engulfment, is key to increasing the clinical applicability of hepatocyte transplantation and improves the odds for successful graft cell repopulation.

The transfusion rates used for clinical and experimental hepatocyte transplantation vary in literature, with cell suspension densities of 0.5–2.0 × 10^7/mL, transfusion rates of 0.5–2.0

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mL/min, and total cell doses estimated based primarily on the disease model and host weight (Strom et al. 1999, Muraca et al. 2002, Yu et al. 2004, Koenig et al. 2005, Dhawan et al. 2009, Meyburg et al. 2010, Goto et al. 2011). There are no definite guidelines for the transfusion rate. Research on hydrodynamic delivery of genes for gene-based liver therapy supports the use of higher transfusion rates (Zhang et al. 2004, Herweijer et al. 2007). It had been reasoned that upon rapid injection into a peripheral vein, the injected fluid enters the vena cava, where the fluid backs up because the large volume cannot be pumped through the heart sufficiently fast (Sawyer et al. 2007). This creates increased pressure in the vena cava and pushes the nucleic acid-containing solution into the draining vasculature, in particular, the large hepatic vein. There, the fluid is forced out of the capillaries into the tissue, and the nucleic acids enter the parenchymal cells. Hydrodynamic injection also induces enlargement of the fenestrae in the hepatic sinusoidal endothelium and enhancement of hepatocyte membrane permeability (Zhang et al. 2004). Effective hydrodynamic delivery is not limited to small (e.g. small interfering ribonucleic acid) and large [plasmid deoxyribonucleic acid (DNA)] nucleic acids (Lewis et al. 2007) but extends to a range of other molecules, including proteins (Herweijer et al. 2007). Red blood cells and platelets were also observed in the space of Disse and even inside rat hepatocytes following hydrodynamic injection (Suda et al. 2007). This suggests that the initial engraftment of hepatocytes may follow similar principles.

In addition, intervention in rats with acute liver injury carries a high mortality rate, and this limitation makes it difficult to compare the results of hepatocyte transplantation with those in other models. The aim of this study was to improve the engraftment and repopulation efficiencies of hepatocyte transplantation for treatment of acute liver failure in a clinically useful way without preconditioning. We hypothesized that the transfusion rate influences the engraftment efficiency of hepatocyte transplantation through the portal vein and that increasing engraftment efficiency by varying the transfusion rate would increase subsequent repopulation.

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(2) Methods

Recipient animals

Male Sprague-Dawley (SD) rats aged 8 weeks and weighing 200–250 g, purchased from BioLASCO Taiwan, were used as the recipient animals. These animals were maintained on standard laboratory chow with a 12-h light/dark cycle. All surgical procedures were performed under anesthesia with Imalgene 1000 (Merial Laboratoire de Toulouse, France)/Rompun (Bayer, Korea). All of the animals received humane care in accordance with the guidelines of the National Science Council of Taiwan (NSC, 1997). The animal experiments were approved by the Institutional Laboratory Animal Care and Use Committee of National Taiwan University.

Donor cells

DsRedT3-emGFP transgenic SD rat hepatocytes were used as the donor cells. The rats (males aged 8–10 weeks and weighing 200–250 g) were purchased from National Laboratory Animal Center, Taiwan. Hepatocytes were purified by in situ liver perfusion, collagenase digestion, and differential centrifugation as previously described (Yu et al. 2004). The viability and purity of each preparation were assessed by evaluating trypan blue (Sigma) exclusion using a Bright-Line™ hemocytometer (Sigma-Aldrich). Isolated hepatocytes were resuspended in phosphate-buffered saline (pH 7.4) (Sigma-Aldrich) at 1×10^7 cells/mL. The preparations contained at least 85% viable hepatocytes, which were transplanted within 1 hour of isolation.

Experimental design

Hepatocyte transplantation for treatment of acute liver failure

One week before transplantation, a 24-G catheter (0.7 mm in diameter, BD, Insyte^{TM}) was placed with the tip in the main trunk of the portal vein before bifurcation via midline laparotomy (Figure 32A), with the lock beneath an undermined skin flap (Figure 32B). The catheter was heparinized and fixed secured with 4-0 silk sutured to the periportal connective tissue first, followed by a loop of knot between the portal vein and the abdominal wall, and finally with the lock reinforced to the abdominal muscle. Acute hepatic injury was induced with D-galactosamine (Sigma) treatment (0.9 g/kg intraperitoneally) 24 hours before transplantation. Hepatocytes (1×10^7/mL) isolated from DsRedT3-emGFP transgenic SD rats were transplanted intraportally through the pre-implanted lock by continuous infusion at 3 steady rates (1/30, 1/70, 1/100 mL/sec) so that the 1-mL volume was infused over 30, 70, or

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100 seconds. Rats were sacrificed and their livers harvested 0, 2, and 7 days after hepatocyte transplantation (Figure 33).

Real-time monitoring of portal pressure during hepatocyte transplantation

Acute hepatic injury was induced in SD rats by D-galactosamine treatment (0.9 g/kg, IP) 24 hours before transplantation. The main portal vein was identified and cannulated with 2 heparinized 24-G catheters (BD, Insyte^{TM}) which were inserted right before the time of transplantation, one for continuous measurement of portal pressure and the other for cell transfusion. The tips of the catheters were at the main trunk of the portal vein before bifurcation. Portal pressures were measured, as described previously (Yu et al. 2004). A total of 1 $\times 10^7$ donor cells in 1 mL were transplanted intraportally through the catheter via continuous infusion at 3 steady rates such that each infusion took place over 30, 70, or 100 seconds, with real-time monitoring of the portal venous pressure. When the portal pressure returned to baseline after hepatocyte transplantation, the rats were sacrificed and their livers harvested (Figure 33).

Immuno-fluoro-histo-chemistry

Transplanted DsRedT3-emGFP hepatocytes in the recipient liver were identified by direct visualization of red fluorescence or staining with antibody against DsRed fluorescence of liver cryosections. Antibodies for staining of DsRed hepatocytes, or for intrahepatic vascular structures against CD31 and vascular cell adhesion molecule 1 (VCAM-1) were used according to the manufacturers' recommendations and detailed in Table 6. Nuclei were revealed with 4',6-diamidino-2-phenylindole (DAPI) staining. Secondary antibodies, including Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes, Oregon, USA), Alexa Fluor 594 donkey anti-goat IgG, and Alexa Fluor 594 donkey anti-rat IgG, were used in immunofluorescence assays.

Hepatic histology and determination of liver engraftment/repopulation

Fresh liver sections were fixed in formalin (Merck, KGaA), embedded in paraffin, sectioned, and stained with hematoxylin and eosin to demonstrate the hepatic histology. Transplanted DsRedT3-emGFP hepatocytes in the recipient liver were identified by direct fluorescence of liver cryosections or immuno-fluoro-histo-chemistry. To analyze the liver repopulation, 3 to 4 sections from multiple liver lobes per rat were examined. Microphotographs were obtained from consecutive adjacent areas to include the whole section under 100× magnification using a digital camera (SPOT™ Imaging Solutions, Diagnostic Instruments, Inc.). The area occupied by the transplanted hepatocytes was quantified using ImageJ software (National

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Cancer Institute, Bethesda, MD).

Serological assay

Hepatic venous blood was sampled when the recipient rat was sacrificed. Biochemical analysis were measured in an animal laboratory by standard automated assays (Hitachi 7080 chemistry analyzer, Hitachi Ltd.) International normalized ratio (INR) of prothrombin time was determined using fresh whole blood without anticoagulants and measured using an automated coagulation monitoring device (CoaguChek XS System, Roche Diagnostic, Mannheim, Germany).

Statistical analysis

The number of animals per treatment was at least 4. Data are presented as the mean ± standard error and analyzed using a student t test or an analysis of variance (ANOVA) followed by the Tukey post hoc test, when appropriate. Data analysis of real-time pressure measurement was performed by using non-parametric Kruskal-Wallis one-way analysis-of-variance-by-ranks test. A $P < 0.05$ was considered significant.

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(3) Results

Pathology of acute liver injury model

Grossly, the liver demonstrated congestion and easily touched bleeding (Figure 34A). The histopathology of our rat model of acute liver injury showed massive hepatocyte necrosis and apoptosis, hemorrhage and sinusoidal dilatation (Figure 34B).

The rate of cell transfusion was associated with the engraftment efficiency and subsequent repopulation efficiency in acute liver injury

In our acute liver injury model, 3 different transfusion rates resulted in different engraftment efficiencies ($P = 0.018$) and subsequent repopulation efficiencies ($P = 0.037$) by ANOVA (Figure 35). Post hoc tests utilized Tukey's procedure revealed that infusion over 70 seconds yielded a higher engraftment efficiency (1.1 ± 0.53%) than the other rates [30-second (0.35 ± 0.32%) and 100-second (0.16 ± 0.18%); $P = 0.072$ and 0.018, respectively]. Infusion over 70 seconds also tended to produce a higher repopulation efficiency (2.06 ± 1.78%) than the other rates [30-second (0.09 ± 0.09%) and 100-second (0.14 ± 0.25%)] (Post hoc tests, $P = 0.063$ and 0.074, respectively) (Figure 35B).

Liver function improvement

The blood chemistry results of serum aspartate aminotransferase (AST) level, serum alanine aminotransferase (ALT) level, serum total bilirubin level, serum albumin level, INR, and blood ammonia level were shown in Figure 36. The serum AST level (2179.1 ± 381.7 U/L), serum ALT level (855.3 ± 352.2 U/L) and INR (4.5 ± 0.3) were highly elevated 24 hours after induction of acute liver injury using D-galactosamine and the success of consistent acute liver injury induction using D-galactosamine based on the comparable biochemical and histological changes 24 hours after the injection D-galactosamine in our previous pilot study (Yu et al. 2004). In the 70-second group, the albumin level was significantly higher after 1 week (3.2 ± 0.2 g/dL) than on day 2 (1.6 ± 0.5 g/dL; P = 0.0044) and the serum AST level was significantly lower after 1 week (67.9 ± 9.1 U/L) than on day 2 (284.6 ± 69.6 U/L; P = 0.036) after hepatocyte transplantation. The serum levels of albumin and AST did not change significantly between day 2 and 1 week in the other groups (30- and 100- second). The ammonia level was significantly higher after 1 week (69.0 ± 6.3 μmol/L) than on day 2 (40.8 ± 3.3 μmol/L; P = 0.013) in the 30-second group and of no significant changes in the other groups. The serum ALT level, serum total bilirubin level, and INR did not change

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significantly between day 2 and 1 week in 3 groups.

Real-time monitoring of perioperative portal venous pressure during hepatocyte transplantation for treatment of acute liver injury demonstrated no significant portal hypertension

Median time to the first peak portal venous pressure was 33, 75 and 100 seconds in the 30, 70, and 100-second groups, respectively, with significance ($P < 0.001$). Real-time monitoring of perioperative portal pressure during hepatocyte transplantation demonstrated no significant difference in baseline, peak pressures and pressures at 10, 20, and 30 minutes among the 3 groups (Figure 37). The mean first peak pressures were 14.8 ± 6.5, 17.7 ± 3.7, and 13.6 ± 3.0 mmHg in the 30, 70, and 100-second groups, respectively. The portal venous pressure achieved second peaks shortly after declined from the first ones, though the second peak portal pressures were smaller compared to the first ones (Figure 37).

Early engraftment of donor cells was observed shortly after hepatocyte transplantation over 70 seconds

Early engraftment of transplanted hepatocytes can be observed shortly after transplantation for treatment of acute liver injury when infused over 70 seconds (Figure 38A), but not seen in the other groups. Transmigrated DsRedT3-emGFP cells occasionally could be noted just outside the wall of the portal vein (Figure 38B).

Potential complications of hepatocyte transplantation at different rates of transfusion

Cell embolus was rarely observed in the lung in all groups 1 week after the procedure (Figure 39). Pulmonary embolism was not observed more frequently after transfusion at higher rates, and transfusion at a slower-than-optimal rate did not prevent pulmonary entrapment of transplanted cells.

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(4) Discussion

Our study demonstrated that transfusion rate is an important determinant of the engraftment and repopulation efficiencies after hepatocyte transplantation for treatment of acute liver injury. Table 7 and 8 summarizes the current literature (animal and human) regarding the transfusion rates used in hepatocyte transplantation for various etiologies (Fox et al. 1998, Muraca et al. 2002, Yu et al. 2004, Weber et al. 2006, Lee et al. 2007, Weber et al. 2009, Meyburg et al. 2010, Goto et al. 2011, Enosawa et al. 2012, Ribes-Koninckx et al. 2012, Stéphenne et al. 2012, Timm et al. 2013). Rates slower than 0.5 mL/min were associated with intraportal accumulation of cells though engraftment in an acute liver injury model is expected to be considerably different from transplant into a confluent, normal liver in many of these referred studies. For example, Fox *et al.* (Fox et al. 1998) transplanted hepatocytes through the portal vein at a rate of 2.5 mL/min in a 10-year-old girl with Crigler-Najjar syndrome type I without significant increase in portal vein pressure and the patient survived for more than 11 months with partial correction of the metabolic disorder. Consideration of our study results altogether imply the significance of rate influence in cell therapy. Although few cases were reported for acute liver failure, it suggested that, in general, the rate of the intraportal transfusion of hepatocytes could be, at least, increased safely for potentially improved efficiency.

In our study, donor cells were observed to extravasate through the sinusoidal endothelial barrier shortly after transplantation with infusion time over 70 seconds (Figure 4), before the development of occlusion-induced inflammation and the destruction by Kupffer cells (Krohn et al. 2009). Acute complete thrombosis of the portal vein (which could cause intestinal congestion and mesenteric venous ischemia) and pulmonary embolism were rarely seen in our study. Increasing the transfusion rate was associated with only transient mild elevation in the portal venous pressure. A wider safe range of the transfusion rate than previously expected is warranted. Intraportal hepatocyte transplantation may cause portal vein thrombosis (Baccarani et al. 2005). Concerns regarding this and portal hypertension might restrict the variation of the rate of cell transfusion. However, the complications of portal hypertension, such as variceal bleeding, or ascites, occur mainly in patients with chronic liver disease (McCormack et al. 1985, Ohta et al. 1994), and the risk would not be high in those with acute liver injury. Koenig *et al*. observed prominent accumulation of donor cells within small portal venules, which occurred but to a less degree in our 100-sec group, and vascular

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transmigration and integration of cells were noted 24 hours after transplantation, with a slow infusion rate (Koenig et al. 2005). Breakdown of the sinusoidal endothelial barrier by ischemic-reperfusion injury allowed live donor cells to migrate to and integrate into the parenchyma (Koenig et al. 2005). Mechanisms of extravasation other than the ischemic-reperfusion model seem ready to come well-predicted. Besides, Timm and Vollmar provided evidence for differential portal blood supply to different areas of the liver using *in vivo* fluorescence microscopy (Timm et al. 2013). Inadequate mixing of portal venous blood and the cell suspension would further increase such heterogeneity. The mixing mechanisms that occur when the cell suspension is infused into the portal vein are molecular and turbulent diffusion. Molecular diffusion is based primarily on the concentration gradient that occurs when a high-concentration cell suspension is infused into the portal vein, whereas turbulent diffusion provides more rapid mixing during processing. The scenario is more complicated when the mixing of the 2 fluids involves momentum. In this context, an adequate transfusion rate is one that produces an environment of rapid mixing of the cell suspension with the portal venous blood. This would result overall in less occlusion of small portal venules and a better chance for engraftment. In patients with acute liver failure, the amounts of hepatocytes are of sudden shortage. Timely supply of more already- transmigrated hepatocytes by the optimal transfusion rate could safely bridge the critical period which allow time for hepatocytes (either native or donor cells) to further proliferate, or transit to liver transplantation, and save the hosts.

Our study provides proof of the principle that the rate of intraportal cell transplantation influences the efficiency of engraftment, although we have not yet clarified the details of the underlying mechanism or the optimal rate adjusting for body size, species, or liver disease. However, given the difference in size between rats and humans, the pressure variation zone, which was tolerable in rats, would likely be of wider safety range in humans. In the clinical transplants, the investigators usually tried to infuse up to 5% of liver mass or 2 x 10^8 cells /kg into a recipient. In the present work we only infused approximately 1/4 or even less of this cell number. The comparison with the clinical studies might be inappropriate because of the model, the infusion rates and the cell number. The summary of infusion rates in Table 7 (animal) and 8 (human), however, suggests a general trend toward up-titration of transfusion rates of hepatocyte transplantation into various backgrounds of liver for larger body sizes. Regardless, the observed phenomenon is easily manipulated and potentially clinically

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applicable. For real-time monitoring of portal pressure, we were able to validate the finding of early vascular transmigration of donor cells after transfusion at an appropriate rate. In addition, portal vein thrombosis and hypertension seem not to be major problems. A wider range of the rate of intraportal hepatocyte transplantation in clinical practice is reasonably expected to yield variable engraftment efficiencies. Our study indicates that the optimal rate(s) of intraportal hepatocyte transplantation should be considered seriously in the clinical setting because it affects graft cell engraftment.

In conclusion, differential hepatocyte transfusion rate contribute to accelerated early engraftment and repopulation in rats with acute liver injury. These findings represent proof of concept but are of clinical significance because they are easy to translate into practice.

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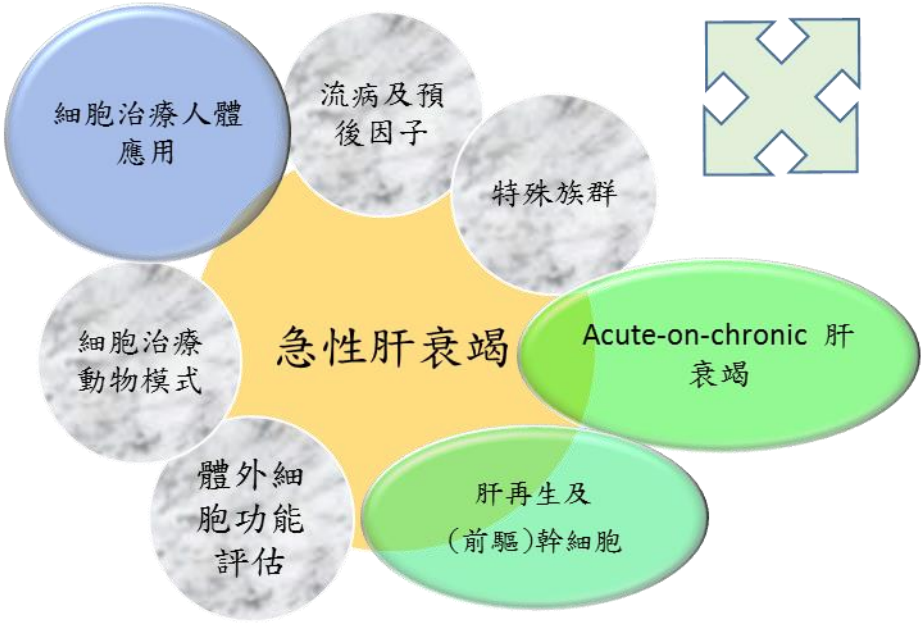
Chapter 6

Future perspectives and Conclusion



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The hallmark of acute liver failure is massive liver cell loss, uncontrolled inflammation and impaired regeneration. If inflammation is not naturally good, why living beings are kept equipped with this arm from generation to generation? As we raise the question, a review from Nature published in 2016 answer part of it (Karin M et al. 2016). Adequate inflammation can result in less overall tissue damage by harmful triggers, such as infection, or toxin while chronic unresolved inflammation will culminates in a host of pathologies, including cancer and fibrosis (Karin M et al. 2016). The ancient signaling of inflammation and repair was actually originated from the same cytokines (such as TNF, IL-6, IL-22, and IL-17) and axes (such as MAPK-AP-1, IKK-NF-κB, Hippo-YAP/Notch, and WNT signaling). Downstream factor (YAP) of another ancient Hippo signaling pathway for growth and development is involved in the convergence of IL-6 and noncanonical WNT signaling pathways (Karin M et al. 2016). The concept of reparative inflammation is formulated and it actually take charge of tissue regeneration, including intestinal cells and liver cells. Theoretically, the host, if not recover itself, will die and pass the problem to the offspring to see potential solution by natural mutation and evolution. Therefore, numerous studies are designed to modulate or balance between inflammation and regeneration. In clinical or medical sense, the intervention in modern age may help solved the unsolved balance in between. Further research on acute liver failure and cell therapy is focused more on the following: modulation of inflammation by stem cells; characterization of the role of macrophage polarization in the process of acute liver failure and repair; establishment of the animal model of acute-on-chronic liver failure, which is more commonly observed clinically in Taiwan. Finally, we hope the further study can be back to clinical application by the modification of clinical processing of liver cells for cell therapy.

(1) Stem cells as the adjuvant cell source in acute liver failure and acute-on-chronic liver failure

Cell therapy for acute liver failure with stem cells

Mesenchymal stem cells (MSCs), a major component of bone marrow cells, were shown to have multiple beneficial effects *in vitro* that were relevant in a therapeutic context of liver injury, including (1) hepatocellular functional support (improved albumin secretion, urea genesis, hepato-specific gene expression, cytochrome P450 activity) (Gómez-Aristizábal et al. 2012), (2) secretion of molecules that inhibit hepatocyte apoptosis (such as stromal-cell-derived factor-1 and vascular endothelial growth factor) (Oritz et al. 2007, Hematti et al. 2008, van Poll et al. 2008, Balber et al. 2011) and stimulate hepatocyte proliferation (such as

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hepatocyte growth factor (HGF), epidermal growth factor, IL-6, and TNF-α), (3) modulation of an acute phase response and suppress inflammatory responses such as IL-1 receptor antagonists and can upregulate anti-inflammatory cytokines such as IL-10 (Oritz et al. 2007), and (4) secrete several extracellular matrix (ECM) molecules, such as collagen, fibronectin, laminin for liver reconstruction (Gómez-Aristizábal et al. 2009, Yagi et al. 2009). The MSC-derived cytokines that potentially protect the liver during injury can be summarized in Meier et al.'s review (Meier et al. 2013).

In vivo, MSC or MSC conditioned media can attenuate inflammation and secrets cytokines and growth factors for cell proliferation and provide significant rescue from fulminant hepatocyte failure (van Poll et al. 2008, Puglisi et al. 2011, Kanazawa et al. 2011, Li et al. 2012). MSC transplantation following solid organ transplantation, both clinically and experimentally, can also reduce rate of acute rejection (Tan et al. 2012, Wan et al. 2008).MSC transplantation alone, however, is not expected to work in acute liver failure because the hostile microenvironment of acute liver failure is not a good niche for MSC to reside, so long-term engraftment rates are low (Yagi et al. 2009). Transplanted hepatocytes are unable to function, or even survive well, without stromal cell support. Bone marrow–derived mesenchymal stromal cells (MSCs), if transplanted too, can support the proliferation and functionality of hepatocytes (Gómez-Aristizábal et al. 2009).

Over 280 clinical trials of MSN are registered, of which 28 focus on the treatment of liver disease (Meier et al. 2013). If no severe side-effects were observed so far, long-term benefits remain uncertain (Meier et al. 2013). Li et al. transplanted human bone-marrow derived MSCs into pigs with acute liver failure induced with D-galactosamine and without use of immunosuppressants (Li et al. 2012). Most (13/15) achieved long-term survival (>6 months) whereas animals without infusion of MSCs were dead (Li et al. 2012). Up to 30% of the hepatocytes, in their study, were bone marrow-derived MSCs (Li et al. 2012). The elucidation of mechanisms involved in the observed effect in these relevant animal experiments were elusive too (Meier et al. 2013). Furthermore, though controversial, MSCs may carry risks of developing fibrotic reaction (Baertschiger et al. 2009, Forbes 2004, Li et al. 2009) or malignant transformation (Casiraghi et al. 2013), and the long-term risks of developing them should be evaluated.

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It is, therefore, reasonably to assume that transplantation of hepatocytes and MSCs would provide enough support for transplanted hepatocytes to better survive and proliferate and, by so doing, enhance repopulation, which is vital for early prompt control of deteriorating acute liver failure. Different flourescent colors of hepatocytes and MSCs will be chosen to transplanted intraportally in D-gal induced acute liver injured SD rat model for tracking hepatocytes and MSCs, and, especially, MSC differentiation. Whether MSC will differentiate into parenchymal (hepatocyte, cholangiocyte) (Lee et al. 2004, Hong et al. 2005, Snykers et al. 2009, Liu et al. 2013), nonparenchymal (sinusoidal endothelial cells, stellate cell) (Oswald et al. 2004, Russo et al. 2006) or just remains as MSC but govern partially differentiated cells (progenitor cells) and their functional role(s) (Wang et al. 2010) will be investigated.

If transplanted hepatocytes can proliferate and double the cell numbers occurs in time in acute liver failure, there will be even better chance to have enough functional cells to cover the rapid loss of native hepatocytes and therefore, rescue the hosts. We will use different fluorescent colors of hepatocytes and MSCs to trace the course of MSC differentiation (parenchymal, non-parenchymal, or remain partially differentiated progenitor cells) post transplantation, interaction of MSC with hepatocytes and microenvironment, and long-term transplanted cell repopulation in the future.

Application of cell therapy in acute-on-chronic liver failure

Acute-on-chronic liver failure (ACLF) is an increasingly-recognized distinct entity from cirrhosis with acute decompensation and has been associated with very poor short-term survival (Moreau et al. 2013, Arroyo et al. 2015, Moreau et al. 2015). ACLF encompasses patients with previously well-compensated liver disease in whom an acute decompensation of liver function occurs because of a precipitating event and is associated with the development of multi-organ failure leading to high in-hospital mortality despite costly intensive care therapy (Graziadei et al. 2011). In chronic injured liver such as chronic viral hepatitis (Marshall et al. 2005) and non-alcoholic fatty liver disease (Yang et al. 2004), the normally efficient renewal from mature hepatocytes becomes impaired when additional liver insult encountered (Boulter et al. 2013). Liver transplantation remains the only curative therapeutic option for the majority of these patients (Finkenstedt et al. 2013). Due to limited

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organ source and high mortality if the patients left untransplanted, alternative therapy is urgently needed.

Progressive hepatic fibrosis as a wound healing response to chronic liver injury leads to accumulation of collagen surrounding liver nodules and further replacement of injured parenchyma by scar tissue, resulting in impaired hepatocyte function (Schuppan et al. 2008, Hernandez et al. 2011). Hepatic stellate cells are the main contributors to the pathogenesis of liver fibrosis (Henderson et al. 2007, Friedman 2008). Therefore, these cells have represented the primary target to reduce or reverse fibrosis by developing specific anti-fibrotic strategies (Schuppan et al 2009, Kisseleva et al 2011). At present, however, therapeutic options in humans are quite limited (Henderson et al. 2007, Friedman 2010, Kisseleva et al. 2011, Arroyo et al 2015, Moreau et al. 2015).

Our previous results demonstrated that adequate rate of cell infusion can have increased engraftment of transplanted hepatocytes in D-gal induced acute liver injured rat model (Ho et al. 2015). However, the engraftment efficiency is about 2.5 % in one week after hepatocyte transplantation without preconditioning. It is difficult for hepatocytes to proliferate effectively in the hostile microenvironment of acute liver failure clinically (Dhawan et al. 2006), let alone in the setting of acute-on-chronic liver failure. Considering the urgent need of large amounts of hepatocytes in acute-on-chronic liver failure, there still much space of improvement exists.

Adipose-derived stem cells (ADSCs) are derived from the stromal vascular fraction (SVF) of adipose tissue which represents an accessible, abundant source of adult stem cells for potential applications in regenerative medicine (Gimble et al. 2007). ADSCs are very similar to bone marrow derived mesenchymal stem cells and share many common character (Gimble et al. 2003, Katz et al. 2005, Prunet-Marcassus et al. 2006), although they can be distinguished from bone-marrow-derived MSCs by their positivity for CD36 and negativity for CD106 (Bourin et al. 2013).

Compared to MSC from bone marrow, ADSCs, in Kim et al's study, even show a significantly greater angiogenic potential (Kim et al. 2007). ADSC transplantation, though in limited studies, was shown to be beneficial in treating acute liver failure (Deng et al. 2014,

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Chen et al. 2015), improving microcirculation and ameliorating liver fibrosis induced by CCl4 in rats (Wang et al. 2012).

The fibrosis induced by temporary ligation of common bile duct (CBD) is being established in our ongoing work (Figure 40). We will use absorbable thread for CBD ligation to produce transient chronic liver fibrosis. Addition of D-galactosamin further makes the model of acute-on-chronic liver failure. We will investigate the effect of transplantation of hepatocytes and ADSCs on this model of acute-on-chronic liver failure. Different fluorescent colors of hepatocytes and ADSCs will be transplanted intraportally in D-galactosamin induced acute liver injured SD rat model for tracking hepatocytes and ADSCs, and ADSC differentiation.

The prevailing school of thought is that MSC do not express CD34, and this sets MSC apart from hematopoietic stem cells, which do express CD34 (Lin et al. 2012). However, the evidence for MSC being CD34(-) is largely based on cultured MSC, not tissue-resident MSC (Lin et al. 2012). In fact, accumulating evidence suggested that CD34 being expressed in tissue-resident MSC, and its negative finding being a consequence of cell culturing (Lin et al. 2012).

Consistently, several papers have shown that CD34 is highly expressed in freshly isolated ADSC (SVF cells), but its expression is quickly lost in cultured ADSC within the first few (<3) passages, probably due to down regulation of CD34 expression rather than death of CD34+ cells (Ning et al. 2006, Gimble et al. 2007, Helder et al. 2007, Lin et al. 2008).

In vitro preliminary results indicated that CD34+ ADSCs were more proliferative and had a greater ability to form colonies, with expression of angiogenic progenitor markers (Flk-1, and FLT1). Further, in our preliminary experiment with rat hepatocyte and human ADSC transplantation for acute-on-chronic liver failure model, ADSC can be observed within rat livers 1 week after transplantation. The rat transplanted with CD34(+) ADSCs was associated with less histological fibrosis, bile duct dilatation and ductular reaction than that with CD34(-) ones, while unsorted ADSCs had intermediate effect. We proposed that CD34+ ADSCs are associated with more beneficial effects than the other SVF cells.

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It is, therefore, reasonably to assume that transplantation of hepatocytes and ADSCs would provide enough support for transplanted hepatocytes to better survive and proliferate and, by so doing, enhance repopulation, which is vital for early prompt control of deteriorating acute-on-chronic liver failure. Further, fibrosis could be ameliorated by ADSC cellular and/or paracrine effects. If transplanted hepatocytes can proliferate and double the cell numbers occurs in time, and ADSCs can ameliorate the fibrotic background by cellular and/or paracrine effects, there will be even better chance to have enough functional cells to cover the rapid loss of native hepatocytes and therefore, rescue the hosts.

(2) Macrophage polarization and its potential application in acute-on-chronic liver failure

Macrophage have remarkable plasticity in response to environmental cues (Biswas 2012, Gao et al. 2014). Depending on the stimulated signals, macrophages undergo classical M1 activation or alternative M2 activation. M1 macrophages, stimulated by TLR ligands and interferon-γ, are characterized by the release of pro-inflammation cytokines and high production of reactive nitrogen and oxygen intermediates. M2 macrophages, stimulated by interleukin 4 (IL-4) or IL-13, are characterized by the release of IL-10, high expression of arginase 1 (Arg-1) and mannose receptors (Mosser et al. 2008). Macrophages of the M2 phenotype are considered to be anti-inflammatory cells and play critical roles in tissue remodeling (Pena et al. 2011, Hematti et al. 2013).

Increasing evidence showed that MSC can induce macrophage M2 activation *in vitro* (Abumaree et al. 2013, Cho et al. 2014). In the liver, resident macrophages (Kupffer cells) fulfill homeostatic functions, orchestrate tissue remodeling in ontogenesis, and regulate metabolic functions (Sica et al. 2014). Polarized Kupffer cells interact with hepatic progenitor cells, integrate metabolic adaptation, mediate responses to infectious agents, orchestrate fibrosis in a yin-yang interaction with hepatic stellate cells (Sica et al. 2014, Tacke et al. 2014).

It is important in the future to investigate the polarization process of hepatic macrophages (mainly Kupffer cells) (from M1 to M2 which was known to express markers of CD206 and CD163) before, during and after transplantation of hepatocytes and MSCs in ameliorating the

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fibrosis of acute (D-gal induced) on chronic (CBD ligation by absorbable thread) liver injured rat model.

(3) Modification of clinical hepatocyte processing in the real-world

The purpose is to investigate the feasibility of modification with simplification in hepatocyte processing for hepatocyte transplantation, and to compare the cellular function and quality in vitro. We plan to isolate the hepatocytes from deceased donor liver with signed informed consents in operation and prepare the cell suspension for ex vivo functional assays (such as cell viability, activity of the urea cycle enzyme, mitochondrial enzymes, and albumin synthesis). As shown in Figure 41, it is feasible to perform hepatocyte isolation in the operation room, although further process modification is needed to achieve better cell quality and quantity.

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Conclusion

ALF in Taiwan is mainly due to viral infection. Patients with malignancy and alcohol exposure have worst prognosis. The use of proton pump inhibitor is associated with improved survival. Half of the ALF survivors have liver cirrhosis. Prominent ductular reaction with at-least partially functional hepatocyte differentiation did not guarantee successful regeneration in acute liver failure and there is demand left for hepatocyte transplantation. With further refinement of ICG could be used to develop a rapid assay for assessment of the function of isolated human hepatocytes. Differential hepatocyte transfusion rate contribute to accelerated early engraftment and repopulation in rats with acute liver injury. These proof-of-concept findings are of clinical significance because they are easy to translate into practice. Further studies are needed for improvement of hepatocyte transplantation for ALF in Taiwan, albeit some problems solved. We plan to investigate on the modulation of ALF microenvironment and the application of cell therapy with hepatocytes and/or MSC in the near future in hope to further improve the prognosis of ALF.

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Figures

Figure 1. Gene expression (mRNA) changes in clinical living liver donors before and after liver resection. The different rows represent gene expression patterns in different donors. Red represents upregulated genes and green represents downregulated genes. Similar patterns of gene expression are noted in cluster analysis. Specific up- and downregulated genes are labeled as shown.

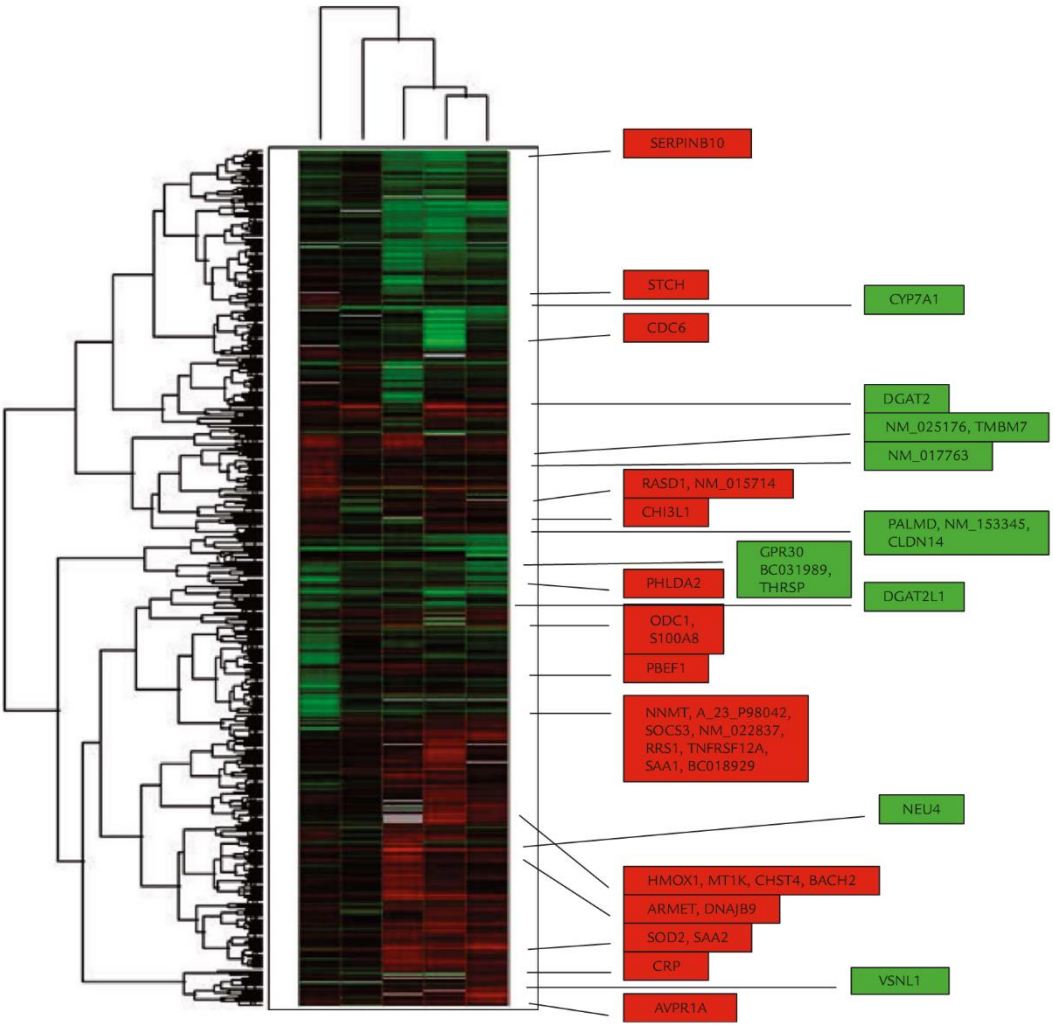

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Figure 2. Process of clinical hepatocyte isolation and transplantation.

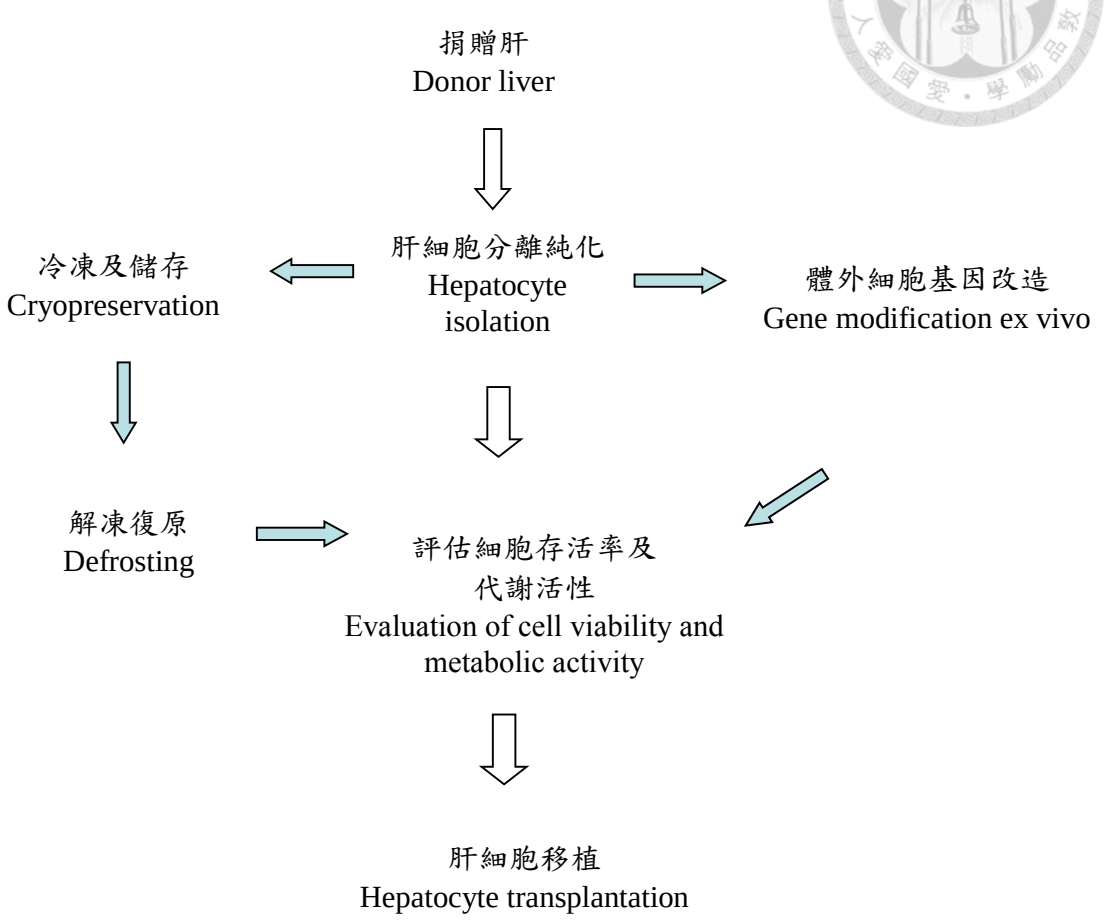

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Figure 3. Clinical hepatocyte isolation in reality. A. Microbacterial monitoring in clinical-grade cell isolation room. B. Transfer perfusate, preservation solution, pippete tips into the clinical-grade cell isolation room though the transit box. C. Liver perfusion in the hood. D. Cut the liver capsule and mince the digested liver parenchyma, and release the cells into ice-cold EMEM. E. Filter cells with gauze. F. Centrifuge cells with 50g. G. Freezing cells for preservation. H. Clinical hepatocyte transplantation during infusion.

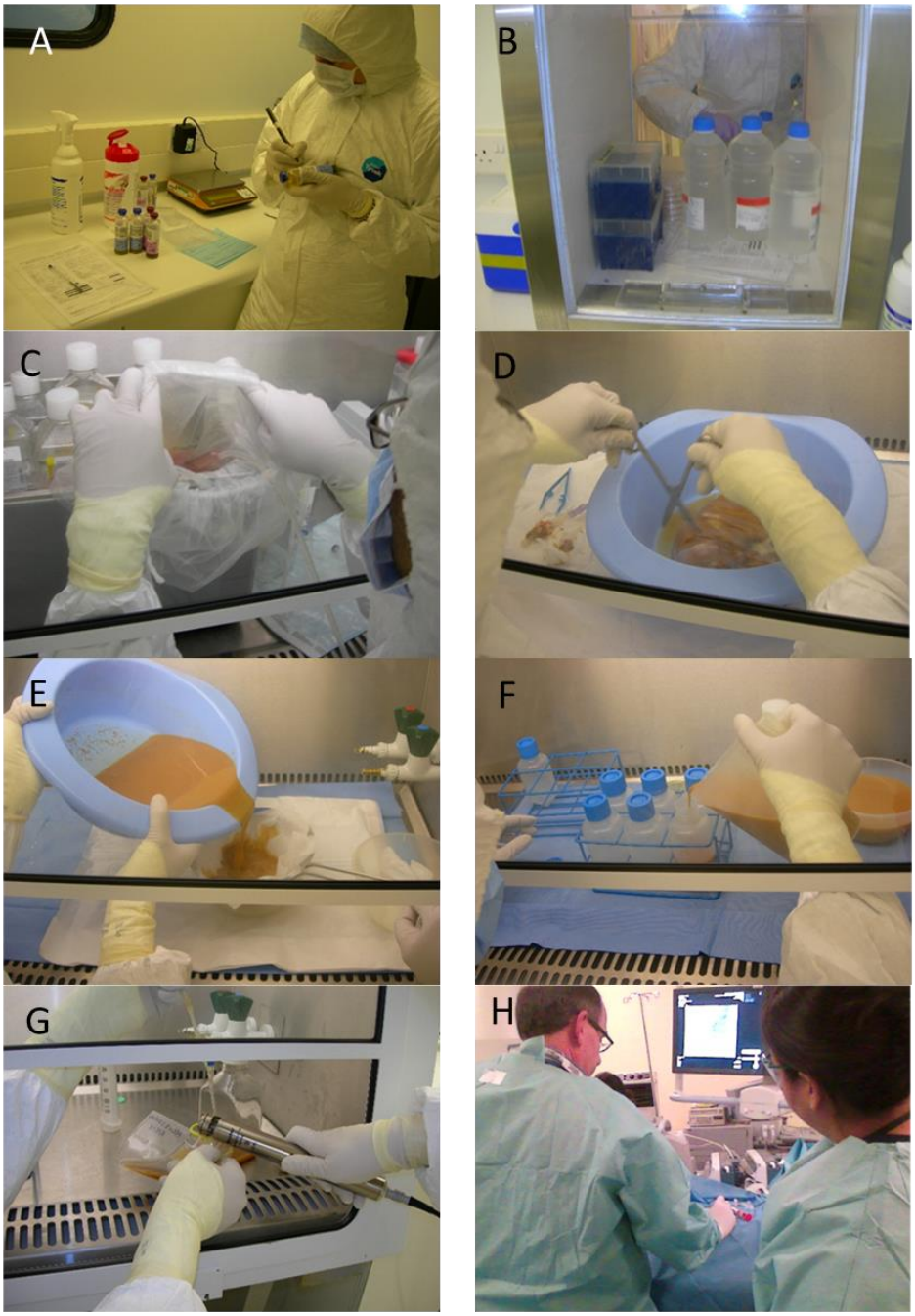

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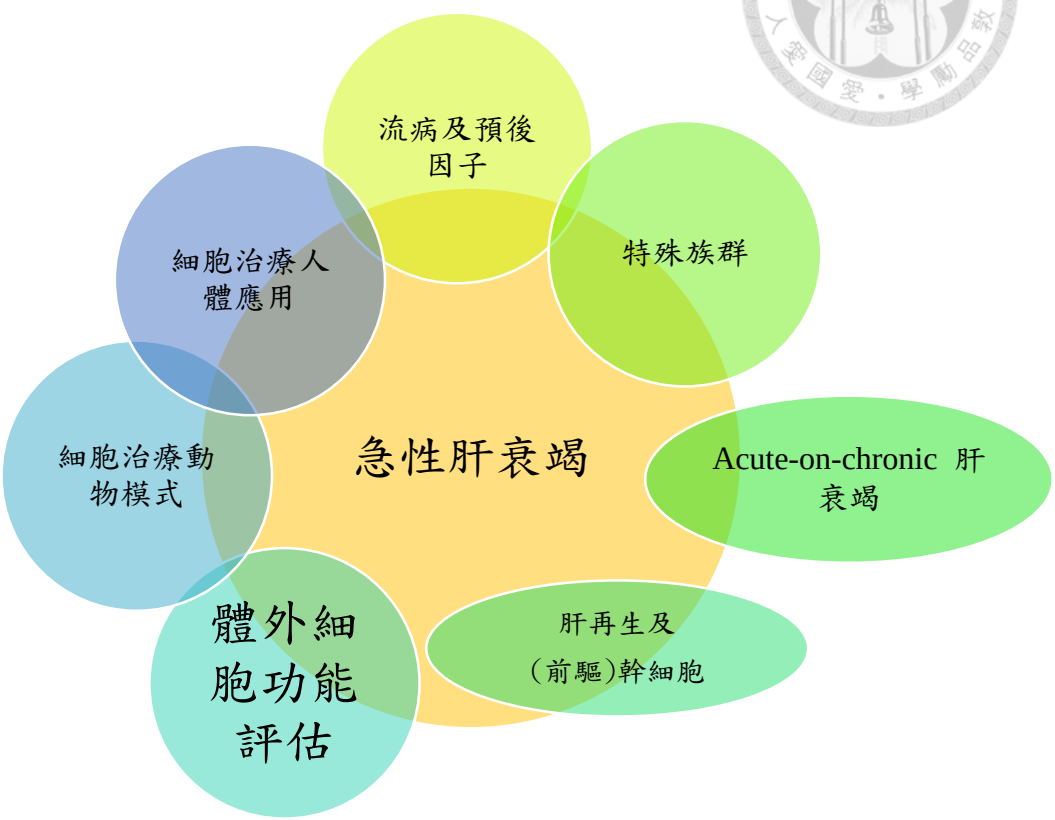
Figure 4. Research infrastructure and association network.



中英對照說明 Chinese-English terms
急性肝衰竭 Acute liver failure
流病及預後因子 Epidemiology and prognostic factor
特殊族群 Special population
Acute-on-chronic 肝衰竭 Acute-on-chronic liver failure
肝再生及(前驅)幹細胞 Liver regeneration and (progenitor) stem cells
體外細胞功能評估 Cellular functional evaluation *ex vivo*
細胞治療動物模式 Animal model of cell therapy
細胞治療人體應用 Clinical application of cell therapy

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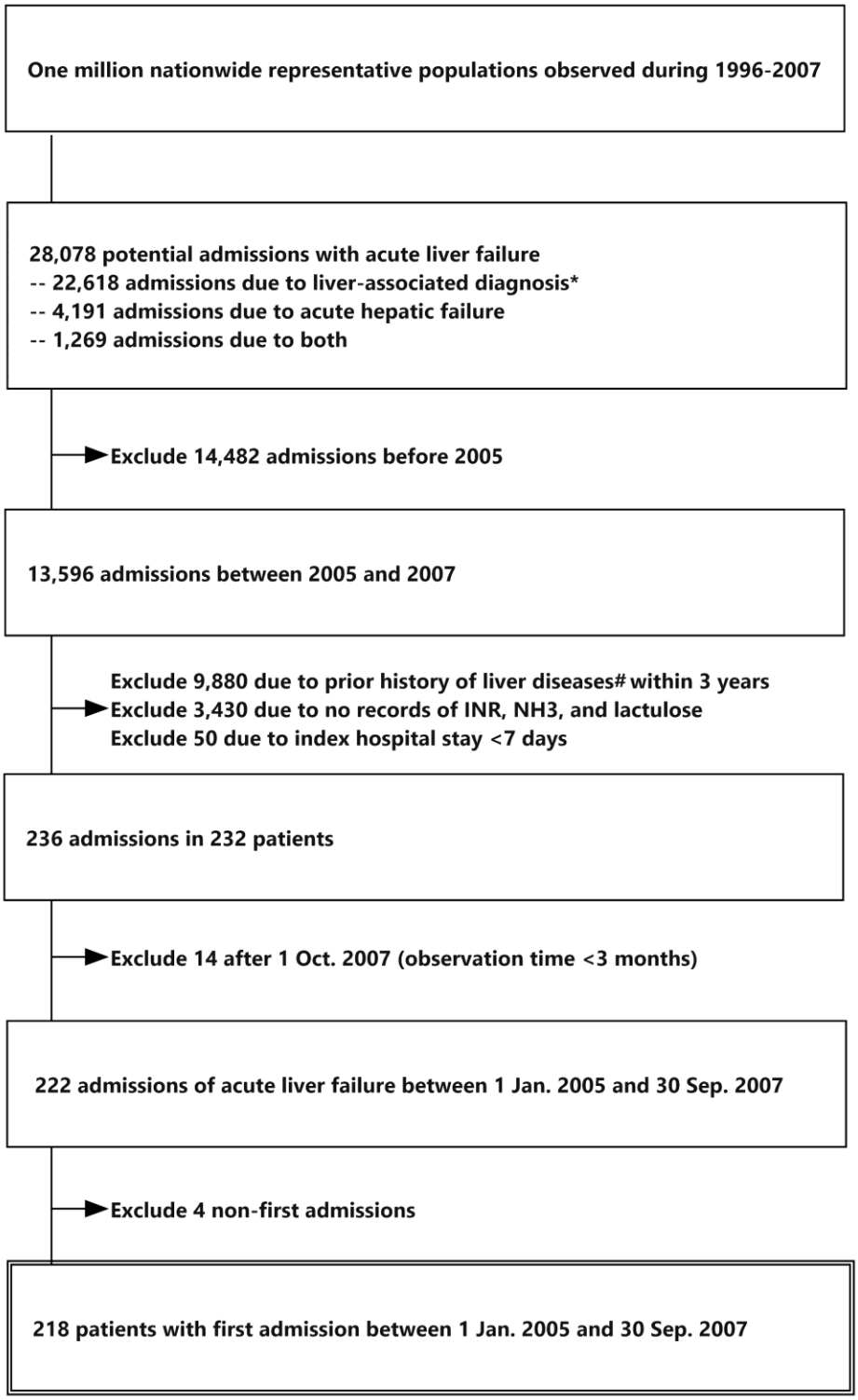
Figure 5. Schematic representation of the patient selection process in choosing target patients of acute liver failure in Taiwan.



Liver-associated diagnosis* included ICD-9-CM (International Classification of Diseases, ninth revision, clinical modification) 070.0-070.9, 571.1, 571.42, 573.3, 782.4, and V02.61. Prior history of liver diseases# included (1) liver-associated diagnoses; (2) chronic hepatitis (ICD-9: 571.4); (3) hepatic stone (ICD-9: 574.5); (4) HCC (ICD-9: 155.0); (5) intrahepatic cholangiocarcinoma (ICD-9: 155.1); (6) malignant neoplasm of gall bladder (ICD-9: 156.0); (7) malignant neoplasm of extrahepatic bile ducts (ICD-9: 156.1); (8) malignant neoplasm of ampulla of vater (ICD-9: 156.2); (9) malignant neoplasm of pancreas (ICD-9: 157.9); and (10) liver metastasis (ICD-9: 197.7).

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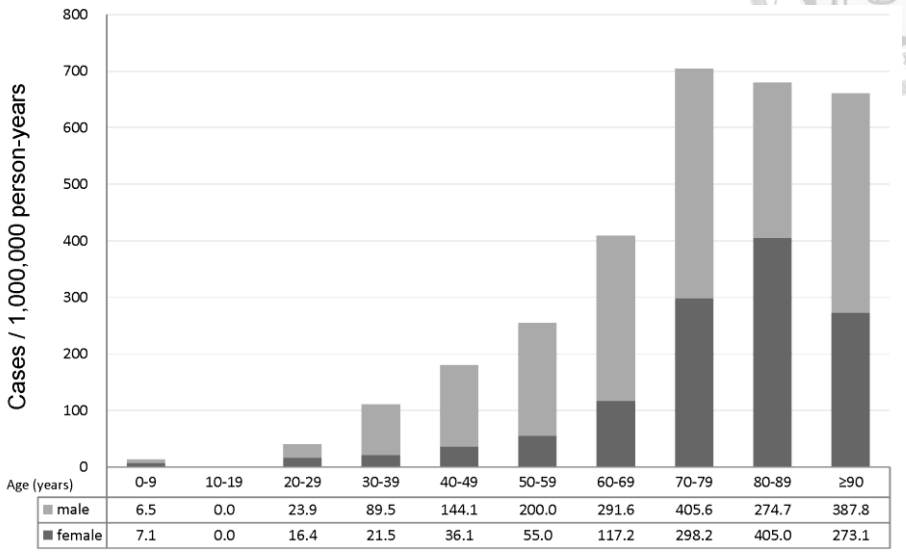
Figure 6. The calculated incidence of acute liver failure in Taiwan from 2005~2007. Note that the incidence increased with age in both genders.



Age (years)	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	≥90
male	6.5	0.0	23.9	89.5	144.1	200.0	291.6	405.6	274.7	387.8
female	7.1	0.0	16.4	21.5	36.1	55.0	117.2	298.2	405.0	273.1

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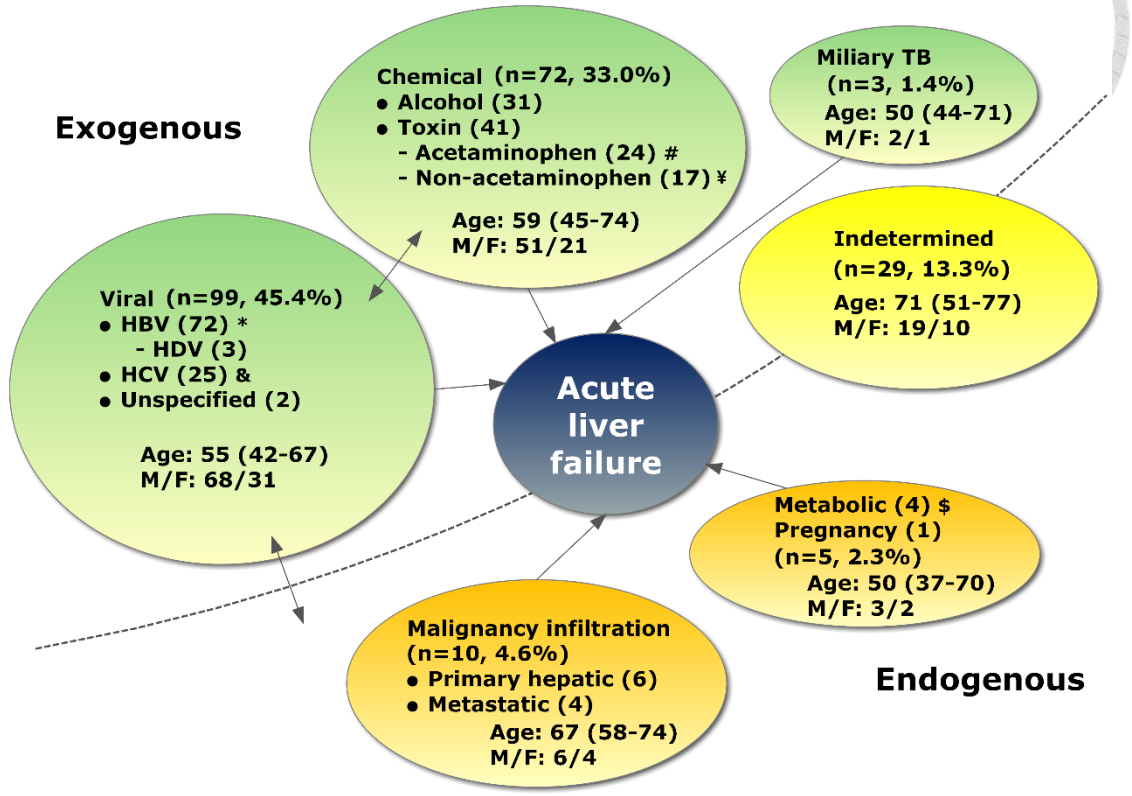
Figure 7. The etiology of acute liver failure in Taiwan from 2005~2007.



* 14 pathologically exposed to alcohol, 5 co-infected with HCV, 2 exposed to TB, and one also exposed to recent anti-TB medications and 1 Wilson disease of age 25. & 4 pathologically exposed to alcohol. ¥ 18 also exposed to non-acetaminophen hepatotoxic agents and 2 exposed to herbal agents. $ 4 also exposed to herbal agents. £ 1 Wilson disease of age 44.

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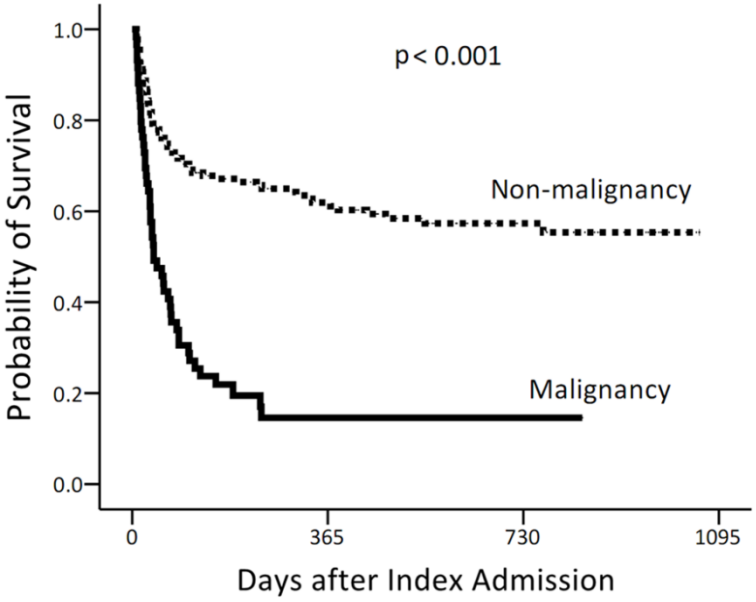
Figure 8. Kaplan-Meier curve of survivals for patients of acute liver failure stratified according to the status of malignancy.



Non-malignant	159	75	30	0
Malignant	59	6	2	0

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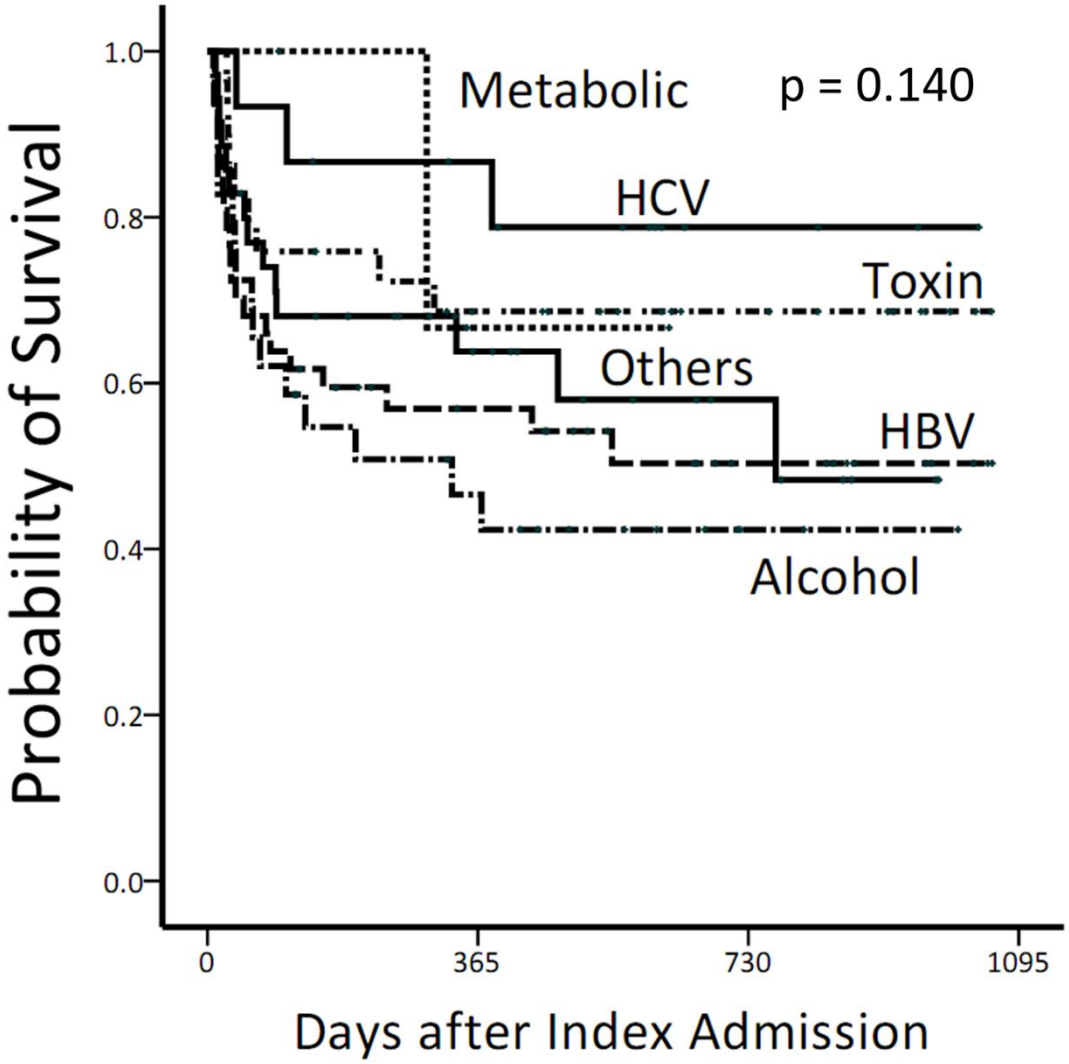
Figure 9. Kaplan-Meier curve of survivals for patients of acute liver failure stratified according to the etiological exposure except malignancy.



HBV	47	21	9	0
HCV	15	11	3	0
Alcohol	29	11	2	0
Toxin	29	17	10	0
Metabolic	4	1	0	
Others	35	14	6	0

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Figure 10. Adjusted survival curve for patients of acute liver failure in Taiwan stratified according to the etiological exposure except malignancy. Adjusted survival curves were plotted based on regression estimates in the Cox model and average covariate values (average covariate method).

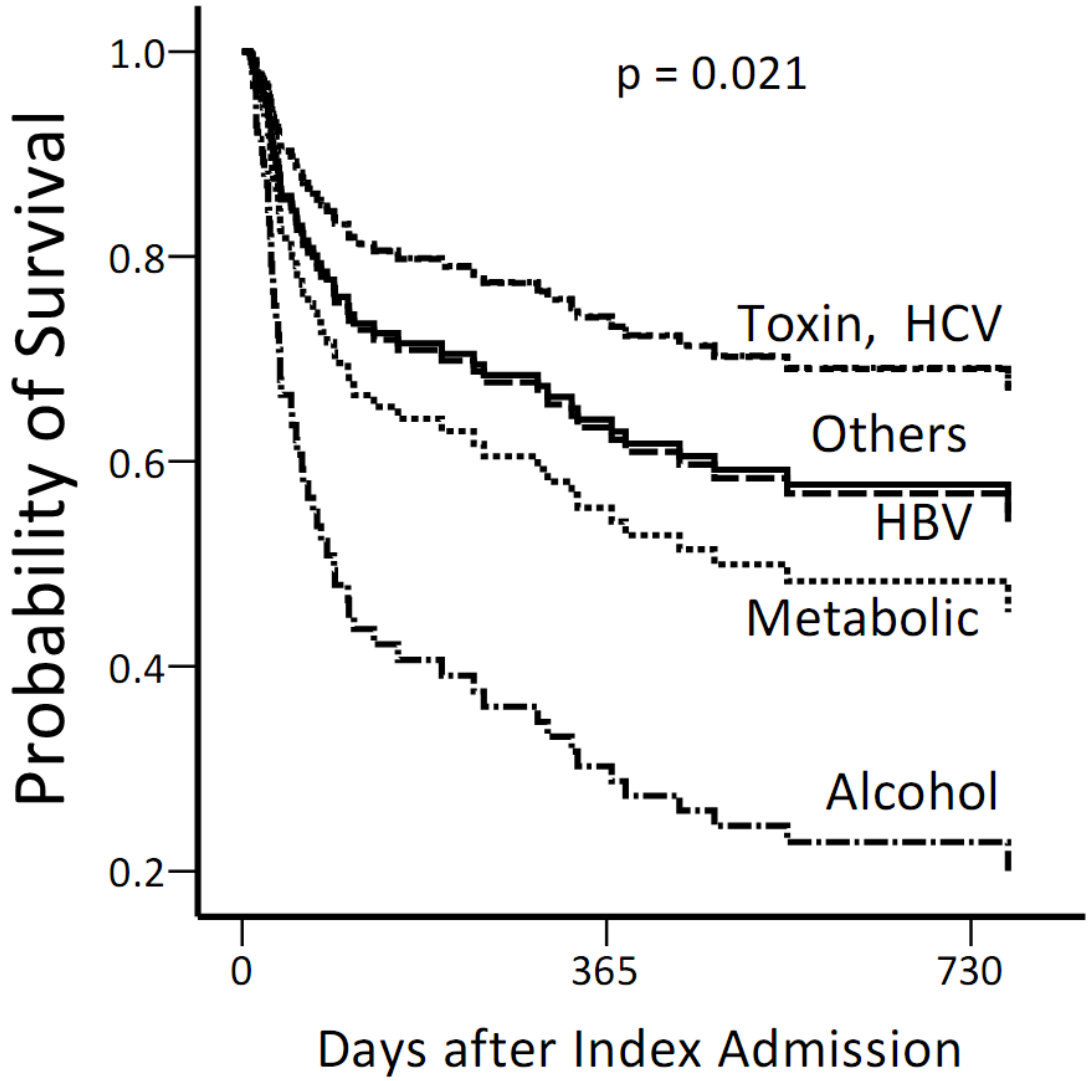

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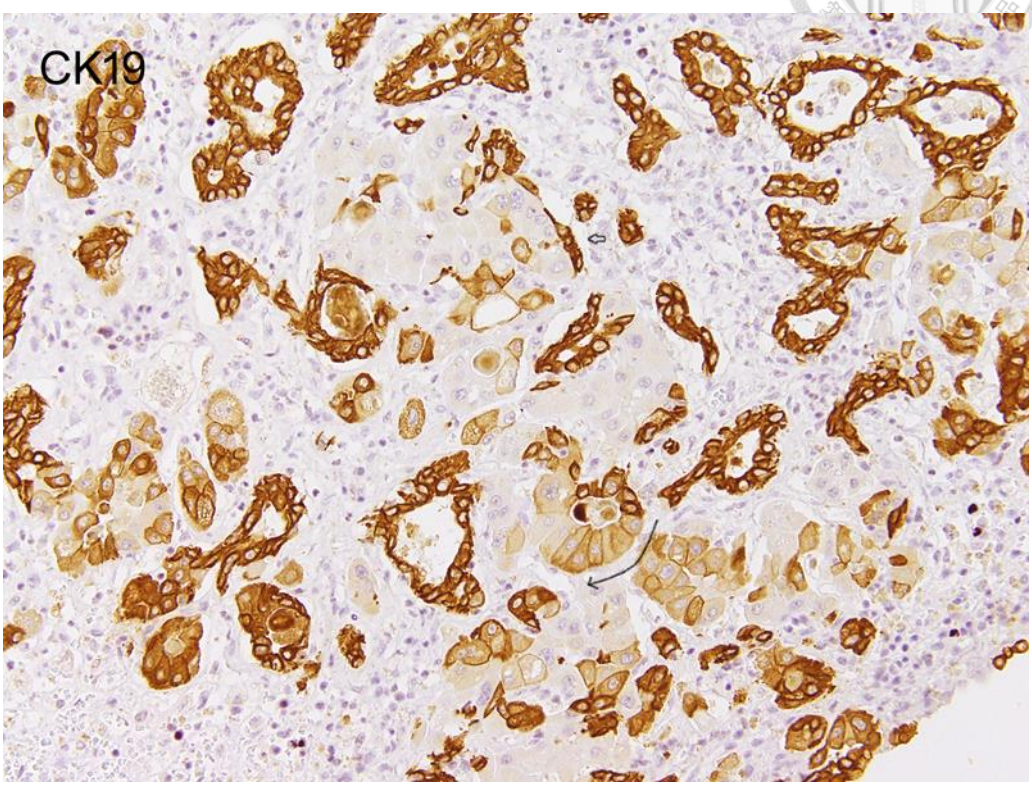
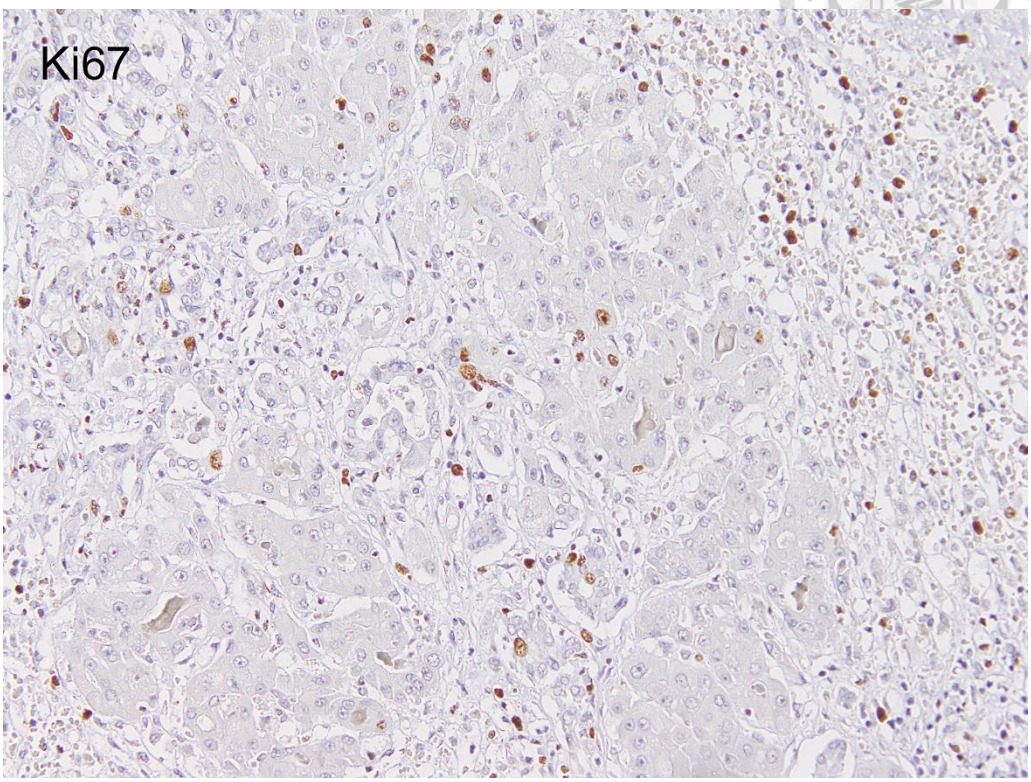
Figure 11. Ductular reaction in acute liver failure characterized by the immunohistochemical examination of cytokeratin19 (200X).



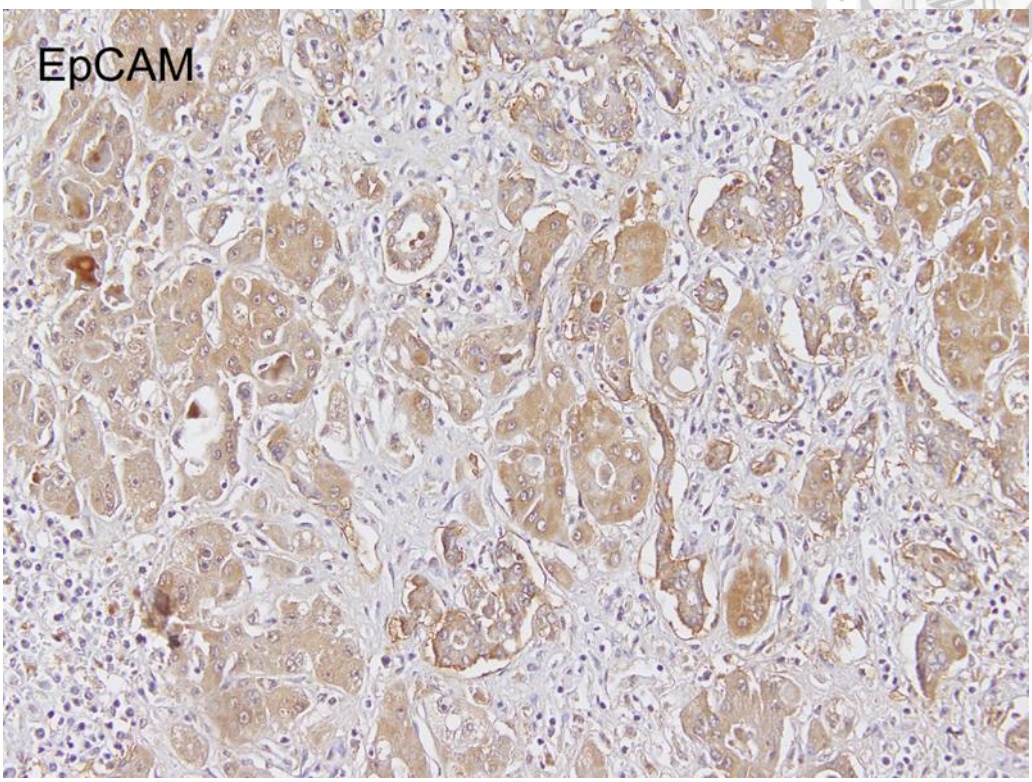
doi:10.6342/NTU201600315

Figure 12. Immunohistochemical examination of Ki-67 in a case of acute liver failure and prominent ductular reaction (200X).



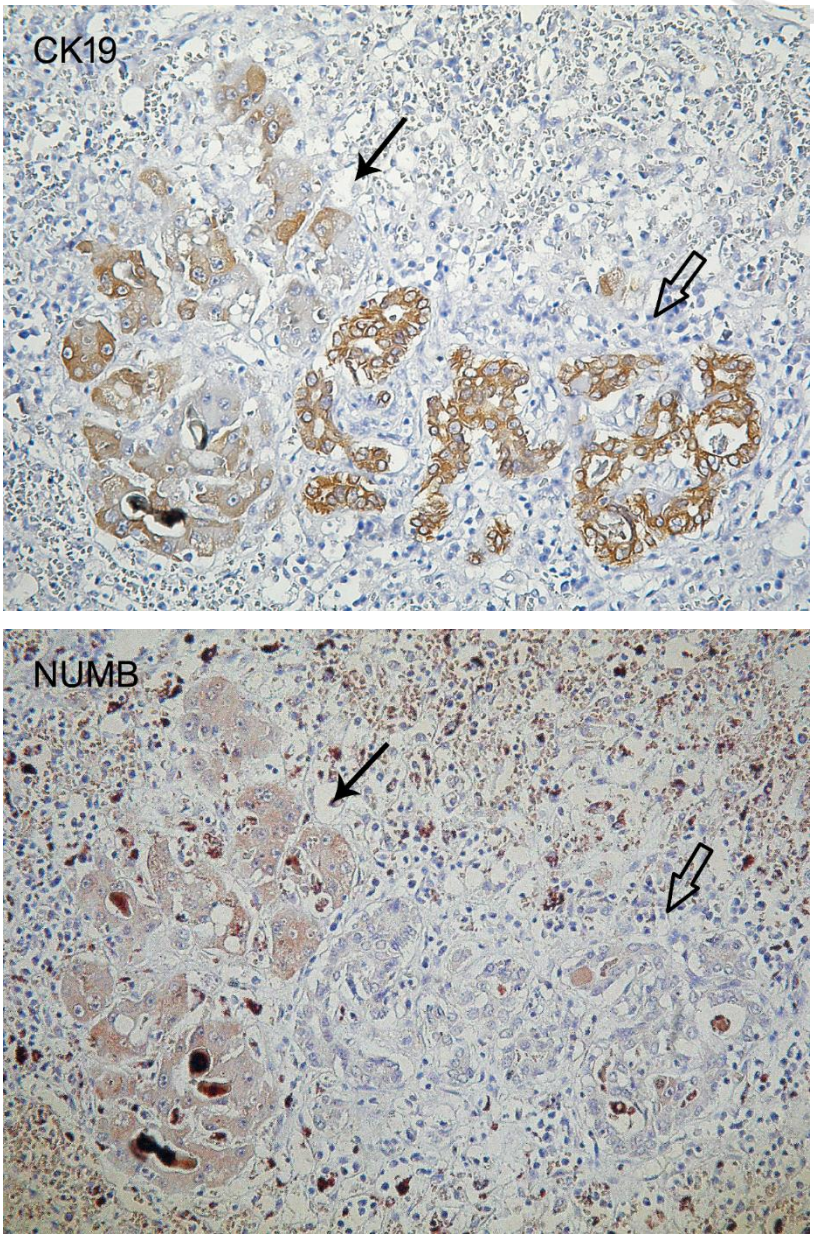
doi:10.6342/NTU201600315

Figure 13. Immunohistochemical examination of epithelial cell adhesion molecule in a case of acute liver failure and prominent ductular reaction. 200X



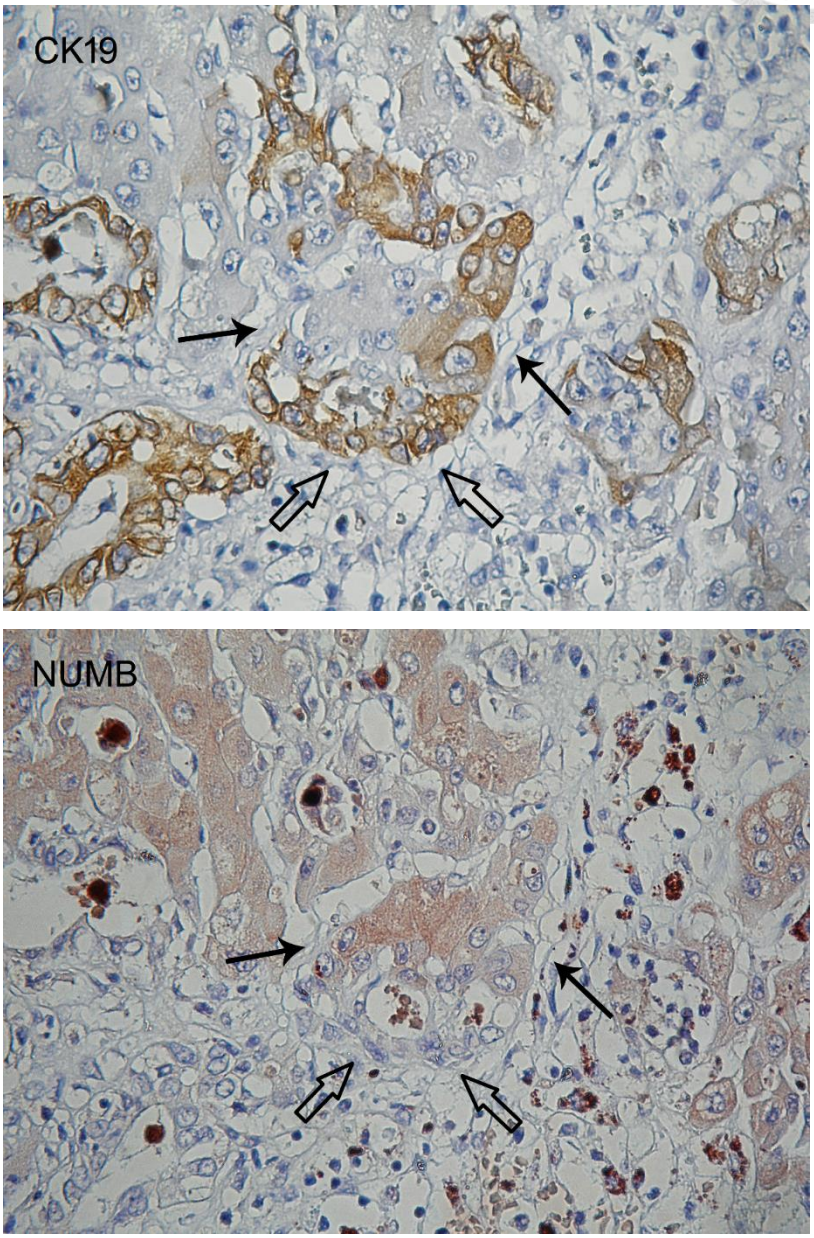
doi:10.6342/NTU201600315

Figure 14. Transitional expression of NUMB in acute liver failure. Compared to the immunohistochemical staining of cytokeratin 19 (A), NUMB expression was shown in cell clusters differentiating toward hepatocytes (arrows, B) but not those toward cholangiocytes (open arrows, B). 200X



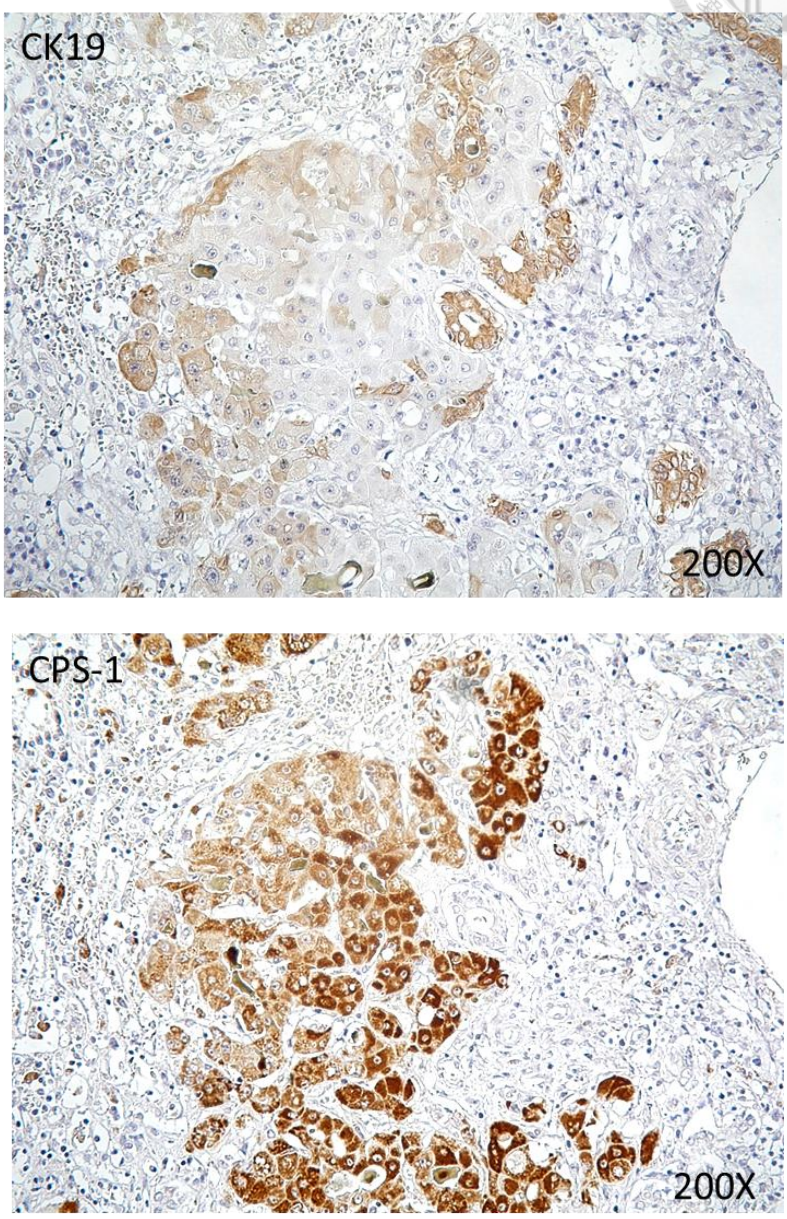
doi:10.6342/NTU201600315

Figure 14. Transitional expression of NUMB in acute liver failure. Compared to the immunohistochemical staining of cytokeratin 19 (A), NUMB expression was shown in cell clusters differentiating toward hepatocytes (arrows, B) but not those toward cholangiocytes (open arrows, B). 400X



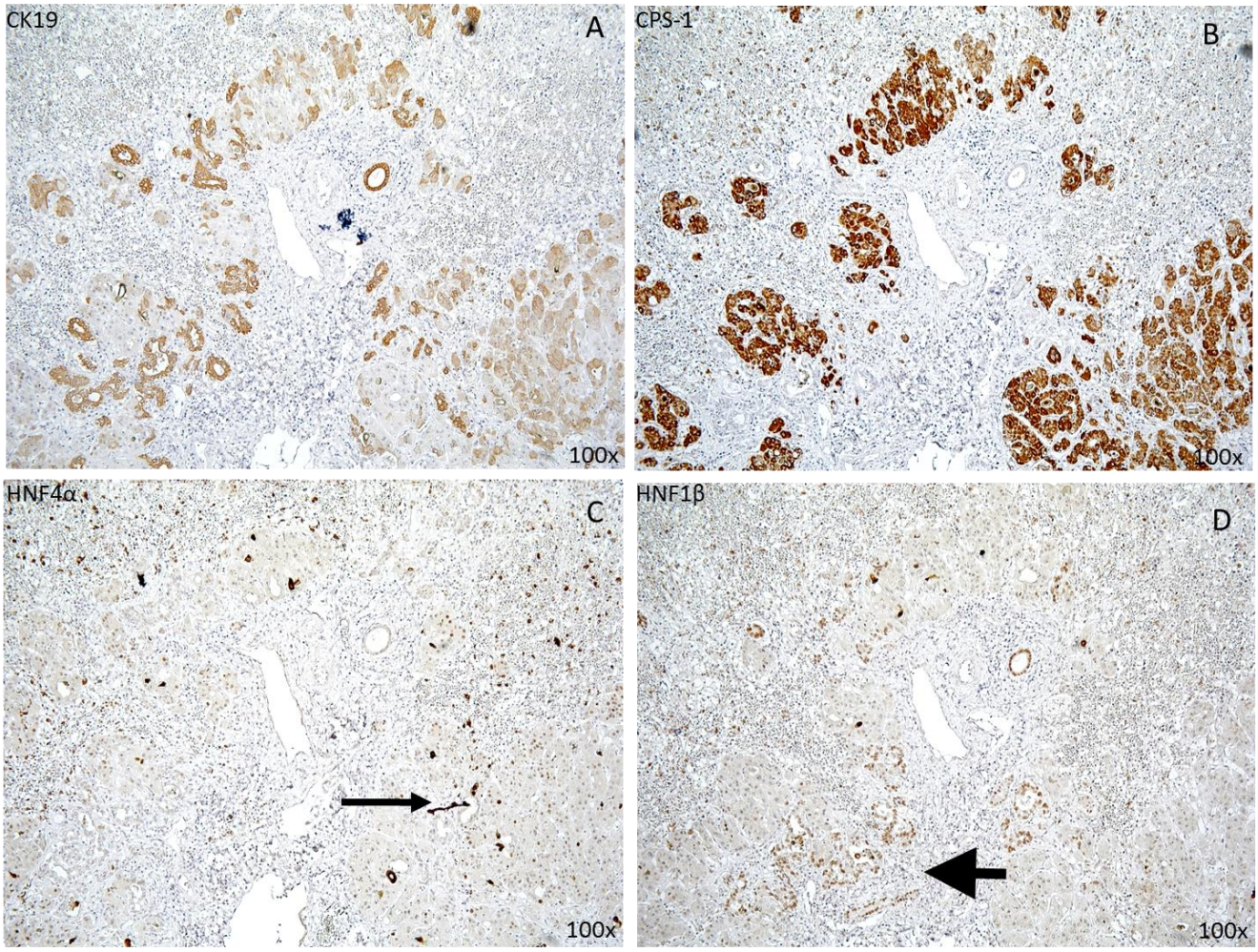
doi:10.6342/NTU201600315

Figure 16. Transitional expression of CPS-1 in acute liver failure. Compared to the immunohistochemical staining of cytokeratin 19 (A), CPS-1 expression was shown in cell clusters differentiating toward hepatocytes (B). 200X



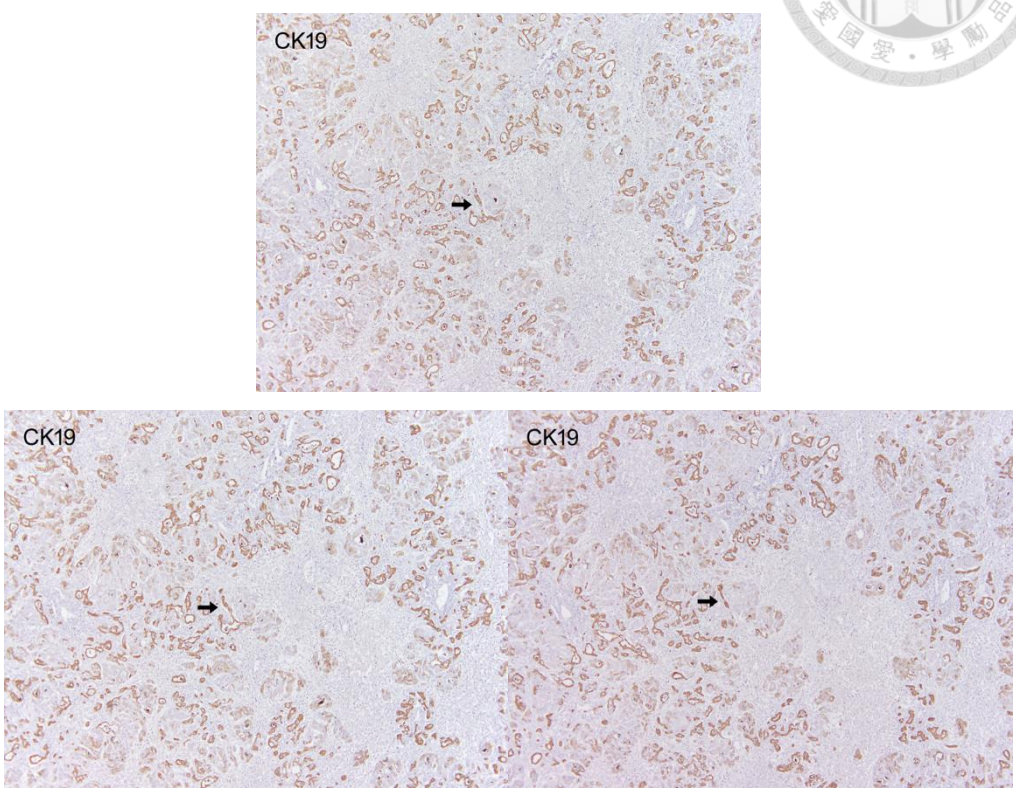
doi:10.6342/NTU201600315

Figure 17. Immunohistochemical examination of CK19, CPS-1, HNF4α, and HNF1β in acute liver failure. Progenitor cells differentiating toward hepatocytes exhibited HNF4α (C, long and thin arrow) and CPS-1 and cholangiocytes, HNF1β without CPS-1 (D, short and thick arrow). Serial sections 100X.



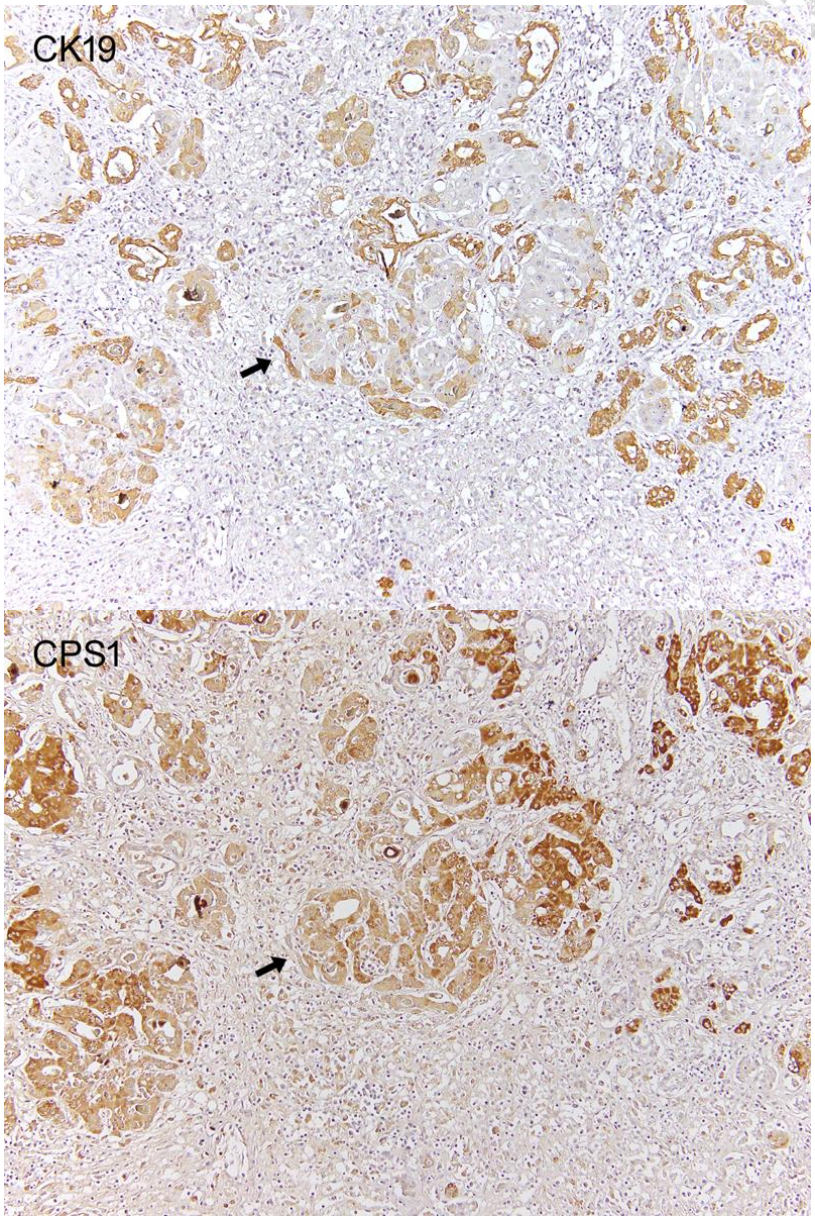
doi:10.6342/NTU201600315

Figure 18. Immunohistochemical examination of CK19 in acute liver failure. Canal of Hering can be identified by CK 19+ sequentially (A-C). 40X



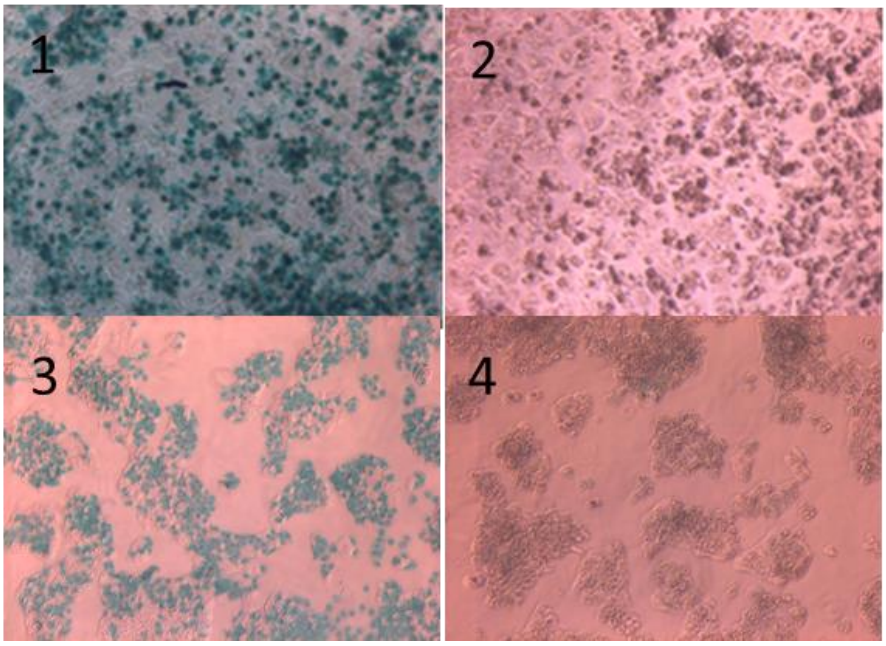
doi:10.6342/NTU201600315

Figure 19. Immunohistochemical examination of CK19 (A) and CPS1 (B) in acute liver failure. Newly formed hepatocytes were CPS1+ (B). Canal of Hering can be identified by as they were CK19+/CPS1- (arrows, A, B). 100X.



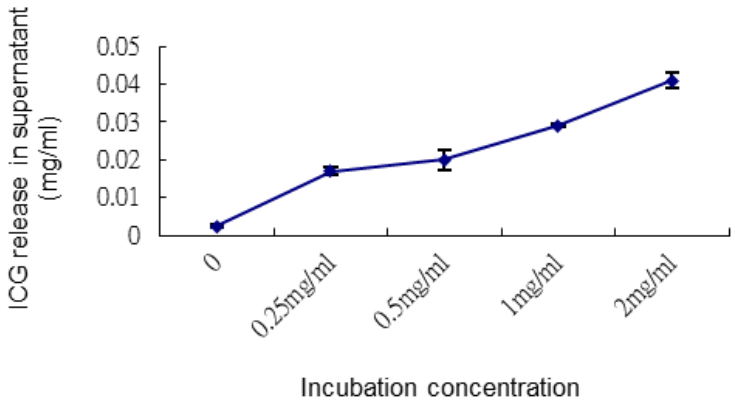
doi:10.6342/NTU201600315

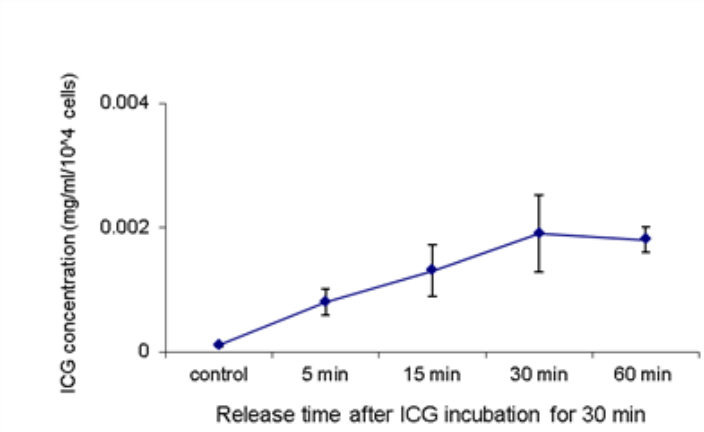
Figure 20. Characteristics of ICG uptake and release by human hepatocytes and HepG2 cells in vitro. Uptake and release of ICG: after ICG incubation (1, 3) and 3 hours release of ICG (2, 4). 1, 2: human hepatocytes; 3, 4: HepG2 cells.



doi:10.6342/NTU201600315

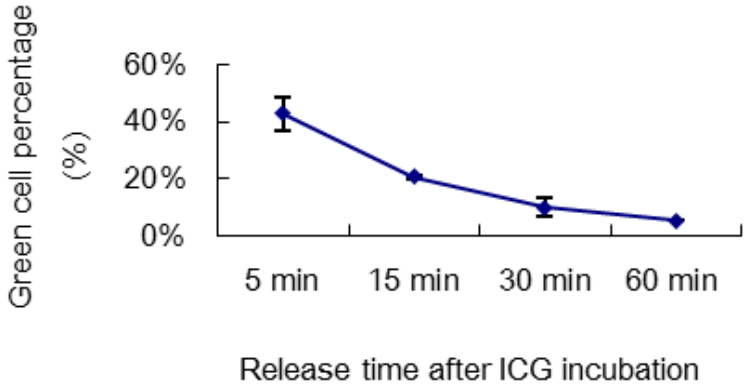
Figure 21. ICG release into the supernatant by human hepatocytes increased with ICG concentration used for incubation.





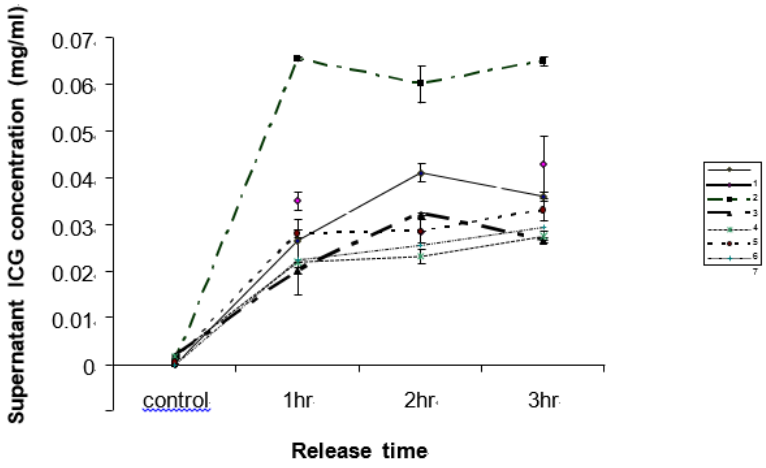
doi:10.6342/NTU201600315

Figure 22. HepG2 cells in plates release ICG with time as shown as decrease in the percentage of green cells.



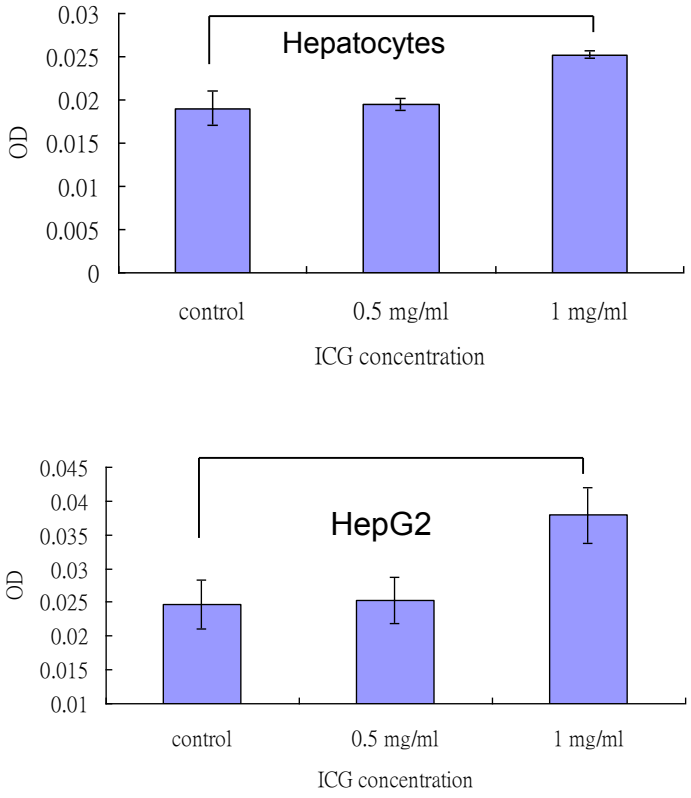
doi:10.6342/NTU201600315

Figure 23. Release patterns of ICG by ex vivo human hepatocytes from different donors.



doi:10.6342/NTU201600315

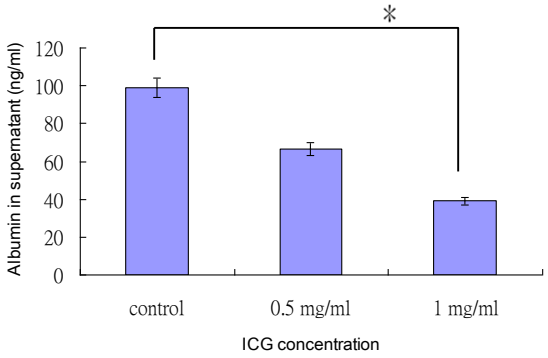
Figure 24. Functional disturbances of human hepatocytes and HepG2 cells following ICG treatment. MTT assay showed increased mitochondrial function in hepatocytes (top) and HepG2 cells (bottom) incubated with 1.0 mg/ml ICG. (* $p<0.05$)



MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ICG, indocyanine green

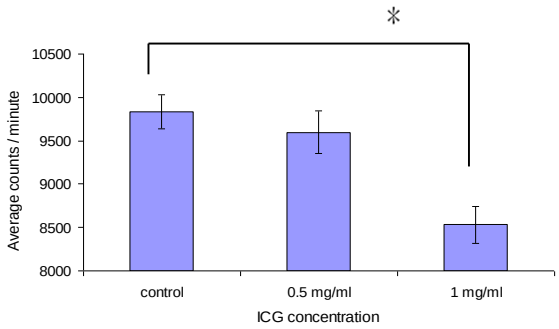
doi:10.6342/NTU201600315

Figure 25. Functional disturbances of HepG2 cells following ICG treatment. Albumin synthesized by HepG2 cells decreased in a dose-responsive relationship as increased ICG incubation concentration. (n=2) (* $p<0.05$)



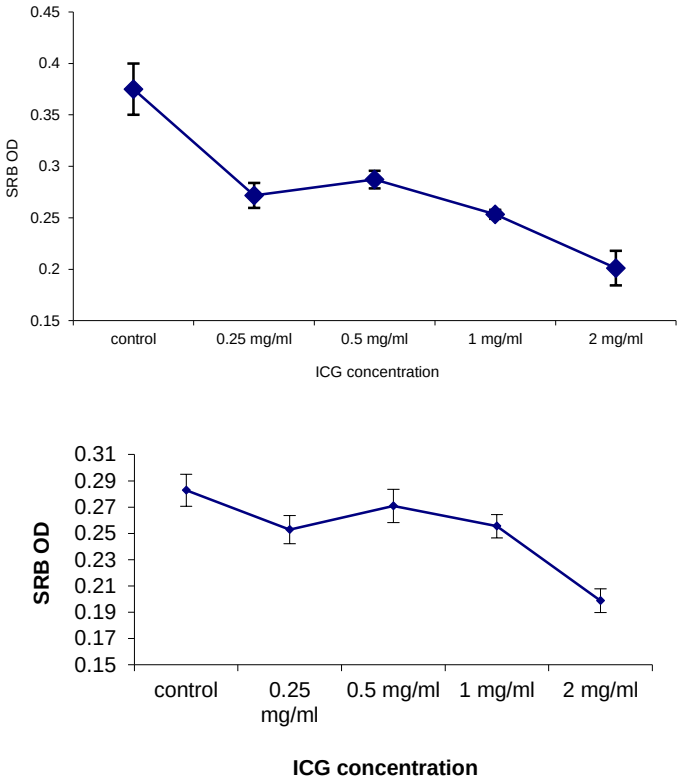
doi:10.6342/NTU201600315

Figure 26. Functional disturbances of HepG2 cells following ICG treatment. Incubation with 1.0mg/ml ICG decreased HepG2 cell proliferative activity. (n=2) (* $p<0.05$)



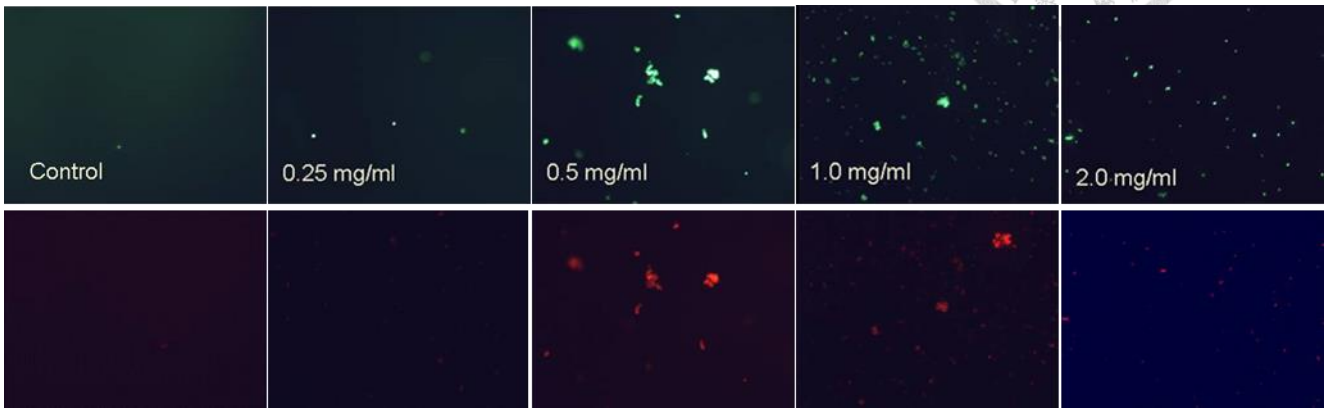
doi:10.6342/NTU201600315

Figure 27. Human hepatocytes and HepG2 cells detach when releasing indocyanine green. Detachment of human hepatocytes (top) and HepG2 cells (bottom) on incubation with increasing ICG concentration.



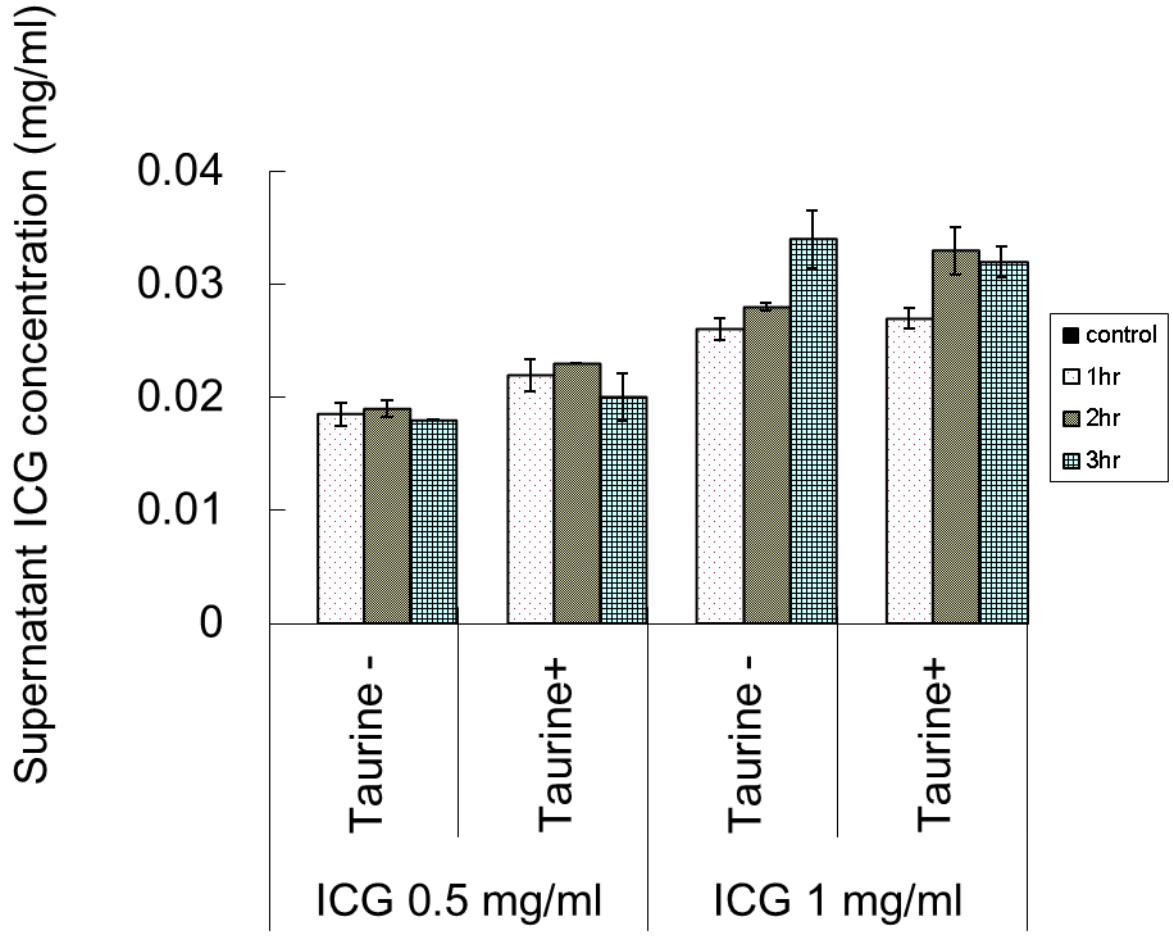
doi:10.6342/NTU201600315

Figure 28. Human hepatocytes and HepG2 cells detach when releasing indocyanine green. Cell viability staining with fluorescein diacetate (FDA)/ethidium bromide (EtBr) showed that more viable cells detached when incubated with higher concentrations of indocyanine green.



doi:10.6342/NTU201600315

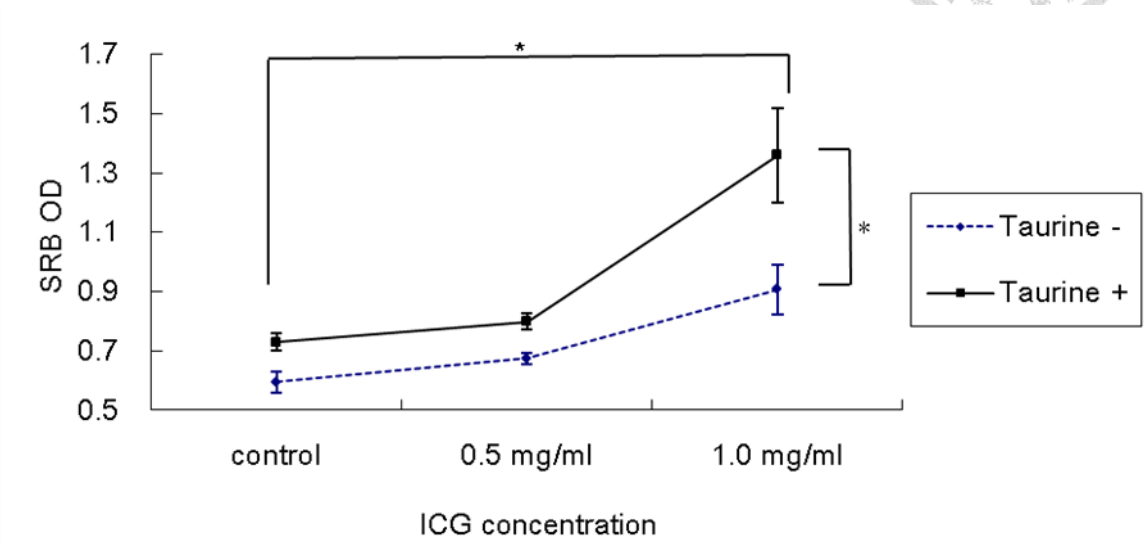
Figure 29. Effect of pretreatment with taurine on human hepatocytes. (A) Human hepatocytes released more ICG when pretreated with taurine. When incubated in 1.0 mg/ml ICG, the pattern of ICG release was maintained in taurine pretreated hepatocytes. (* $p<0.05$)



ICG, indocyanine green; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

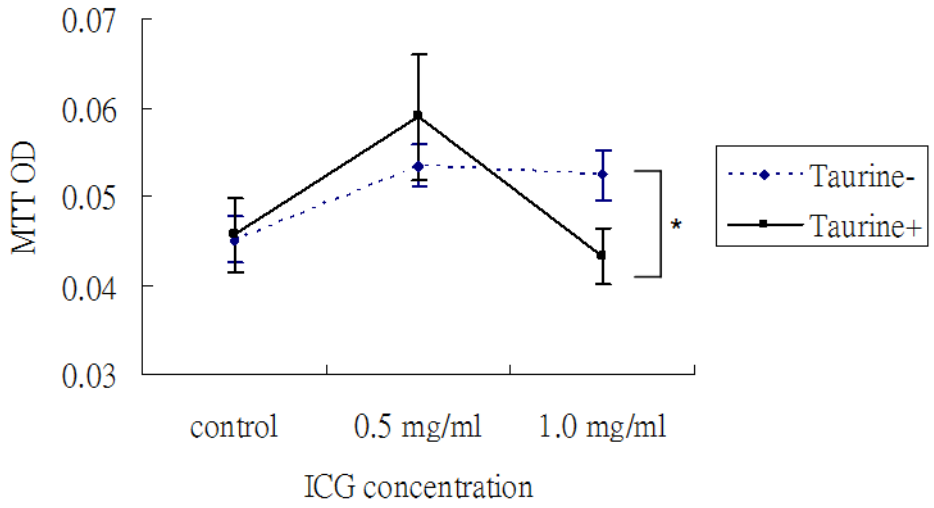
doi:10.6342/NTU201600315

Figure 30. Pretreatment with taurine increases hepatocyte attachment. Following incubation with ICG for 30 minutes, plates are reincubated overnight to study cell attachment study. Cells pretreated with taurine are attached better with 1.0 mg/ml ICG concentration.



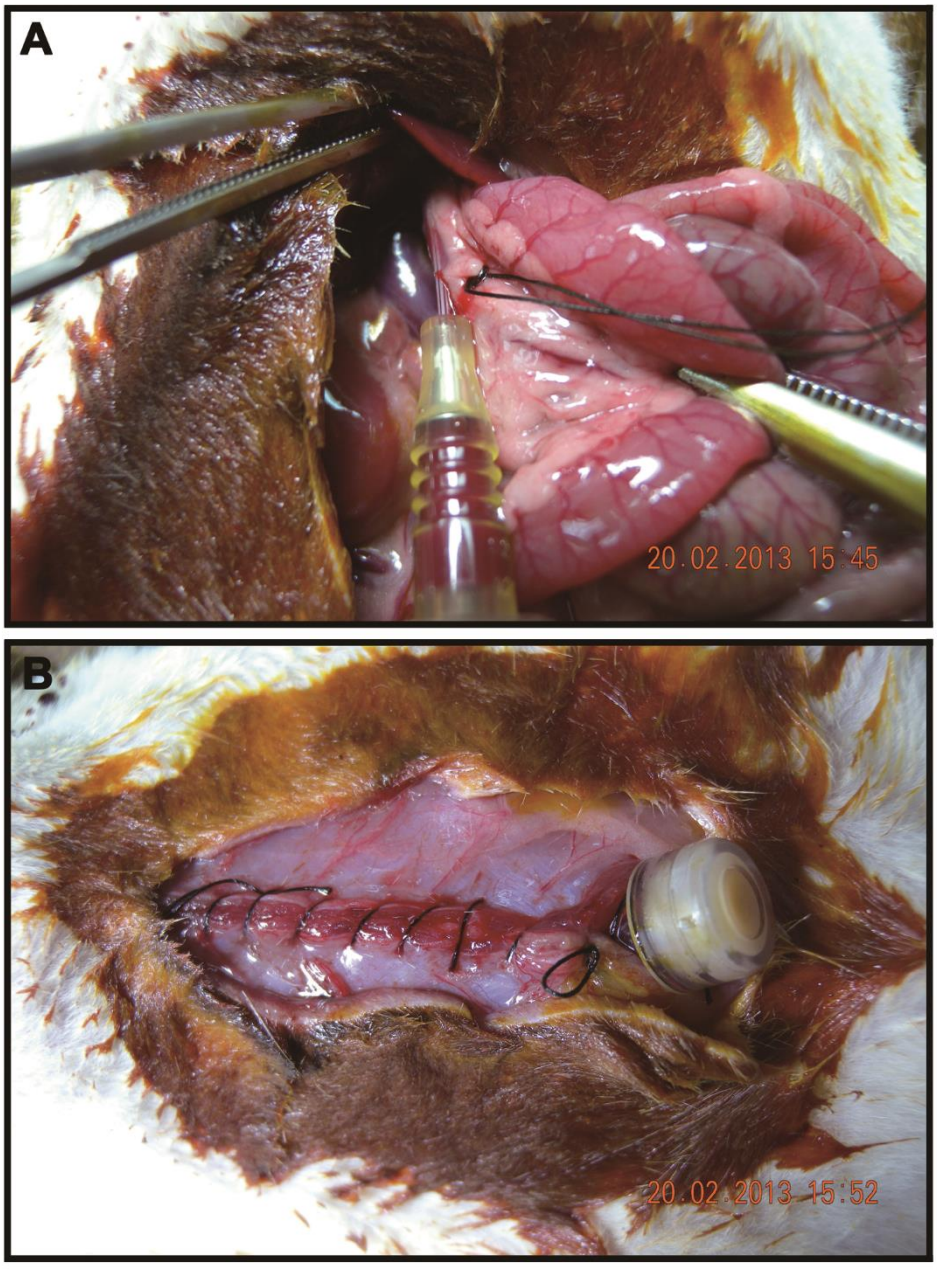
doi:10.6342/NTU201600315

Figure 31. Taurine-pretreated hepatocytes had lower MTT activity on incubation with 1.0 mg/ml than with 0.5 mg/ml ICG.



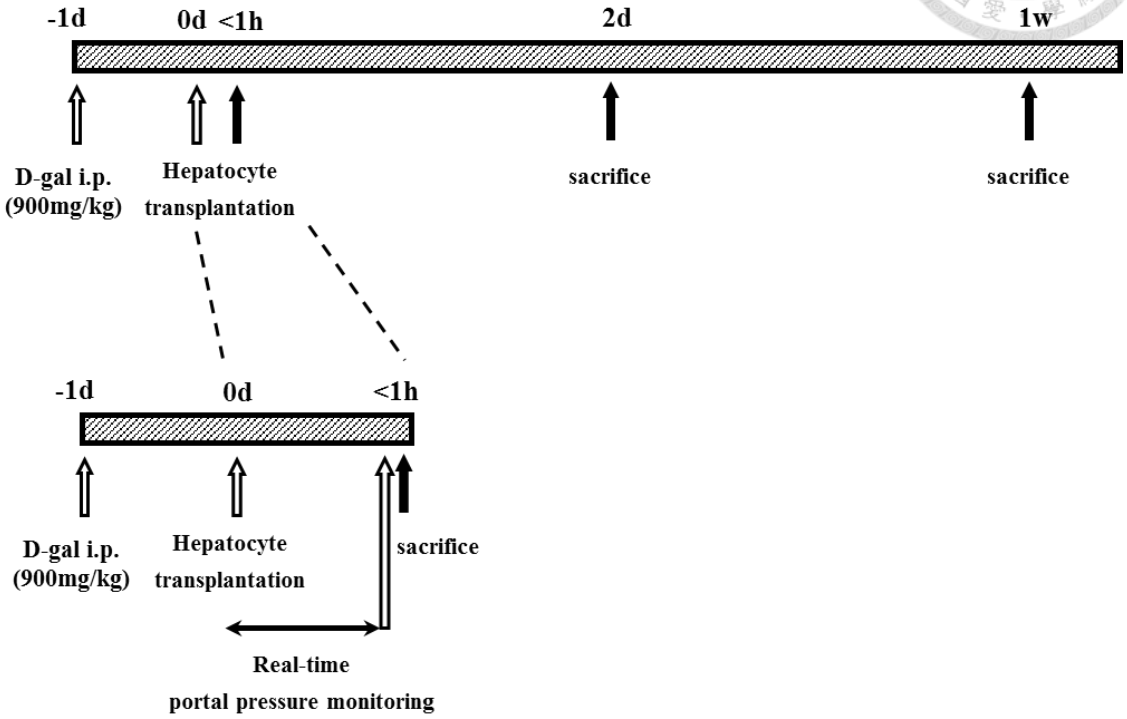
doi:10.6342/NTU201600315

Figure 32. Rat model of acute liver injury and hepatocyte transplantation. Catheterization of the portal vein for cell infusion. (A) A 24-G catheter placed in the main portal vein via midline laparotomy. (B) The lock of the catheter beneath an undermined skin flap.



doi:10.6342/NTU201600315

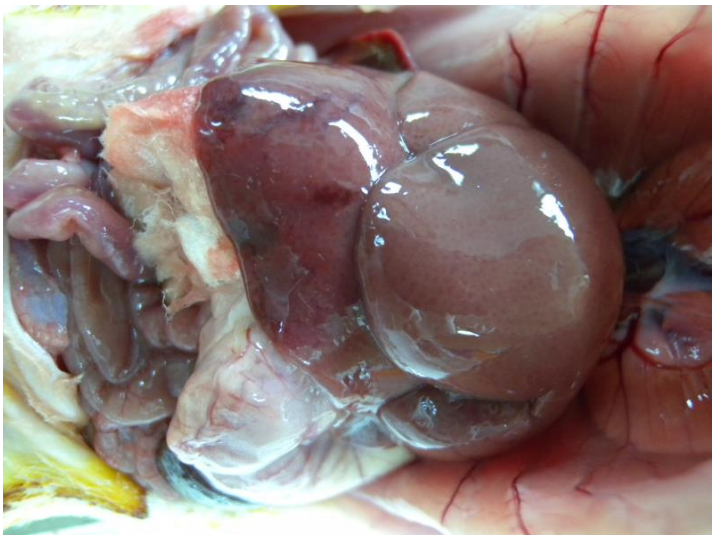
Figure 33. Schematic design of animal experiment of hepatocyte transplantation for acute liver failure.



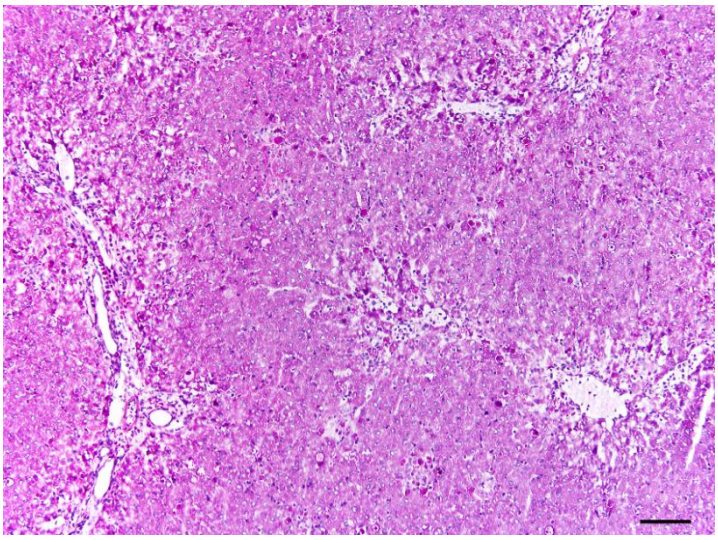
doi:10.6342/NTU201600315

Figure 34. Representative images of acute liver injury. Gross appearance of acute liver injured rat 24 hours after D-galactosamine (0.9 g/kg) treatment (A). Representative images of acute liver injury. Histopathology of the acute liver injury rat showed massive hepatocyte necrosis and apoptosis, hemorrhage, and sinusoidal dilatation. (scale bar: 100μm)

A



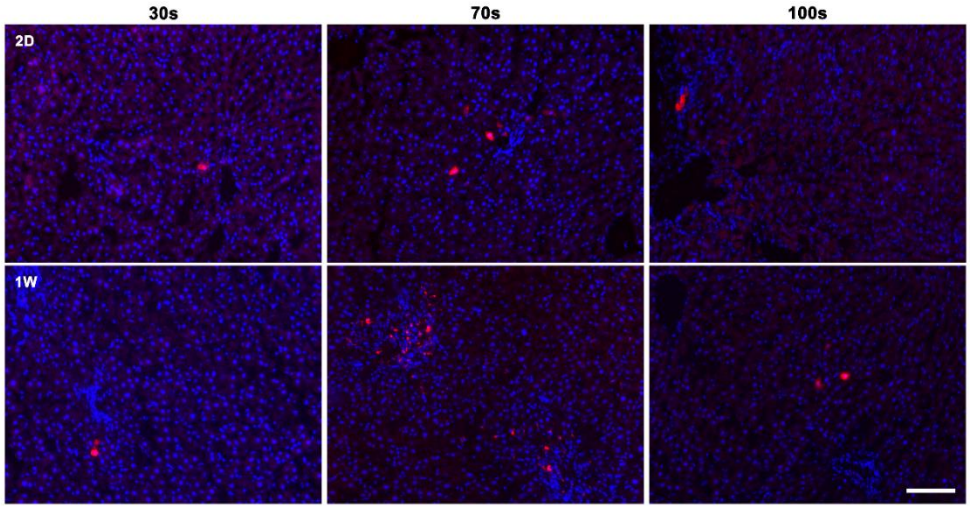
B



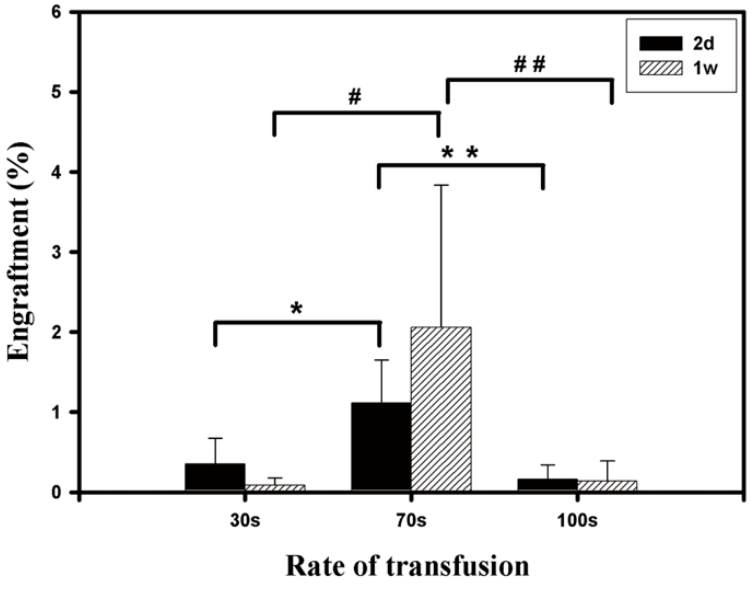
doi:10.6342/NTU201600315

Figure 35. Early engraftment (2 days) and repopulation (7 days) after transplantation of hepatocytes from DsRedT3-emGFP transgenic rats into recipients with acute liver injury. A total of 1 × 107 hepatocytes in 1 mL were transfused intraportally over a period of 30, 70, or 100 sec. Three groups had significantly different hepatocyte engraftment (P = 0.018) and repopulation (P = 0.037) efficiencies. (A) Both 2 and 7 days after transplantation, the transplanted hepatocytes (identified by direct visualization of red fluorescence) were observed more frequently in the 70-sec group. (B) Average engraftment and repopulation with respect to the transfusion rate. The 70-sec group had superior engraftment and repopulation efficiencies to the other groups. (Tukey post hoc tests; p: * 0.072; # 0.063; ** 0.018; ## 0.074) (scale bar: 100μm)

A

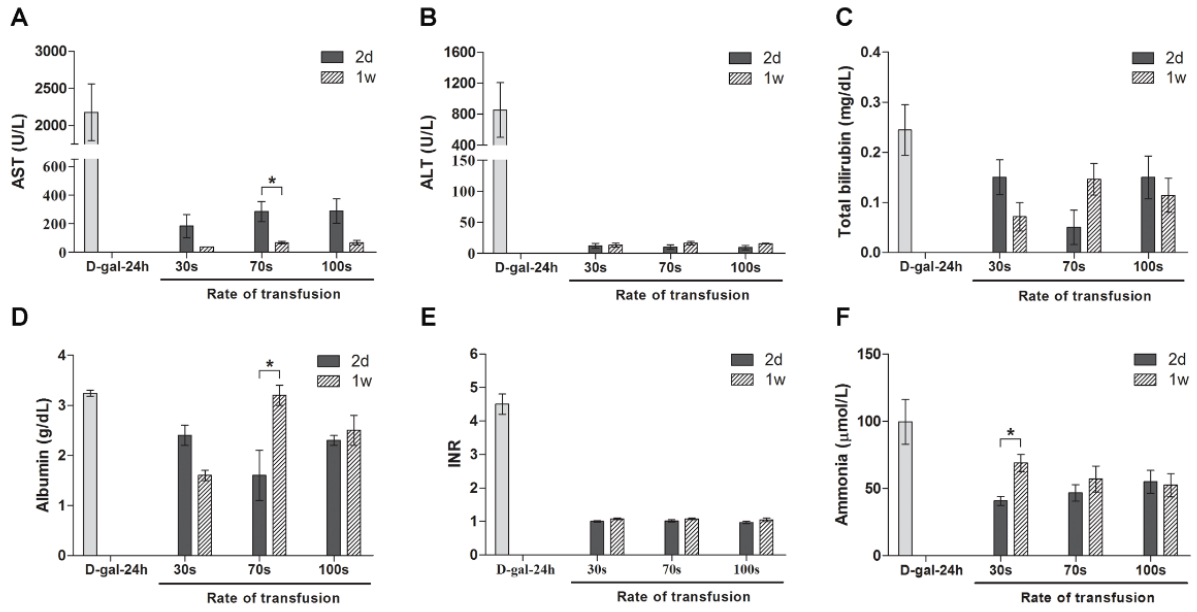


B



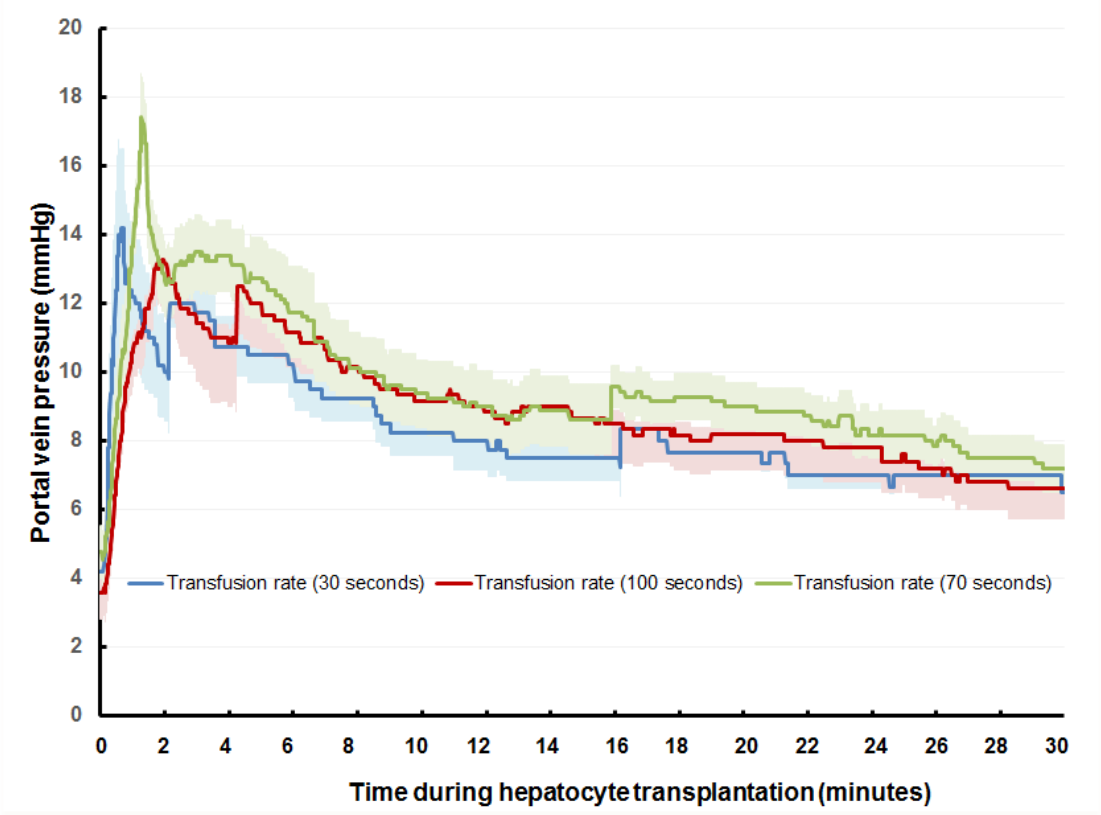
doi:10.6342/NTU201600315

Figure 36. Effect of hepatocyte transplantation on biochemical indexes of acute liver injury. Blood biochemistry of liver function tests was evaluated by (A) serum aspartate aminotransferase (AST) level, (B) serum alanine aminotransferase (ALT) level, (C) serum total bilirubin level, (D) serum albumin level, (E) international normalized ratio (INR) of prothrombin time, and (F) blood ammonia level. *p < 0.05. D-gal-24h, 24 hours after D-galactosamine treatment.



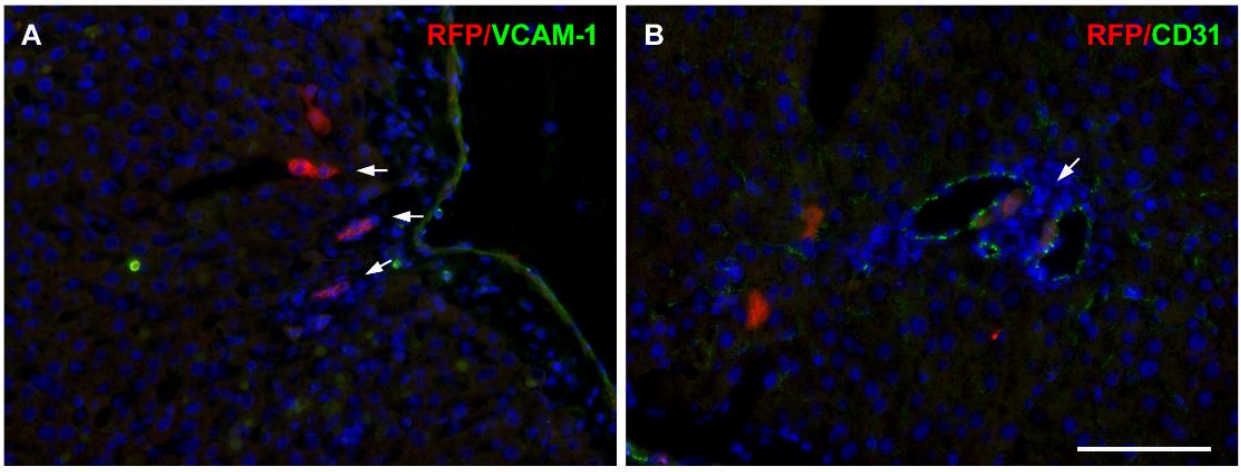
doi:10.6342/NTU201600315

Figure 37. Real-time monitoring of perioperative portal venous pressure during rat hepatocyte transplantation for treatment of acute liver injury at different rates of transfusion. Three groups reached the first peaks of pressure at different time with significance ($p < 0.0001$). Transfusion rate of 70 seconds achieved higher pressure than the other groups though without statistical significance. The second lower peaks than the first ones of portal pressure were observed in all three groups. Shaded zones are 95% confidence interval.



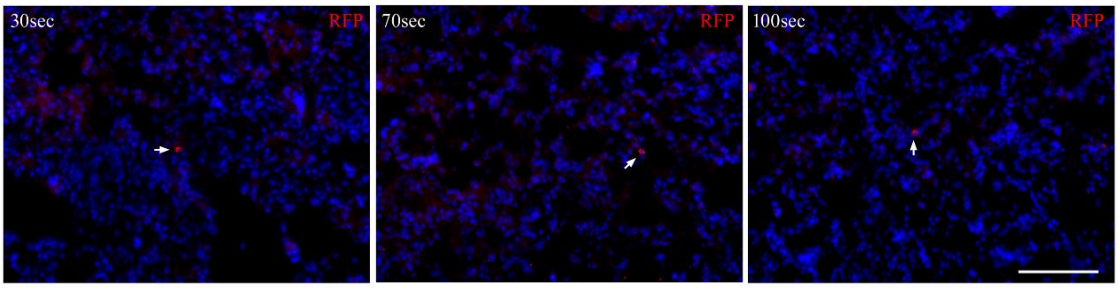
doi:10.6342/NTU201600315

Figure 38. Early vascular transmigration of donor cells (DsRedT3-emGFP hepatocytes, identified by immune-fluorescence) was observed 20 minutes after hepatocyte transplantation at the optimal rate of transfusion in rats with D-galactosamine-induced acute liver injury. (A) Early engraftment was seen in liver parenchyma (arrow). Vascular wall was stained with the antibody against vascular cell adhesion molecule 1 (VCAM-1). (B) Vascular transmigration of donor cells (arrows) was detectable just outside the wall of the portal vein (arrow). Portal vein was stained with the antibody against CD31. (scale bars: 100μm)



doi:10.6342/NTU201600315

Figure 39. Cell embolus was scatterly noted at day 2, but rarely observed in the lung in all groups 1 week after intra-portal hepatocyte transfusion in acute liver injured rats. Scattered donor cells (DsRedT3-emGFP hepatocytes) (arrow) were marked. (scale bars: 200μm)



doi:10.6342/NTU201600315

Figure 40. Liver fibrosis induced by temporary ligation of common bile duct in SD rat [first (2 weeks) and second (3 weeks) rows]. Acute-on-chronic liver failure was further induced by add-on D-gal (500 mg/kg) and rescued by hepatocyte and stem cell co-transplantation (3rd row). H&E (left panel), trichrome (middle panel) and GGT (right panel) staining for rat liver sections. (100X).

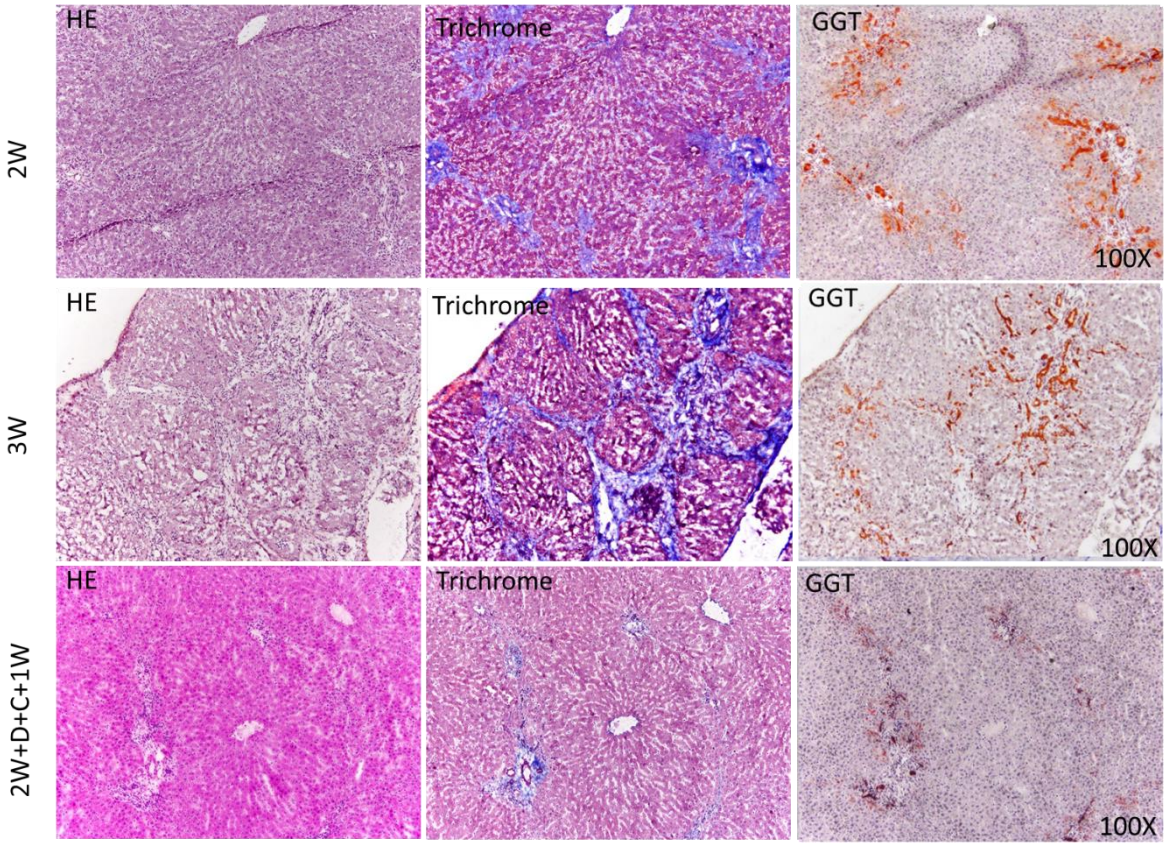


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Figure 41. Clinical in vivo isolation of hepatocytes from a dead donor at operation room.

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Tables

Table 1. Concomitant malignancy in index admission for acute liver failure and major etiologic exposures in Taiwan from 2005~2007

Primary focus of malignancy	No.	HBV	HCV	Alcohol	Toxin
Hepatocellular carcinoma	37	17	13	6	3
Extra-hepatic malignancy	25	10	1	0	10
Head and neck cancer	4	1	0	0	2
Lung cancer	4	1	0	0	3
Colorectal cancer	4	1	0	0	1
Cholangiocarcinoma and gall bladder carcinoma	3	1	0	0	2
Breast and cervical cancer	3	2	0	0	1
Leukemia	3	2	0	0	0
Pancreatic cancer	2	1	0	0	1
Bladder cancer	1	1	0	0	0
Unknown primary	1	0	1	0	0

Abbreviations: No., total patient numbers; HBV, hepatitis B virus; HCV, hepatitis C virus

Data were numbers of patients

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Table 2. Characteristics of patients with acute liver failure in Taiwan from 2005~2007

	Survived ≥90 days (n=130)	Died within 90 days (n=88)	Overall (n=218)
Follow-up days*	458.5 [45-1059]	29 [7-93]	171 [7-1059]
Hospital stay*	19.5 [7-574]	26.5 [7-93]	22 [7-574]
ICU admission*	89 (69)	73 (83)	162 (76)
Age, years*	56.5±18.4	60.0±14.7	57.9±17.1
Male	91 (70)	59 (67)	150 (69)
Underlying co-morbidity			
Diabetes mellitus	46 (35)	27 (31)	73 (34)
Peptic ulcer	29 (22)	16 (18)	45 (21)
Concomitant malignancy*	18 (14)	41 (47)	59 (27)
Prior to index admission*	4 (3)	11 (13)	15 (7)
COPD	17 (13)	8 (9)	25 (12)
End-stage renal disease	2 (2)	4 (5)	6 (3)
Autoimmune disease	0	3 (3)	3 (1)
Low income	6 (5)	4 (5)	10 (5)
Etiology of ALF			
Hepatitis B virus exposure	39 (30)	33 (38)	72 (33)
Hepatitis C virus exposure	18 (14)	7 (8)	25 (11)
Toxin exposure	24 (19)	17 (19)	41 (19)
Acetaminophen[#]	15 (12)	9 (10)	24 (11)
Alcohol exposure	19 (15)	12 (14)	31 (14)
Metabolic disorder	4 (3)	0	4 (2)

Abbreviations: COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; ICU, intensive care unit
Data were either number (%), median [inter-quartile range], or mean±standard deviation.
*Significant difference (p<0.05) between patients who died within 90 days and those who did not, by *chi*-square test or *t* test
[#]Mean±standard deviation of the defined daily dose of acetaminophen in the three groups were 16.3±13.6, 12.2±10.8, and 14.6±12.5, respectively.

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Table 3. Severity and complications of acute liver failure during index admission in Taiwan.

	Survived ≥90 days (n=130)	Died within 90 days (n=88)	Overall (n=218)
Presence of ascites	49 (38)	46 (52)	95 (45)
Presence of esophageal varices	27 (21)	11 (13)	38 (18)
Frequency of check-ups per week			
Aspartate transaminase	1.4±1.6	1.7±1.3	1.5±1.5
Alanine transaminase	1.4±1.6	1.7±1.2	1.6±1.5
Total bilirubin*	0.4±0.7	1.9±1.5	1.0±1.3
Direct bilirubin	0.9±1.1	1.1±1.1	1.0±1.1
Ammonia*	1.0±0.9	1.4±1.4	1.2±1.1
INR of prothrombin time	1.2±1.8	1.6±1.5	1.4±1.7
Lactulose dose (ml/week)	321±319	397±486	351±396
Undergoing procedure			
Panendoscopy	61 (47)	34 (39)	95 (45)
Computed tomography	59 (45)	57 (65)	116 (55)
Brain MRI	10 (8)	9 (10)	19 (9)
Plasmapheresis*	10 (8)	13 (15)	23 (11)
Sepsis*	26 (20)	34 (39)	60 (28)
Local infection			
Pneumonia	17 (13)	12 (14)	29 (14)
Urinary tract infection	15 (12)	7 (8)	22 (10)
Hemorrhage	22 (17)	20 (23)	42 (20)
Unspecified GI tract	9 (7)	10 (11)	19 (9)
Peptic ulcer	6 (5)	5 (6)	11 (5)

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Varices	2 (2)	0 (0)	2 (1)
Brain	4 (3)	4 (5)	8 (4)
Unspecified	1 (1)	1 (1)	2 (1)
Blood transfusion (U/week)			
Packed red blood cell	1.2 ± 2.1	1.7 ± 2.4	1.4 ± 2.2
Platelet	2.9 ± 8.9	2.7 ± 6.1	2.8 ± 7.9
Fresh frozen plasma*	3.6 ± 6.3	8.1 ± 11.5	5.4 ± 9.0
Organ damage			
Renal insufficiency	28 (22)	18 (20)	46 (22)
Prescription of diuretic[#]	87 (67)	77 (88)	164 (77)
Prescription of vasopressin[§,]*	15 (12)	21 (24)	36 (17)
Requiring HD/HF*	6 (5)	16 (18)	22 (10)
Requiring ventilator support*	35 (27)	44 (50)	79 (37)
Seizure	9 (7)	2 (2)	11 (5)

INR, international normalized ratio; MRI, magnetic resonance imaging; HD/HF, hemodialysis or hemofiltration; GI, gastrointestinal

Data were either mean±standard deviation or number (%).

*Significant difference (p<0.05) between patients who died within 90 days and those who did not, by *chi*-square test or *t* test

[#]Mean±standard deviation of the defined daily dose (DDD) of diuretics in the three groups was 26.5±56.4, 35.2±49.9, and 30.0±53.9, respectively

[$]Mean±standard deviation of vasopressin DDD in the three groups was 0.4±1.3, 0.2±0.9, and 0.3±1.2, respectively

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Table 4. Risk factors of survival in all patients with acute liver failure and in those without malignancy on index admission in Taiwan.

	Overall (n=218)			Patients without malignancy (n=59)		
	HR	95% CI	p	HR	95% CI	p
No. of hepatitis B virus	1.00	0.61-1.64	0.990	0.99	0.52-1.86	0.960
No. of hepatitis C virus	1.76	0.99-3.16	0.056	1.74	0.64-4.72	0.280
No. of alcohol consumption	1.67	1.01-2.77	0.046	2.43	1.31-4.53	0.005
No. of toxin	1.25	0.70-2.24	0.450	0.75	0.33-1.68	0.480
No. of malignancy	2.90	1.92-4.37	<0.001	-	-	-
No. of check-ups per week for total bilirubin	1.57	1.40-1.76	<0.001	1.91	1.63-2.23	<0.001
No. of sepsis	1.85	1.20-2.85	0.005	1.79	1.03-3.11	0.039
No. of hemorrhage	1.25	0.77-2.03	0.370	0.92	0.46-1.85	0.820
No. of ventilator use	1.00	0.65-1.55	>0.999	1.64	0.95-2.83	0.076
No. of hemodialysis/hemofiltration	2.12	1.15-3.91	0.015	2.38	1.19-4.79	0.015
No. of proton pump inhibitor (7DDD)	0.94	0.90-0.98	0.005	0.95	0.91-0.99	0.025

CI, confidence Interval; DDD, defined daily dose; HR, hazard ratio; No., frequency number.

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Table 5. Details of donor livers used for ex vivo ICG experiments.

Liver	Age (years)	Gender	Cause of death	Status at retrieval	Cold ischemia (h)	Warm ischemia (min)	Viability (%)
1	31	F	ICH	Cadaveric	10	-	70.0
2	57	M	ICH	NHBD fatty liver	12	15	79.8
3	52	M	ICH	NHBD	13	20	52.1
4	26	M	Cardiac arrest	NHBD	12.5	22	76.0
5	76	M	ICH	Cadaveric	10	=	78.6
6	60	F	Heart attack	NHBD	12	19	39.7
7	28	M	Cardiac arrest	Cadaveric	11.5	=	67.0

ICH, intracerebral hemorrhage; NHBD, non-heart beating donor

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Table 6. Antibodies used in immuno-fluoro-histo-chemistry in animal experiment of hepatocyte transplantation.

Name	Company	Cat. No.	Dilution
RFP	MBL, Nagoya, Japan	PM005	1: 300
CD31	Millipore, Temecula, CA	MAB1393	1: 125
Vascular cell adhesion molecule 1 ; VCAM-1	Serotec, Oxford, UK	MCA4633GA	1: 100

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Table 7. Literature review of transfusion rate in intraportal hepatocyte transplantation of animals

Species	Transfusion rate	Transfusi-on time	Cell density	Total cells	Outcome (reference)
DPPIV (-/-) rat and D-gal injury	0.67–0.5 mL/min	3–4 min	10^7/mL	2×10^7	Portal hypertension (McCormack et al. 1985)
DPPIV (-/-) rat	0.5 mL/min	1 min	2×10^7/mL	10^7	0.26% engraftment efficiency, mainly in zone 1 (Dagher et al. 2009)
Wistar rat	0.25 mL/min	2 min	2×10^6/mL	10^6	Cells pile up in distal portal vein, small amounts in sinusoids (Stéphenne et al. 2012)
Infant pig (2.5 kg)	0.67 mL/min	37.3 min	1×10^7/mL	2.5×10^8	No marked anomaly of physiological parameters, positive engraftment after 1 day (Dhawan et al. 2010)
Pig	1 mL/min	16–32 min	2.5×10^7/mL	$4–8 \times 10^8$	Vascular thrombosis, portal hypertension, and pulmonary cell emboli (Herweijer et al. 2007)
Non-human primates (*Macaca mulatta*, 3–5.5 kg) with liver partial portal branch embolization	2 mL/min	20 min	1×10^7/mL	4×10^8	Proliferation of transplanted hepatocytes (Fox et al. 1998, Krohn et al. 2009)

DPPIV, dipeptidyl peptidase IV; D-gal, D-galactosamine

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Table 8. Literature review of transfusion rate in intraportal hepatocyte transplantation of human

Case	Transfusion rate	Transfusi-on time	Cell density	Total cells	Outcome (reference)
Children (1 acute liver failure, 2 urea cycle disorders, and 1 Crigler-Najjar syndrome; 3–40 kg)	2–6.1 mL/min	21–33 min	5.7–9.5 × 10^6/mL	1.4–7.2 × 10^9	No clinical signs of portal vein thrombosis, pulmonary embolism, or anaphylactic reactions; portal pressure increased 24–36% and remained elevated for 6 hours (Strom et al. 1999)
Children (1 ornithine transcarbamylase deficiency, 1 Crigler-Najjar syndrome, 1 glycogen storage disease Ia, and 1 tyrosinemia type I; age, 45 days to 12 years)	1 mL/min	40–60 min	1–1.5 × 10^7/mL	3–10 × 10^7/kg of body weight per dose for 2–15 doses	Improvement in clinical status (Suda et al. 2007)
Child (phenylketonuria in a 6-year-old boy)	1 mL/min	63 min on day 1 and 110 min on day 2	10^7/mL	63 × 10^7 cells, 110 × 10^7 cells on days 1 and 2	Second transplant (1.4 mL/min) yielded greater improvement in clinical status than the first series (1 mL/min). Better donor cell quality in second transplant (Weber et al. 2006)
	1.4 mL/min	1 hour	10^7/mL	85 × 10^7	
Child (Crigler-Najjar syndrome type I in a 10-year-old girl	2.5 mL/min	30 min for 3 separate infusions separated by 4–6 hours	1.3–2 × 10^7/mL	7.5 × 10^9 cells over 15 hours	Portal vein pressure did not increase by more than 4 mm Hg for more than 5 minutes. Survived for more than 11 months with partial correction of the metabolic disorder (Soltys et al. 2010)
Young adult (glycogen storage disease type Ib in an 18-year-old man)	4 mL/min	5 min	10^8/mL	2 × 10^9	Improvement in clinical status (Yu et al. 2004)

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Appendix 附錄

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