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Reactivity of human fetal and adult immortalized hepatocytes to potentially toxic bilirubin and bile acid species

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Reactivity of human fetal and adult immortalized hepatocytes to potentially toxic bilirubin and bile acid species

Dissertação apresentada para a obtenção do Grau de Mestre

em Genética Molecular e Biomedicina, pela Universidade

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NOTA DE ABERTURA

A presente dissertação destina-se à obtenção do grau de Mestre em Genética Molecular e Biomedicina, pela Faculdade de Ciências e Tecnologia, da Universidade Nova de Lisboa.

O trabalho prático foi realizado no grupo "Neuron Glia Biology in Health and Disease" do Centro de Patogénese Molecular (CPM)/iMed da Faculdade de Farmácia da Universidade de Lisboa (FFUL), tendo a orientação sido da responsabilidade da Professora Doutora Dora Maria Tuna de Oliveira Brites, Investigadora Coordenadora do referido grupo e da Doutora Adelaide Maria Afonso Fernandes.

O trabalho prático teve início dia 1 de Setembro de 2008, com a integração nas actividades desenvolvidas no Laboratório e após uma fase de aprendizagem das técnicas laboratoriais utilizadas.

O presente trabalho de investigação (não integral) foi apresentado, em comunicação oral, no XXIX Congresso Nacional de Gastrenterologia e Endoscopia Digestiva e publicado na forma de resumo no Jornal Português de Gastrenterologia.

[1]Vizetto Duarte C, Fernandes A, Brites D. A icterícia como factor de risco da colestase crónica: manifestações citopatológicas numa linha humana saudável de hepatócitos.
XXIX Congresso Nacional de Gastrenterologia e Endoscopia Digestiva, Porto, 2009.

[2]Vizetto Duarte C, Fernandes A, Brites D. A icterícia como factor de risco da colestase crónica: manifestações citopatológicas numa linha humana saudável de hepatócitos.
GE-Jornal Português de Gastrenterologia (Suplemento), 2009, 16 (4): P8 (13A).

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CONTENTS

ABBREVIATIONS

AFP	α-fetoprotein
AGQDC	ácido glicoquenodesoxicólico [*N*-(3α,7α-dihydroxy-5β-cholan-24-oyl)glycine]
BC	bilirrubina conjugada
BNC	bilirrubina não conjugada
BSA	bovine serum albumin
CA	cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid)
CB	conjugated bilirubin
CDCA	chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid)
DCA	deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid)
DMEM	Dulbecco's modified Eagle's medium
GCDCA	glycochenodeoxycholic acid [*N*-(3α,7α-dihydroxy-5β-cholan-24-oyl)glycine]
HHL-5	human hepatocyte line 5
HSA	human serum albumin
HSC	hepatic stellate cell
IL	interleukin
IκB	inhibitory protein of NF-κB
JNK 1/2	c-Jun *N*-terminal kinases 1 and 2
LCA	lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid)
LDH	lactate dehydrogenase / lactato desidrogenase
MAPKs	mitogen-activated protein kinases
MEKKs	members of the MAPK kinase family
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NF-κB	nuclear factor κB
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulphate
TBS	trisbuffered saline
T-TBS	trisbuffered saline containing Tween-20
TCDCA	taurochenodeoxycholic acid [2-[(3α,7α-dihydroxy-24-oxo-5β-cholan-24-oyl)amino]ethanesulfonic acid]
TNF-α	tumor necrosis factor α

UCB	unconjugated bilirubin
UDP	uridine diphosphate
WRL-68	hepatic fetal human epithelial cell line

ABSTRACT

Cholestasis is the reduction or stoppage of bile flow. When bile flow is interrupted, the bile compounds, namely bile acids and bilirubin, accumulate into the hepatocyte causing cellular injury and cell death. The hepatocyte function under cholestasis must be studied using liver cell lines closely resembling human primary hepatocytes. However, most of the cell lines used is derived from hepatic tumors which have altered gene expression. In this project, we used the novel non-neoplasic cell line, HHL-5, which retain primary adult hepatocyte phenotype. In addition, we used the fetal hepatocyte cell line WRL-68, which was shown to present similar morphological properties and antigenic profile of human fetal hepatocytes *in situ*. Using these two cell lines, we evaluated whether fetal and adult hepatocytes respond differently to conditions that mimic cholestasis with associated jaundice, never evaluated *in vitro* before. The hepatocytes were exposed to 100 μM glycochenodeoxycholic acid (GCDCA), 100 μM conjugated bilirubin (CB), 100 μM unconjugated bilirubin (UCB), 100 μM GCDCA + 100 μM CB + 100 μM UCB or vehicle alone, in the presence of 100 μM human serum albumin (HSA), at various time points. After, we assessed cellular toxicity analyzing cytolysis by lactate dehydrogenase (LDH) release and apoptosis by caspase-3 activity and nuclear fragmentation. There was a significant increase of all these parameters in hepatocytes stimulated with the GCDCA+CB+UCB mixture. LDH release significantly increased after 48 h incubation for GCDCA+CB+UCB treatment (~40%, $P<0.01$) in the adult cell line. This increase was also statistically significant when compared to GCDCA, CB or UCB incubation alone ($P<0.05$). Relatively to WRL-68 cells, the release of LDH also increased significantly (~22%) after 48 h incubation with GCDCA+CB+UCB when compared to control ($P<0.05$), GCDCA ($P<0.05$) or UCB ($P<0.01$) incubations. Regarding caspase-3 activity, the increase was evident for GCDCA+CB+UCB treatment. In the adult cell line, it increased ~1.5-fold after 6 h ($P<0.01$ *vs.* control, $P<0.01$ *vs.* GCDCA, $P<0.05$ *vs*. UCB), peaking at 12 h (~3.5-fold, $P<0.05$ *vs.* control, $P<0.05$ *vs.* GCDCA) and remaining elevated after 24 h ($P<0.01$ *vs.* control, $P<0.01$ *vs.* GCDCA, $P<0.05$ *vs*. CB, $P<0.01$ *vs*. UCB). In the fetal cell line, the peak of caspase-3 activity with the same treatment occurred earlier and in a higher magnitude. Indeed, caspase-3 activity increased 4-fold after GCDCA+CB+UCB treatment at 6 h incubation ($P<0.05$) and this activation was sustained until 12 h incubation ($P<0.01$). Concerning nuclear fragmentation, in both cell lines, hepatocytes incubated with GCDCA+CB+UCB exhibited profound changes in nuclear morphology, consistent with

apoptosis, in a more marked way than when incubated with GCDCA, CB or UCB alone. The results showed similar data to those previously obtained for caspase-3 activity. Next, we evaluated the ability of hepatocytes to reduce the MTS compound which can either identify loss of cell viability or cellular proliferation upon a stimulus. In general, all the treatments slightly elevated MTS reduction. Having verified that the exposure to GCDCA in combination with CB and UCB promoted cellular death, we decided to evaluate the activation of JNK1/2 and p38 MAPKs (by western blot assay) and NF-κB (by immunocytochemistry). These molecules are typical main effectors of the inflammatory response and key mediators of specific intracellular programs that coordinate the cellular response to a variety of extracellular stimuli, also involved in the modulation of cell fate. In both cell lines, JNK1/2 were activated mainly after CB and UCB treatment, and also after GCDCA+CB+UCB. In general, GCDCA alone did not exert any effect in JNK1/2 activation. Activated p38 was, on the contrary, consistently reduced by CB and UCB incubations, in the two cell lines. P-p38 was also reduced in GCDCA+CB+UCB treatment in WRL-68 cell line, but not in HHL-5 cell line, where it slightly increased after 24 h. Regarding the NF-κB translocation to the nucleus, it was evidenced that CB was the main activator of NF-κB in our study model for both HHL-5 and WRL-68 cell lines, and that the activation of this transcription factor increased when cells were co-treated with GCDCA+CB+UCB. In addition, a major and sustained effect was observed in HHL-5 cells when compared to WRL-68 cells. As NF-κB has been considered to have anti-apoptotic functions in hepatocytes, by inducing transcription of survival genes, these data contribute, at least in part, to explain the higher levels of UCB-induced cell death in our model comparing to CB. Higher NF-κB activation in the adult cell line may also indicate less predisposal to cell death by apoptosis. Altogether, it is demonstrated that the fetal cells generally respond rapidly and in a more marked manner to the various stimuli, suggesting a more immature phenotype. Collectively, our results point out that the poor prognosis related with the presence of bilirubin in a chronic cholestatic situation is due to the marked toxicity that these molecules exert together, literally destroying hepatocytes by processes of cellular death. In this regard, the prevention of cellular demise or the induction of survival processes by means of pharmacological intervention will be of great interest in the clinical approach of jaundice-associated cholestasis.

Keywords: bile acids, conjugated and unconjugated bilirubin, cellular death, cholestasis, fetal and adult hepatocytes, cell reactivity.

RESUMO

A colestase é definida como uma redução do fluxo biliar devida à diminuição ou interrupção do mesmo. Quando o fluxo biliar é reduzido, os componentes da bílis (nomeadamente ácidos biliares e bilirrubina) acumulam-se dentro do hepatócito podendo causar dano e morte celular. As funções dos hepatócitos numa situação de colestase devem ser estudadas em linhas celulares que se assemelhem, tanto quanto possível, a hepatócitos humanos primários. Neste trabalho, utilizámos duas linhas humanas de hepatócitos não malignas, uma linha que conserva o fenótipo do fígado adulto (HHL-5) e outra linha que apresenta características morfológicas e antigénicas típicas de hepatócitos fetais *in situ* (WRL-68). A investigação com estas linhas que não têm origem cancerígena é importante uma vez que a maioria dos estudos são realizados em linhas celulares neoplásicas que possuem, consequentemente, a expressão génica alterada. Assim, o estudo da toxicidade induzida pela bilirrubina, conjugada (BC) e não conjugada (BNC), e pelo ácido glicoquenodesoxicólico (AGQDC), mimetizando uma colestase crónica associada a icterícia, nunca antes avaliada *in vitro*, foi avaliado nestas duas linhas. As células foram incubadas com o AGQDC (100 μM), BC (100 μM), BNC (100 μM), 100 μM AGQDC + 100 μM BC + 100 μM BNC ou apenas com o veículo, na presença de 100 μM de albumina sérica humana, durante vários períodos de tempo. A morte celular por citólise foi avaliada pela libertação de lactato desidrogenase (LDH), enquanto que a indução de apoptose o foi por activação da caspase 3, utilizando substracto específico, e por condensação de cromatina e fragmentação nuclear, recorrendo à marcação nuclear com Hoescht. A exposição das células HHL-5 à mistura AGQDC+BNC+BC provocou um aumento significativo de todos os parâmetros estudados relativamente à incubação unicamente com o AGQDC. A libertação de LDH aumentou significativamente nas células adultas com a incubação com AGQDC+BC+BNC após 48 h (~40%, $P<0.01$). Este aumento foi também estatisticamente significativo quando comparado com as incubações com AGQDC, BC ou BNC isoladamente ($P<0.05$). Relativamente às células WRL-68, a libertação de LDH também aumentou significativamente (~22%) após 48 h de incubação com AGQDC+BC+BNC quando comparado com o controlo ($P<0.05$), com o AGQDC ($P<0.05$) e com a BNC ($P<0.01$). Quanto à actividade da caspase-3, esta aumentou maioritariamente com o tratamento com AGQDC+BC+BNC. Na linha celular adulta, houve um incremento de ~1.5 vezes após 6 h com esta incubação ($P<0.01$ *vs.* controlo, $P<0.01$ *vs.* AGQDC, $P<0.05$ *vs.* BNC), atingindo um pico às 12 h (~3.5 vezes, $P<0.05$ *vs.* controlo, $P<0.05$ *vs.* AGQDC) e

permanecendo elevada até às 24 h (P<0.01 *vs.* controlo, P<0.01 *vs.* AGQDC, P<0.05 *vs*. BC, P<0.01 *vs*. BNC). Na linha celular fetal, o pico da actividade da caspase-3 com a mesma incubação ocorreu mais cedo e de uma forma mais marcada. De facto, a actividade da caspase-3 quadruplicou às 6 h (P<0.05) e esta actividade permaneceu elevada até às 12 h de incubação (P<0.01). Em relação à fragmentação nuclear, em ambas as linhas celulares, os hepatócitos incubados com AGQDC+BC+BNC apresentaram mudanças profundas na morfologia nuclear, características do processo de apoptose, e de uma forma mais notória que quando incubados com AGQDC, BC ou BNC, isoladamente. Estes resultados confirmam os obtidos em relação à actividade da caspase-3. Posteriormente, avaliámos a capacidade dos hepatócitos de reduzir um composto, o MTS. Este teste pode identificar perda de viabilidade celular ou aumento da proliferação celular. No geral, todas as incubações aumentaram ligeiramente a redução do MTS. Tendo verificado que a exposição dos hepatócitos à mistura AGQDC+CB+BNC promovia a morte celular das células, decidimos avaliar a activação das MAPKs JNK1/2 e p38 (por *Western blot*) e do NF-κB (por imunofluorescência). Estas moléculas são efectoras da resposta inflamatória, sendo consideradas como apresentando um papel chave na regulação de programas intracelulares específicos que coordenam a resposta celular relativamente a uma panóplia de estímulos extracelulares, modulando desta forma o destino da célula. Nas duas linhas celulares estudadas, houve activação da MAPK JNK1/2 principalmente após incubação com BC e BNC, mas também após tratamento com a mistura AGQDC+BC+BNC, não apresentando o AGQDC qualquer efeito na activação da JNK1/2. A activação da p38, pelo contrário, foi reduzida pela incubação com BC e BNC, nas duas linhas celulares, ao longo de todos os tempos de incubação estudados. Também a forma fosforilada/activada da p38 se apresentou reduzida na incubação com a mistura AGQDC+BC+BNC na linha celular fetal, mas não na adulta onde a sua activação aumentou ligeiramente após 24 h. Em relação à translocação do NF-κB para o núcleo, a BC aparece como a principal activadora do NF-κB no nosso modelo de estudo, tanto para a linha fetal como para a linha adulta. Muito provavelmente será a BC a responsável pela activação deste factor de transcrição no tratamento com AGQDC+BC+BNC. De referir, ainda, que a activação deste factor é mais elevada e mantida ao longo do tempo na linha celular HHL-5 que na linha celular WRL-68. Como o NF-κB tem sido identificado nos hepatócitos como tendo funções anti-apoptóticas através da indução de genes de sobrevivência, estes resultados podem apontar para uma razão para a BNC induzir mais morte celular que a BC no nosso modelo e para que os hepatócitos sejam mais susceptíveis à lesão causada pelo AGQDC,

quando associado com a BC+BNC. A maior e mais persistente actividade do NF-κB nos hepatóctios adultos pode também sugerir uma menor susceptibilidade destas células à morte celular por apoptose. No conjunto, é demonstrado que os hepatócitos fetais apresentam uma resposta mais rápida e mais marcada aos vários estímulos, confirmando a sua maior susceptibilidade como células mais imaturas. Colectivamente, os resultados alcançados evidenciam que o mau prognóstico atribuído à presença da bilirrubina nas colestases hepáticas crónicas se deve à marcada toxicidade que estas moléculas exercem em conjunto, levando à destruição dos hepatócitos por processos de morte celular por apoptose e citólise. Desta forma, a prevenção da morte celular ou a indução de processos de sobrevivência mediante indução farmacológica será de grande interesse na abordagem clínica da icterícia colestática.

Palavras-chave: ácidos biliares, bilirrubina conjugada e não conjugada, apoptose, citólise, colestase, hepatócitos humanos fetais e adultos, reactividade celular.

1. INTRODUCTION

1.1. The liver

The liver is one of the largest as well as one of the most important organs inside the body. Indeed, it is considered a vital organ and there is currently no effective approach to compensate for the absence of liver function leading to the need of liver transplantation.

1.1.1. Liver development

Organogenesis of the fetal liver begins in the third to fourth week of gestation with the development of an outpouching of endodermal epithelium from the ventral surface of the posterior foregut (Diehl-Jones and Askin, 2002). At this point, these endodermal cells are already capable of secreting proteins such as α-fetoprotein (AFP) and are specified to enter the liver lineage (determination). The morphology of the cells then changes to that of the hepatoblast (an early progenitor cell) and form the hepatic diverticulum (Zaret, 1996). The hepatic diverticulum, or liver bud, consists of rapidly growing hepatoblasts that penetrate mesodermal mesenchymal cells arising from the septum transversum of the diaphragm (Shafritz and Dabeva, 2002). The resulting interaction between these two types of cells forms cords of hepatocellular tissue, separated by sinusoids, which receive blood from the vitalline vessels in the yolk sac. These vessels are eventually incorporated into the growing liver and form the portal and hepatic venous systems (Kaufman, 1992). As development continues, the connection between the hepatic bud and the duodenum narrows, originating the common bile duct (Sadler, 2000).

By the end of the first month of gestation, hepatoblasts secrete specific proteins such as AFP, albumin and cytokeratin-19. As the liver begins the process of hematopoiesis, or blood cell production, liver tissues further develop into two types of cells (Fig.1.). Most cells mature into hepatocytes (albumin positive) and the remainder develops into intrahepatic bile ducts cells or cholangiocytes (cytokeratin-19 positive).

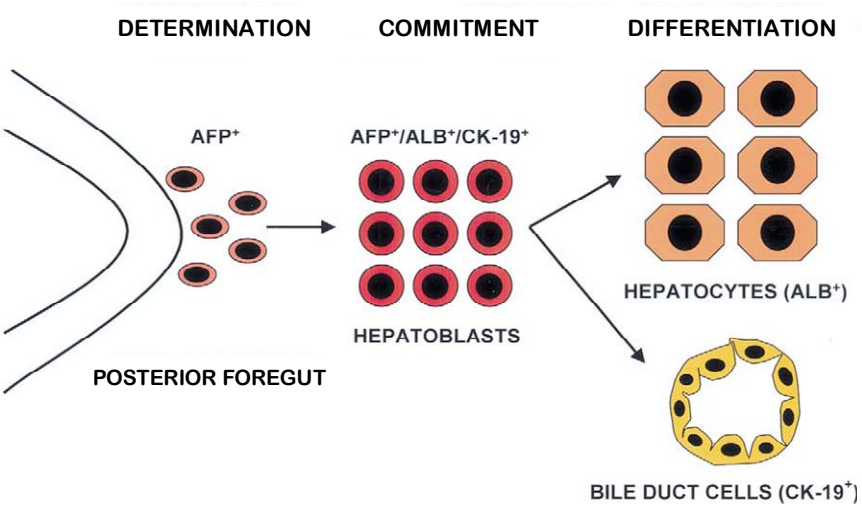

Fig.1. Schematic diagram of hepatocytes and bile duct cells differentiation. A bud of endodermal epithelium cells, already AFP^+, is primarily formed from the ventral surface of the posterior foregut. Then, these endodermal cells are specified to enter the liver lineage (determination). Their morphology changes to that of the hepatoblast which are AFP^+, ALB^+ and $CK\text{-}19^+$, identifying the commitment stage. As the cells begin to differentiate they follow one of two patterns, they may differentiate into fully mature hepatocytes (which are only ALB^+) or differentiate into bile duct cells (which do not produce AFP nor ALB, but only CK-19). AFP, α-fetoprotein; ALB, albumin; CK-19, cytokeratin-19. Adapted from Shafritz and Dabeva (2002).

In the following weeks (weeks 5–6), hematopoietic stem cells arising from the mesoderm of the septum transversum can be found in the liver. This is the beginning of the shift in hematopoiesis, from the yolk sac to the liver. The liver continues to be the main site of hematopoiesis until approximately six months of gestation, after which the bone marrow becomes the primary site of blood cell formation.

In the second month of gestation, the structure responsible for bile secretion, the canaliculus, develops. At week ten of embryonic life, the liver constitutes approximately 10 percent of the total body weight and by the third month of gestation, cholesterol and glycogen synthesis can be detected in the liver tissue (Sadler, 2000).

During the second trimester, the development of new liver cells by the process of mitosis peaks, continuing in the third trimester. However, in this last stage enlargement of individual hepatocytes (cell hypertrophy) becomes the more common process in liver growth. At the time of birth, the architecture of the liver is well established resembling an adult one.

1.1.2. Liver structure

The adult liver is a voluminous organ (1200–1500 g), highly vascularized (Young *et al*., 2006), and it can be functionally divided into structures termed hepatic lobules. The hepatic lobule (Fig. 2A) is a polyhedral prism with its boundaries limited by six portal triads (Fig. 2B) prolonged by connective tissue. The portal triads contain the hepatic portal vein and the hepatic artery, the main blood vessels running into the liver, as well as a lymphatic vessel. The liver is therefore an unusual organ having both arterial and venous blood supplies. Besides the hepatic portal vein and the hepatic artery, the portal triad also contains a bile duct that transports bile away from the liver to be secreted and stored in the gallbladder. The centre of the lobule contains the terminal hepatic venule (centrolobular vein) that drains the blood from the liver. The portal triads are connected to the central veins by plates of hepatocytes separated by the sinusoids.

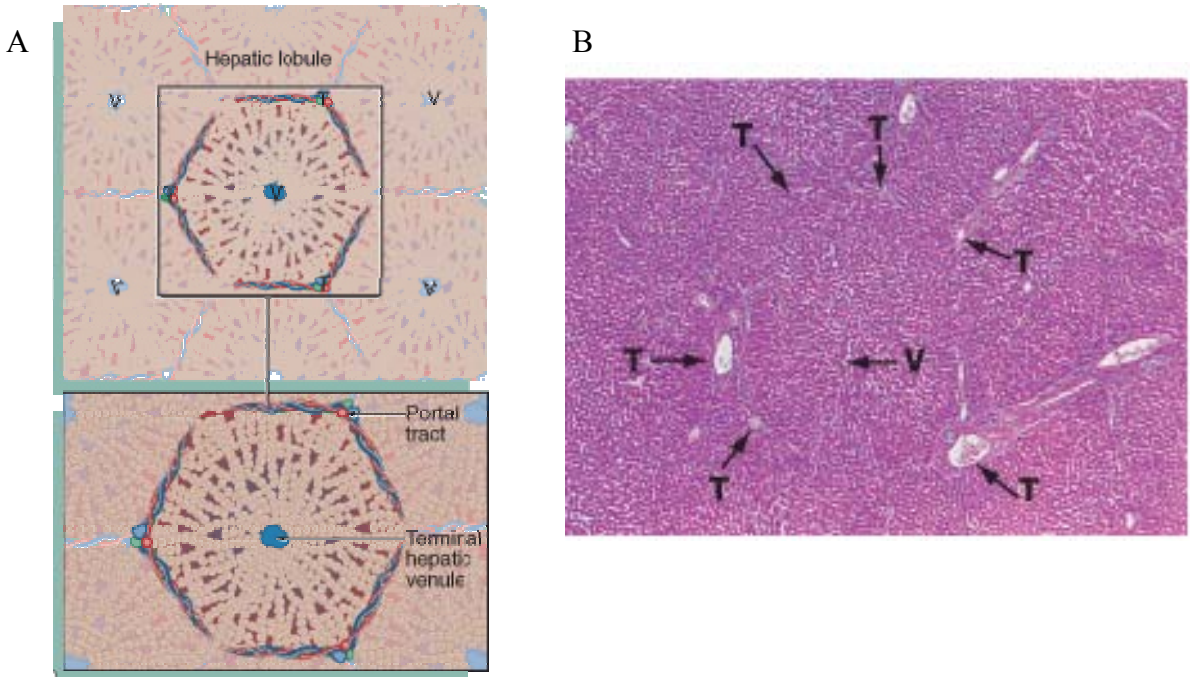

Fig. 2. The hepatic lobule is the smallest functional unit of the liver, a mass of liver parenchyma that is supplied by terminal branches of the portal vein and hepatic artery and drained by a terminal branch of the bile duct. (A) Schematic diagram showing the definition of a lobule, outlined by a hexagonal array of portal triads (T) arranged around a central hepatic venule (V). (B) Micrograph showing the overall structure of the human liver which is a solid organ composed of tightly packed plates of epithelial cells termed hepatocytes. In the human liver, a well-defined structural definition does not exists, although it can be seen the portal triads roughly defining a hexagon around the central hepatic vein. H & E staining (× 20). Adapted from Young *et al.* (2006).

Hepatocytes, the chief parenchymal cells of the liver, are responsible for maintaining a wide range of specialized functions including storage, synthesis and detoxification/excretion of various molecules (Khan *et al.*, 2007). These cells are large, multifaceted and polyhedral cells, arranged in plate-like cords separated by adjacent vascular sinusoids (Weibel *et al.*, 1969). Within the hepatic cords, between adjacent hepatocytes, lies a network of bile canaliculi, allowing the passage of bile through intercellular channels, which drain into the nearest branch of the bile duct system (Fig. 3).

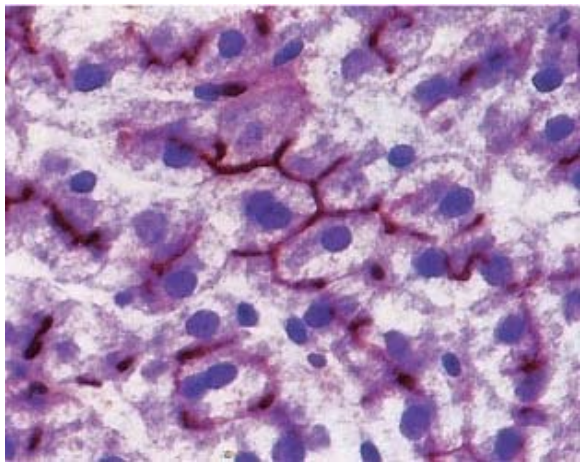

Fig. 3. Bile is secreted into a system of canaliculi which form a network within the hepatocyte plates. Bile then drain into the bile ductules of the portal tracts. The canaliculi are merely formed by the plasma membranes of adjacent hepatocytes. Enzyme histochemical method for ATPase of the bile canalicular membranes (stained in brown). Original magnification × 480. From Young *et al.* (2006).

This specialized architecture optimizes the liver's parallel functions as an exocrine gland, an endocrine gland as well as a blood filter. Owing to the liver's unique vascular organization, whereby blood percolates through the sinusoids from the place of inflow (the portal triad) to outflow (the terminal hepatic vein system), hepatocytes are exposed to a gradient of oxygen, nutrients, toxins and other biologically active molecules.

Between the incoming vessels of the portal tracts and the central veins lie the hepatic sinusoids which allow exchange between blood and hepatocytes. Sinusoids, as represented in Figure 4, are special capillaries with: (i) a fenestrated endothelial barrier; (ii) resident macrophages (Kupffer cells) for destroying bacteria and other particles in the sinusoidal blood; (iii) liver-associated lymphocytes, some of which are large, granular lymphocytes; and (iv) stellate cells (considered as pericytes) that store fat and vitamin A, and produce collagen

(Wake, 1995). Blood in the sinusoids is separated from hepatocytes by endothelial cells in the space of Dissé (Greenwel and Rojkind, 2001).

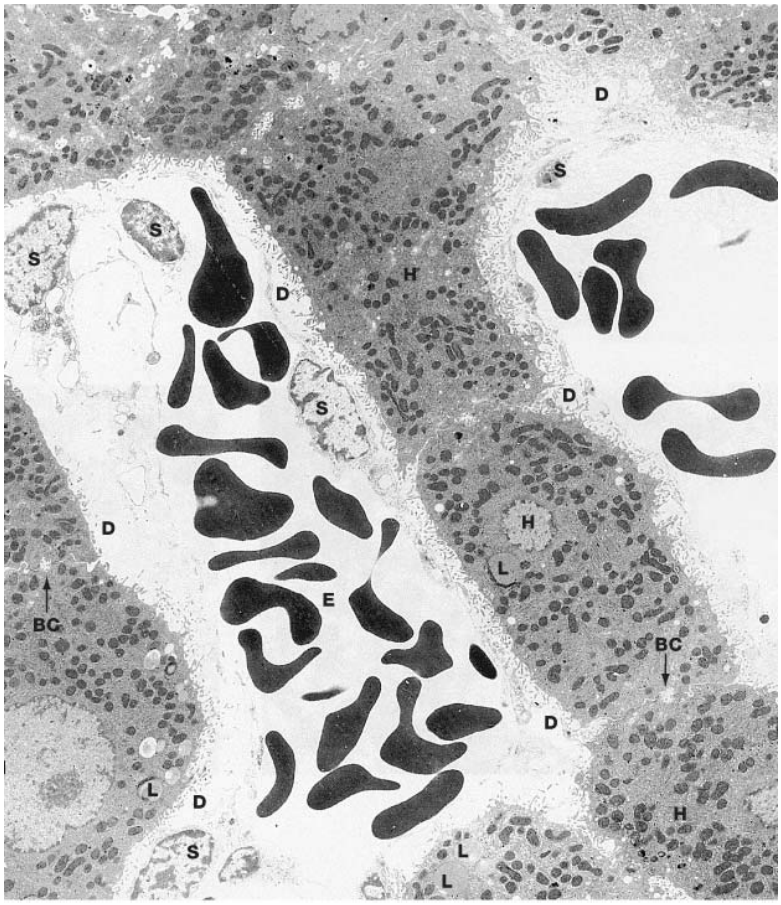

Fig.4. Micrograph demonstrating the main ultrastructural features of liver. Erythrocytes (E) can be seen within the liver sinusoids. Hepatocytes (H) are in contact to the sinusoids by a discontinuous layer of sinusoid lining endothelial cells (S). Space of Dissé (D) is located between the lining cells and the hepatocyte surface. Via the gaps in the sinusoid lining, the space of Dissé is continuous with the sinusoid lumen, thus bathing the hepatocyte surface with plasma. Numerous irregular microvilli extend from the hepatocyte surface into the space of Dissé, greatly increasing the surface area for metabolic exchanges. The hepatocyte cytoplasm is crowded with organelles, particularly mitochondrias and lysosomes, thus reflecting their range of biosynthetic and degradative activities. Lipid droplets (L) are present in variable numbers depending on nutritional status. Bile canaliculi (BC) are formed from the membranes of adjacent hepatocytes. From Young *et al.* (2006).

1.1.3. Liver functions

The liver performs multiple diverse functions essential for life including: (1) lipid metabolism; (2) carbohydrate metabolism; (3) protein metabolism; (4) storage; (5) conjugation and elimination of metabolites and toxins; (6) bile synthesis and secretion; and (7) excretion of bilirubin (Stevens and Lowe, 2005; Young *et al.*, 2006).

The liver is involved in synthesis of cholesterol, lipoproteins and phospholipids. It also oxidizes fatty acids to provide energy. Lipids and aminoacids are converted into glucose in the liver by gluconeogenesis. It synthesizes many proteins, including most of the plasma proteins such as albumin and blood clotting factors (for instance, fibrinogen and prothrombin). The liver is also the main site of detoxification of exogenous compounds such as drugs and toxins. The hepatocytes' smooth endoplasmic reticulum possesses a large number of enzymes that breakdown and conjugate metabolites or toxic substances (e.g. alcohol, barbiturates, etc). This process, known as biotransformation, is able to convert lipophilic substances to more hydrophilic ones for subsequent elimination. Another major function of the liver is the production of bile, which is an alkaline secretion containing water, ions, phospholipids, bile acids and bile pigments (mainly bilirubin diglucuronide). Most attractive is the ability of the liver to regenerate, a unique property among solid organs in mammalian species. Following two-thirds partial hepatectomy, there is a compensatory growth by the remaining liver, resulting in restoration of the total parenchymal cell number and mass within 1-2 weeks (Michalopoulous and DeFrances, 1997). Liver transplantation is currently the only successful treatment for acute hepatic failure or end stage liver disease. At the present time, however, a serious donor shortage is a major limitation to its use. Hepatocyte transplantation may have the potential to solve this problem. Several studies using rat models of primary hepatocyte transplantation revealed that transplantation leads to efficacious donor chimerism that can rescue animals from lethal hepatic failure (Rajvanshi *et al.*, 1996; Gagandeep *et al.*, 2000). Additionally, human cell lines have also been shown to improve the survival rate in an acute liver failure model (Kobayashi *et al.*, 2000). Hepatic stem cells from fetal livers may also have the ability to repopulate the liver successfully and promote long-term engraftment, given that they possess active proliferative capacity and the competence for differentiate into hepatic and cholangiocytic lineages (Kakinuma *et al.*, 2009a). It has been demonstrated that defined populations in mid-gestational fetal liver contain hepatic stem cells (Kakinuma *et al.*, 2009b).

After birth, with cessation of placental function, the neonatal liver must assume many different tasks. The physiologic development of normal hepatic function is characterized by rapid maturation of some processes at the end of gestation; however, for other processes, including bile formation, a ‘‘physiologic immaturity’’ remains for several months after birth (Emerick and Whitington, 2002). This ‘‘physiologic immaturity’’ is manifested in early life as inefficient lipid digestion, delayed hepatic clearance, slow metabolism of exogenous

substances (drugs) and endogenous compounds (bile acids and bilirubin), and a cholestatic phase of liver development (physiological cholestasis) (Belknap *et al.*, 1981; Suchy *et al.*, 1981). In fact, evidence shows different patterns of perinatal hepatic enzymatic activity, which can affect the infant's capacity for normal metabolic processes such as oxidation, reduction, hydrolysis, and conjugation, therefore influencing its ability to metabolize, detoxify, and excrete xenobiotics (Heubi *et al.*, 1982; Emerick and Whitington, 2002). Some of these differences may have relevance to understanding neonatal susceptibility to liver disease.

1.2. Pathophysioly of cholestasis

The term "cholestasis" was generalized by Hans Popper in order to describe the retention of biliary constituents (Popper, 1981). Thus, cholestasis can also be defined as a decrease or cessation of canalicular bile flow that results in accumulation of bile components in hepatocytes and canaliculi (Elferink, 2003). This condition may result either from a functional defect in bile formation at the level of the hepatocyte (hepatocellular cholestasis) or from an impairment in bile secretion and flow at the level of bile ductules or ducts (extrahepatic cholestasis) (Trauner *et al.*, 1999). Hepatocellular cholestasis may be caused by acute inflammation (hepatitis), cancer that has spread to the liver, inflammation or blockade of the bile ducts, genetic disorders, hormonal effects on bile flow during pregnancy (a condition called intrahepatic cholestasis of pregnancy) and/or drugs (Ling, 2007; Lee and Brady, 2009). On the other hand, causes of extrahepatic cholestasis are usually diseases of the bile ducts due to stones, abnormal narrowing of a bile duct (strictures) or tumors. Indeed, cholestasis is a common feature of many chronic human liver diseases leading to impaired bile formation and damage of target liver cells such as hepatocytes.

Cholestasis is also a frequent symptom of liver disease in newborns. The neonate develops cholestasis in response to a wide variety of insults, hepatic and systemic, indicating a relative sensitivity of the mechanisms of bile formation and excretion compared with children and adults.

The enterohepatic circulation in newborn animals of various species is characterized by a decrease of bile acid secretion, bile flow, bile acid synthesis, bile acid pool size, uptake of portal bile acids, and inefficient ileal uptake of bile acids (Balistreri *et al.*, 1983). At birth, basal bile acid secretion is decreased significantly compared with the mature animal and progressively increases after weaning (Shaffer *et al.*, 1985; Tavoloni *et al.*, 1985). In newborn

humans, studies have also demonstrated that duodenal bile acids concentrations do not reach the critical micellar concentration and are particularly low in premature infants, reflecting immature bile acid secretion (Heubi *et al.*, 1982; Suchy *et al.*, 1987).

Strong evidence also shows that hepatocyte uptake of bile acids and of other anions from the portal blood is decreased in the immature liver (Suchy *et al.*, 1981; Suchy *et al.*, 1987). Also, bile acid synthesis is decreased in neonates compared with adults (Balistreri *et al.*, 1983). Altogether, decreased rate of bile secretion with decreased bile acid synthesis is likely due to the immaturity of several steps of the bile acid synthetic pathway and enzymes involved in bile acid conjugation (Subbiah and Hassan, 1982). Furthermore, major changes occur during development in the volume densities of the cellular organelles that are involved in bile acid metabolism. The volume density of the smooth endoplasmic reticulum is markedly less in the neonate than in the adult rat liver and proliferates rapidly postnatally (Rohr *et al.*, 1971; Daimon *et al.*, 1982).

The immaturity of the bile acid synthetic pathway of normal infants is also evidenced by the presence of ‘‘atypical’’ bile acids in the meconium and stool of infants. These ‘‘atypical’’ bile acids are characterized by multiple hydroxylations and completely novel species. Some of these species may be hepatotoxic (Back and Walter, 1980; Strandvik and Wikstrom, 1982).The presence of potentially hepatotoxic bile acids could be a potentiating factor that may cause amplification of any cholestatic process in the infant.

Moreover, the developing liver also has immature mechanisms for hepatoprotection as animal studies reveal that detoxification mechanisms, such as sulfation, are not fully developed at birth (Balistreri *et al.*, 1984; Suchy *et al.*, 1985), and glucuronidation is also reduced in the developing liver (Klinger, 1982). The immaturity of these processes may theoretically play a role in the pathophysiology of cholestasis during childhood.

1.2.1. Main clinical biomarkers of chronic cholestasis: from diagnosis to prognosis

During cholestasis, components normally excreted into bile, including bile acids and bilirubin, accumulate in liver cells and biliary passages (Elferink, 2003). In this context, the elevation of their concentrations in the serum may be viewed not only as diagnostic biomarkers but also as prognosis indicators due to their toxicity at the hepatocytes.

1.2.1.1. Bile acids

Bile acids, the major constituents of bile, are synthesized from cholesterol by a complex series of chemical reactions (for review see Björkhem, 1985; Russell and Setchell, 1992; Setchell and Russell, 1994). As represented in Figure 5, primary bile acids are synthesized from cholesterol by the addition of hydroxyl groups and the oxidation of its side chain to form a more water soluble end product. The two primary bile acids synthesized in humans and most animal species are cholic acid (CA) and chenodeoxycholic acid (CDCA). Prior to secretion and storage in the gallbladder bile, CA and CDCA are conjugated to the aminoacids glycine and taurine at a 3:1 ratio (Chiang, 2003), resulting in glycocholic acid (GCA) and taurocholic acid (TCA) or glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA), respectively. Conjugation significantly alters the physiochemical properties of bile acids, markedly increasing the polarity of the molecule and, thereby, facilitating renal excretion (Hofmann and Roda, 1984). Furthermore, the greater hydrophilicity of the conjugated species minimizes the membrane-damaging potential of the more hydrophobic unconjugated species (Scholmerich *et al.*, 1984).

After secretion in the intestine, where they play crucial biological roles such as emulsifiers of lipids, a fraction of bile acids is converted to the secondary bile acids, deoxycholic (DCA) and lithocholic (LCA) acids by bacterial biotransformation of CA and CDCA respectively (Björkhem, 1985; Setchell and Russel, 1992; Setchell and Russel, 1994). While emulsified nutrients are taken up by enterocytes in proximal segments of the gut, bile acids continue to move distally until absorbed in the ileum. Subsequently, bile acids re-enter the liver via the portal vein, pass through the liver sinusoids and are taken up by hepatocytes and then resecreted into bile (van Berge Henegouwen *et al*., 2000). Approximately 95% of secreted bile acids are recovered by the enterohepatic circulation and the lost 5% is replenished by *de novo* synthesis. In addition, CDCA can also undergo oxidation to 7-oxolithocholic acid, followed by reduction yielding the 7β-isomer, originating the tertiary ursodeoxycholic acid (UDCA), present in trace amounts in human bile (Hofmann and Hagey, 2008).

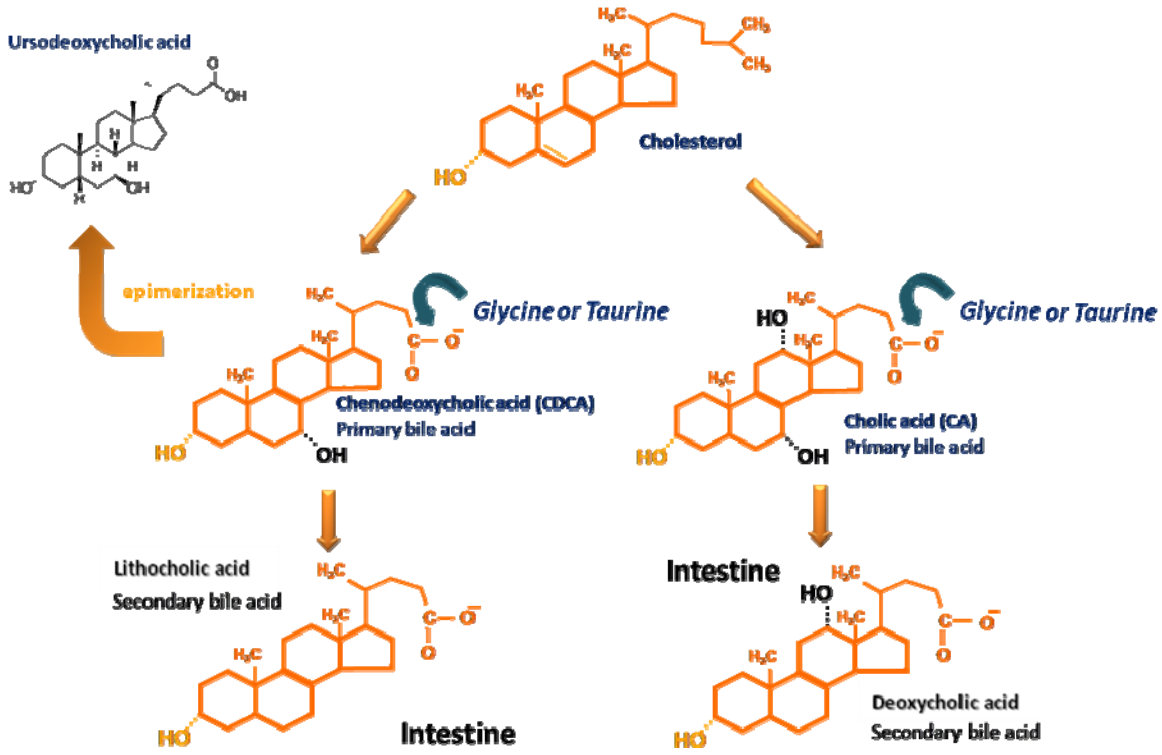

Fig. 5. Schematical representation of the biochemical pathways involved in the conversion of cholesterol to bile acids. Cholesterol is transformed into the "primary" bile acids cholic and chenodeoxycholic acids by a complex series of chemical reactions. Prior to secretion and storage in gallbladder bile, cholic and chenodeoxycholic acids are conjugated at C-24 to the aminoacids glycine and taurine. The bile acids referred to as "secondary", the lithocholic acid and the deoxycholic acid are formed from chenodeoxycholic acid and cholic acid respectively, in the intestine. Chenodeoxycholic acid can also undergo 7β-epimerization to originate the tertiary ursodeoxycholic acid, used in the treatment of hepatobiliary disorders. Although the described pathways are considered the most important for bile acid synthesis in humans, there are several alternative pathways (for review, see Setchell and Russell., 1994).

The production of bile acids occurs early in development being detected in human fetuses by 14 weeks gestation (Little *et al.*, 1975; Wahlen *et al.*, 1989). However, there is evidence that the bile pool in neonates differ from that in adults. Compared to the mature organism, in fetal bile, there is an increased ratio of chenodeoxycholic acid to cholic acid, an increased number of cholic acid conjugates, and differences in specific oxidation sites (Bucuvalas, 1992). These altered bile acids can be detected in meconium and, interestingly, are similar to bile acids found in adults presenting cholestasis. Taurine conjugates predominate in fetal life. This pattern is maintained to approximately six months of age (Balistreri, 1983). Secondary bile acids are also present in fetal bile and although the source is

unknown once fetuses do not contain intestinal bacteria, it has been suggested that they are derived either from maternal bile via transplacental transport or from primary synthesis through an alternative pathway (Balistreri, 1991).

It is worth noting that not all bile acids are toxic and previous studies suggest that this may be related to slight changes in their chemical structure (Hofmann and Roda, 1984). Hydrophobicity is an important determinant of the toxicity and protection of bile acids, two biological properties of these compounds. Bile acids hydrophobicity depends on the number, position and orientation of the hydroxyl groups, as well as amidation at the C-24 position. Therefore, the magnitude of bile acids hydrophobicity and consequently their toxicity are UDCA < CA < CDCA < DCA < LCA as ilustrated in Table 1 (Carulli *et al.*, 2000). Thus, UDCA is the more hydrophilic and the most universally used in the treatment of hepatobiliary disorders (Beuers and Paumgartner, 2002). Since conjugation with taurine and glycine increases their hydrophilicity, the conjugated species also show increased protective properties.

Table 1. Hydrophobic index of bile acids and their conjugated species.

Hydrophobicity & Toxicity

Bile acids	Glycoconjugate	Tauroconjugate	Free
UDCA	-0.43	-0.47	-0.31
CA	-0.07	0	-0.13
CDCA	-0.51	-0.46	-0.59
DCA	-0.65	-0.59	-0.72
LCA	-1.05	-1.00	

Hydrophilicity & Protection

Adapted from Heuman (1989).

The retention of hydrophobic bile acids within the hepatocyte during a condition of cholestasis causes hepatotoxicity. In this regard, when intracellular concentrations of bile acids exceed certain limits, their effects can be damaging to cell structure and function, ultimately causing cell death. Indeed, bile acids accumulation may induce hepatocyte swelling and disrupt cell membranes resulting in necrotic cell death and release of intracellular constituents (Schölmerich *et al.*, 1984). Bile acids have also shown to modulate apoptosis of

hepatocytes as several studies in models of cholestasis have demonstrated mitochondrial dysfunction and caspase activation (Rodrigues *et al.*, 2003; Maher, 2004).

1.2.1.2. Bilirubin

Bilirubin synthesis starts with the lysis of senescent or hemolyzed erythrocytes in the reticuloendothelial system. When erythrocytes are degraded, heme is released from hemoglobin and following its catabolism unconjugated bilirubin (UCB) is produced (Berk, 1994). This molecule presents a nearly symmetrical tetrapyrrolic structure, consisting of two rigid planar dipyrrole units (dipyrrinones) joined by a methylene bridge at carbon 10 and stabilized by intracellular hydrogen bonds (Fig. 6).

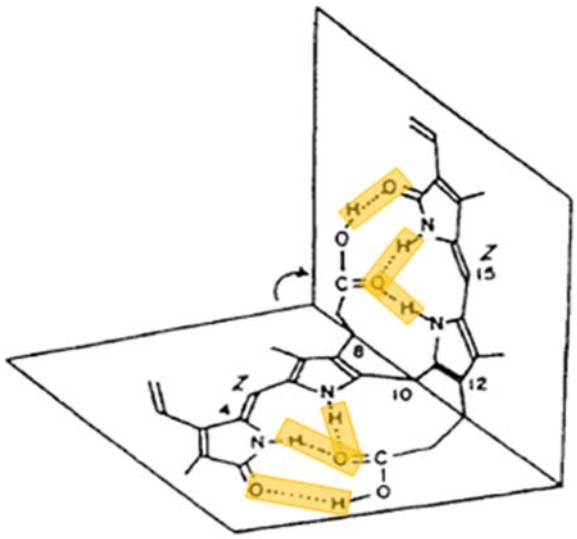

Fig. 6. Structure of unconjugated bilirubin (UCB). The molecule consists of two rigid, planar dipyrrole units joined by a methylene (-CH_2) bridge at carbon 10, and is stabilized by hydrogen bonds (highlighted in yellow). Adapted from Ostrow *et al.* (1994).

The orange-yellow bilirubin isomer is, due to its structure, poorly soluble in aqueous medium (<70 nM) (Berk, 1994; Ostrow *et al.*, 1994). Therefore, it requires a carrier molecule to be transported in the blood and further biotransformation to be excreted from the body. Upon released from the reticuloendothelial system, UCB binds reversibly with albumin, a carrier molecule with a single high affinity binding site for one bilirubin molecule (Berk, 1994), for its journey to the liver, where it is conjugated. When the UCB/albumin complex reaches the plasma membrane of the hepatocyte, UCB detaches from albumin and enters the liver cell. Inside the hepatocyte, UCB binds with other carrier proteins, such as protein Y (or

glutathione S-transferase) or protein Z during times of increased bilirubin load to the liver, to be carried into the endoplasmic reticulum for conjugation (Brito *et al*., 2006). Conjugation occurs inside the smooth endoplasmic reticulum, where each molecule of bilirubin combines with one or two molecules of glucuronic acid by the enzyme uridine diphosphate (UDP)-glucuronosyl transferase to produce bilirubin monoglucuronide and diglucuronide pigments (Fig. 7). This conjugation renders a higher solubility in aqueous medium to the UCB molecule.

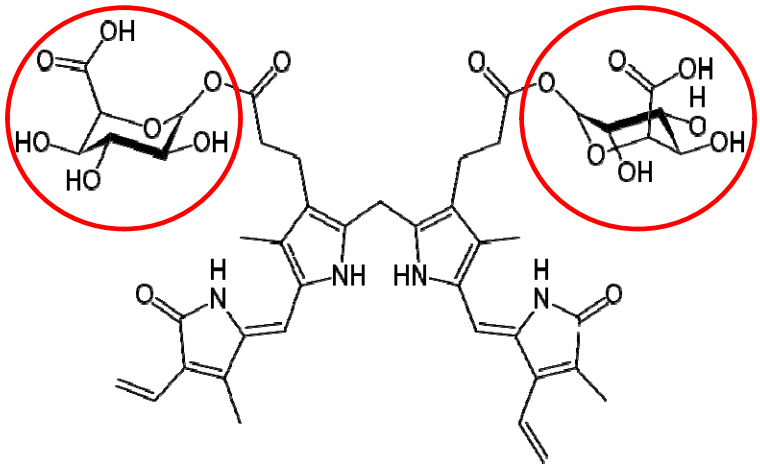

Fig. 7. Structure of bilirubin diglucuronide. The pigment structure remains the same with the addition of two molecules of glucuronic acid (highlighted with red circles). Adapted from http://en.wikipedia.org/wiki/Bilirubin_diglucuronide.

Conjugated bilirubin (CB) is then excreted in bile and passes through the small intestine via the common bile duct without significant absorption. At the intestinal level, β-glucuronidase turns mono- and diglucuronides to UCB which are either reduced and oxidized by intestinal flora or in its absence absorbed by the intestine, entering enterohepatic circulation. Catabolization by colonic flora originates urobilinogen, some of which is oxidized to stercobilin that is excreted in the stool giving it its brown color (Fig. 8). Remaining urobilinogen is reabsorbed and excreted in the urine as urobilin, giving it its yellow color.

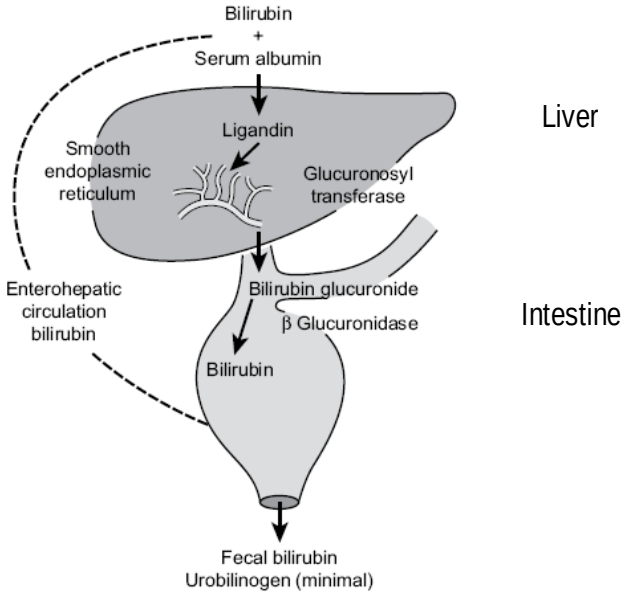

Fig. 8. Bilirubin uptake, transport and secretion by the liver, followed by intestinal excretion. Adapted from Watson (2009).

In fetal life, bilirubin production begins as early as 12 weeks' gestation. In children and adults, approximately two thirds of the monoglucuronides are conjugated to diglucuronides. However in neonates, monoglucuronide is the predominante conjugate specie. Owing to the immaturity of the fetal liver, the fetus has a limited ability to conjugate bilirubin and limited excretory function, and therefore physiologically relevant hepatobiliary elimination of bilirubin does not occur (Briz *et al.*, 2006). The circulating fetal UCB readily crosses the placenta to the maternal circulation, where it is excreted by the maternal liver. However, the concentrations of UCB are higher in fetal than in maternal serum (Monte *et al.*, 1995). Two facts contribute to this difference: a very active heme catabolism together with a very low UDP-glucuronosyl transferase activity in the fetal liver (Kawade and Onishi, 1981). Thus, during intrauterine life, the placenta is the major route for the excretion of fetal biliary pigments (for a review, see Marin *et al.*, 2003).

At birth, this placental protection is suddenly lost. At the same time, an increase in production of UCB occurs, due to the shorter erythrocyte life span of newborns (70-90 vs. 120 days in adults), especially if prematures. In addition, the newborn has to use its own immature mechanisms for hepatic uptake, conjugation and biliary secretion of bilirubin, reason why a significant retention of UCB occurs, even in healthy term neonates (Gourley, 1997; Reiser, 2004). Such retention is further enhanced by the absence of anaerobic ileo-colonic flora in the newborn infant, leading to more unmetabolized UCB available for

intestinal absorption, thus increasing the enterohepatic circulation of UCB (Vitek *et al.*, 2000). As a result, virtually all newborn infants will have mild to moderate UCB levels within the first days of life, a condition known as "physiologic jaundice". Therefore, this neonatal jaundice reflects the transition from intrauterine to extrauterine bilirubin metabolism and is linked to normal development; it is considered benign, and is usually resolved by the end of the first week of life with no treatment requirement (Reiser, 2004). However, it is important to confirm that plasma bilirubin is reducing after 14 days (Beath, 2003). The duration of exposure to overstated hyperbilirubinemia is believed to represent increased risks for neurologic sequelae (Dennery *et al.*, 2001; Hansen, 2002) and is one of the most common factors related with the readmission of term and near-term infants (Brown *et al.*, 1999).

Yellow coloration of skin and eyes, dark urine and light-colored stools are also characteristic symptoms of cholestatic or obstructive jaundice. Jaundice results from excess bilirubin deposited in the skin and dark urine results from excess bilirubin that accumulates in systemic circulation and is excreted by the kidneys. Usually, during cholestasis, jaundice occurs as a consequence of insufficient bile flow (Elferink, 2003).

UCB and CB have also been demonstrated to promote hepatocyte and canalicular toxicity. Indeed, both species have been implicated in the inhibition of biliary phospholipid secretion (Labori *et al.*, 2002; Labori *et al.*, 2009), while UCB showed to induce canalicular membrane damage and consequently to promote intrahepatic cholestasis (Labori *et al.*, 2009), which may aggravate the already established cholestatic condition. In accordance, elevation of serum bilirubin concentration is now considered a poor prognosis indicator in acute liver failure (Hadem *et al.*, 2008) and primary biliary cirrhosis (Krzeski *et al.*, 1999).

1.2.2. Acute *vs.* chronic cholestasis

During a cholestatic condition the concentration of each bile acid in the patient serum may vary according to the type of cholestasis that is present. In fact, results obtained in our laboratory have demonstrated that during acute cholestasis CA is the predominant bile acid while in chronic cholestasis CDCA is the most prevalent (Fig. 9). Therefore, it is possible for the clinicians to diagnose the type of cholestasis based on the serum bile acid profile, allowing a more proper treatment.

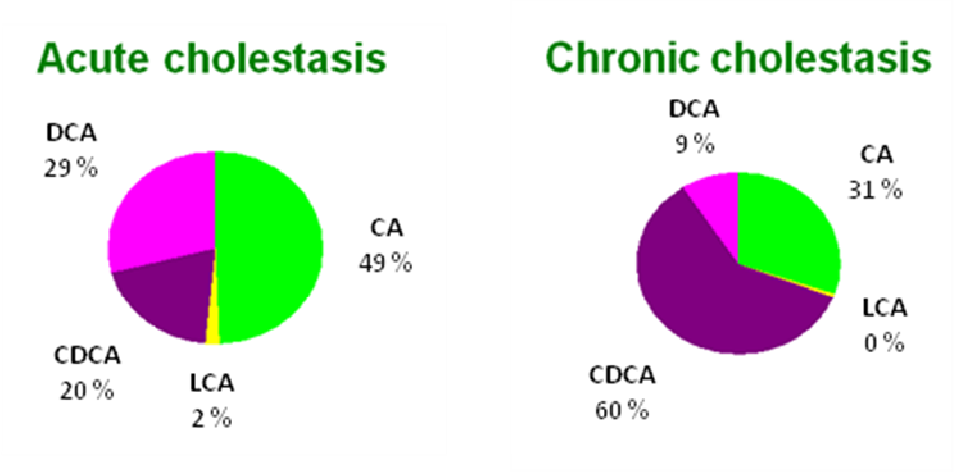

Fig. 9. Pie charts demonstrating the distribution of each bile acid during acute and chronic cholestasis (Brites, personal communication). CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid.

Hepatocyte damage by toxic bile acids is assumed to represent a key event for progression of cholestatic liver diseases (Hofmann, 2002). In this regard, the GCDCA and the TCDCA, predominant dihydroxy bile acids present in chronic cholestatic patients due to conjugation of CDCA, have been held responsible for cholestasis associated liver injury (Schmucker *et al.*, 1990). Exposure of hepatocytes to GCDCA, at concentrations representative of those found in cholestatic human liver injury, are thought to induce hepatocyte necrotic and apoptotic cell death (Patel *et al.*, 1994; Gonzalez *et al.*, 2000; Yerushalmi *et al.*, 2001). Moreover, engulfment of the hepatocyte apoptotic bodies by hepatic stellate cell (HSC) and Kupffer cells enhances their expression of pro-fibrogenic genes and death ligands. Persistent activation of these cells promotes further hepatocyte death, which culminates in hepatic inflammation, with sustained HSC activation. If liver injury continues chronically, hepatic fibrosis develops as a result of the activation of HSC, which are the main cellular elements involved in extracellular matrix deposition (Friedman, 2000).

Nowadays, cholestatic liver diseases account for a large proportion of chronic liver disorders in adults, children and infants, and are one of the most frequent and destructive manifestations of liver diseases (Pauli-Magnus and Meier, 2005). Moreover, long-term cholestasis can lead to development of cirrhosis due to stimulation of fibrotic process. Unfortunately, all too often, progression to end-stage liver disease is either fatal or requires liver transplantation.

1.3. Mechanisms of hepatocyte injury during cholestasis

1.3.1. Major modes of cell death in the liver

The balance between cell division and cell death is a basic feature in the development and maintenance of liver homeostasis. Disturbances in this balance can cause liver diseases: too much cell death can cause liver injury; too little cell death is a prerequisite for the development of hepatocellular carcinoma. Thus, a tight control of the equilibrium between life and death in the liver is necessary.

During cholestatic liver diseases, hepatocytes are exposed to increased levels of cytotoxic compounds like bile acids, bilirubin or even cytokines if inflammation is present. Although hepatocytes have an enormous capacity to defend themselves against these agents, excessive exposure will result in cell death (Schoemaker and Moshage, 2004). Cell death is typically discussed dichotomously as either apoptosis or necrosis (Fig. 10), although recently there have been described alternate types of cell death (for review see Fink and Cookson, 2005).

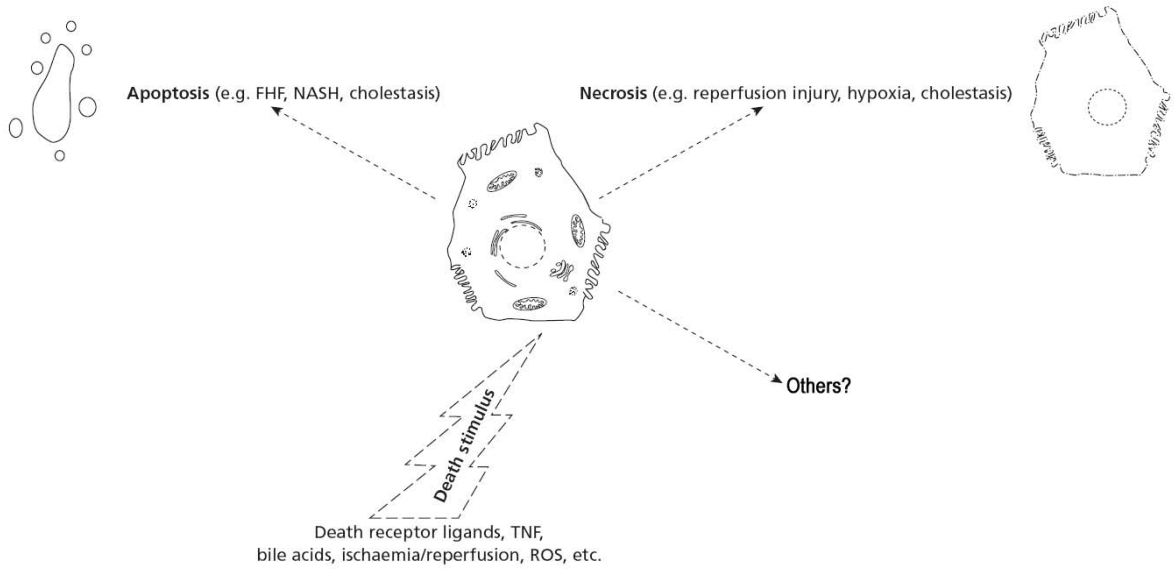

Fig. 10. Common cell death pathways in the liver. Hepatocytes can die from different modes of cell death. Apoptosis occurs in physiological as well as pathological conditions and represents a highly organized and genetically controlled type of cell death leading to shrinkage of the cell and disintegration into small apoptotic bodies. Necrosis (or oncosis/oncotic necrosis) leads to cellular edema and disruption of the cell membrane. FHF, fulminant hepatic failure; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; TNF, tumor necrosis factor. Adapted from Schulze-Bergkamen (2007).

The word apoptosis was proposed by Kerr *et al.* (1972) to describe a controlled physiologic process of removing individual unnecessary components of an organism without destruction or damage to the organism. Apoptosis was initially confirmed as a specific form of cell death that served to eliminate excessive or unwanted cells during embryonic development and normal tissue growth (Williams, 1991), but had been clarified to be also induced in cellular injury with inflammatory disease (Haslett, 1992).

Samali *et al.* (1999) and others (Blagosklonny, 2000) have proposed that apoptosis should be defined as caspase (asparate-specific cysteinyl protease)-mediated cell death with the following morphological features: cytoplasmic and nuclear condensation, chromatin cleavage, formation of apoptotic bodies, maintenance of an intact plasma membrane, and exposure of surface molecules targeting cell corpses for phagocytosis. More specifically, the molecular definition of apoptosis can logically be based on the proteolytic activity of some caspases (caspase-2, -3, -6, -7, -8, -9, and -10) since they mediate the process of apoptotic cell death. Among them, caspase-3 has been identified as being a key mediator of apoptosis of mammalian cells (Samali *et al.*, 1999). Caspase-3 is one of the effectors' caspases from apoptosis that are activated by upstream initiator caspases and are responsible for the cleavage of the key cellular proteins, such as cytoskeleton proteins, that leads to the typical morphological changes observed in cells undergoing apoptosis.

Although apoptosis of hepatocytes can be triggered by several different stimuli, apoptotic signaling is mainly transduced by two major molecular pathways, an extrinsic pathway mediated by death receptors (i.e. TNF-α/TNFR1 signaling) on the cell surface and an intrinsic pathway, which is triggered at the mitochondrial level. Both pathways culminate in the activation of caspases and endonucleases, which ultimately degrade the cellular constituents. Deregulation of the apoptotic program is pathophysiologically involved in acute as well as in chronic liver diseases, including cholestasis (Miyoshi *et al.*, 1999).

If liver injury chronically persists along time, necrotic cell death may occur. Contrary to the controlled cellular death program in apoptosis, necrosis (or recently renamed oncosis or oncotic necrosis) is a more chaotic mechanism of cell death. For designating any cell death characterized by cellular swelling, organelle swelling and increased membrane permeability the term oncosis has been used (Van and Van Den, 2002). Oncosis occurs when a cell is stressed beyond its tolerance. Contrary to apoptosis, this type of cell death does not involve the activation of molecular mechanisms specialized in contributing to cell death. Instead, under extreme circumstances, normal cellular activities are destabilized with devastating

consequences for the cell (Majno and Joris, 1995). It results from metabolic disruption with energy depletion and loss of adenosine triphosphate (ATP), ion deregulation and enhanced degradative hydrolase activity.

The term necrosis has been generally used to portray non-apoptotic accidental death with features of oncosis. However, the distinction between the structural and biochemical processes occurring in a dying cell and the endpoint of death itself is a subject that has been disregarded in the literature and must be clearly distinguished. Pathologists use the word necrosis to designate the presence of dead tissue or cells, being considered the sum of changes that have occurred in cells after they died, regardless of the prelethal process (Levin *et al.*, 1999). Necrosis, therefore, involves cell destruction with extravasation of intracellular components, an event observed after a cell has already died. By definition, this is manifested biochemically as the release of cytosolic enzymes including lactate dehydrogenase (McCarthy and Evan, 1998). The release of cellular contents triggers an inflammatory response in the surrounding tissue (Kerr *et al.*, 1972).

Multiple types of death can be observed simultaneously in tissues or cell cultures exposed to the same stimulus (Fink and Cookson, 2005). An understanding of the processes leading to liver cell death will be important for development of effective interventions to prevent hepatocellular death and consequent liver failure (Malhi *et al.*, 2006).

1.3.2. Cell death and the development of inflammation

In necrosis, in which membrane integrity is lost, the resulting cytolysis and released contents elicits an inflammatory response (Rosser and Gores, 1995). In contrast, the uptake of apoptotic bodies suppresses secretion of inflammatory mediators from activated macrophages (Fadok *et al.*, 1998). Therefore, a critical component of the definitions of apoptosis is its anti-inflammatory outcome. However, in pathological conditions, hepatocellular apoptosis may also cause inflammatory reactions such as infiltration of neutrophils (Guicciardi and Gores, 2005). Upon persistent inflammation, fibrogenesis (the development or proliferation of fibrous tissue) can occur, mainly as a consequence of the activation of HSC following their transdifferentiation into myofibroblasts (Canbay *et al.*, 2004). In addition, Kupffer cells, the major phagocytes of apoptotic bodies in the liver, can also express and release death ligands and proinflammatory cytokines, thereby accelerating hepatocyte apoptosis and inflammatory reactions. Importantly, HSC also engulf apoptotic bodies in the liver, a process that is

associated with their activation and with production of transforming growth factor (TGF)-β, a potent profibrogenic cytokine, ultimately leading to fibrosis (Canbay *et al.*, 2003).

1.3.3. Inflammatory signaling pathways

The hepatocyte, early injured in the course of cholestasis, is in part responsible for many of the subsequent inflammatory and fibrinogenic responses of nonparenchymal cells. Damaged hepatocytes may secrete molecules (cytokines, chemokines, growth factors, lipid peroxide products, etc.) that amplify the inflammatory response, stimulate fibrogenesis by HSC, or directly injure other nearby cells. Thus, understanding the events that initiate liver injury during cholestasis should focus to a large extent on the hepatocyte and the effects of toxic compounds such as bile acids and bilirubin on hepatocyte response and survival (Sokol *et al.*, 2006).

Cytokines are multifunctional pleiotropic proteins that play crucial roles in cell-to-cell communication and cellular activation. Functionally, cytokines have been classified as being either proinflammatory or anti-inflammatory depending on the final balance of their effects on the immune system (Mosmann *et al.*, 1986). They are mediators that initiate multiple signaling pathways that, although independent, may interact with each other and influence the magnitude and duration of the inflammatory response. In some cases, different cytokines may present synergistic, redundant or even opposite actions. In healthy liver, constitutive production of cytokines is absent or very low. Irrespective of its etiology, inflammation-induced cholestasis is mediated by cytokines (Trauner *et al.*, 1999), and hepatocytes are exposed to increased levels of cytokines such as tumor necrosis factor alpha (TNF-α) and various interleukins (IL) (Wullaert *et al*., 2007). *In vivo* studies have shown that bile acids are capable of inducing Kupffer cells to release proinflammatory cytokines and subsequently affect transcriptional alterations in the neighboring parenchymal cells (Miyake *et al.*, 2000).

Bile acids have been shown to also activate the mitogen-activated protein kinases (MAPKs) signaling cascade (Grambihler *et al.*, 2003), which are usually intracellularly activated upon an inflammatory stimulus. MAPKs are important enzymes involved in cellular signaling, apoptosis, carcinogenesis and pathogenesis of variety of diseases (Dhillon *et al.*, 2007). Some of the most prominent members of MAPKs family are c-Jun-N-terminal kinases 1 and 2 (JNK1/2) (Weston and Davis, 2007) and p38 kinase (Bradham and McClay, 2006). MAPKs can be activated by a wide variety of different stimuli, but in general, p38 and JNK1/2 are known for being more reactive to environmental stresses and inflammatory

cytokines (Kyriakis and Avruch, 2001). MAPKs activation is catalyzed by members of the MAPK kinase family such as the MAP kinase kinase kinases (MEKKs) and it consists in the phosphorylation of tyrosine and threonine residues (Cobb and Goldsmith, 1995). Once activated, MAPKs phosphorylate target substrates including some transcription factors or other molecules involved in transcription factors activation such as the IκB/NF-κB signaling (Johnson and Lapadat, 2002).

MAPK cascade selectivity is conferred by specific interaction motifs located on physiological substrates, allowing distinct biological functions for each activated MAPK. Thus, the p38 activity is considered critical in normal immune and inflammatory responses (Ono and Han, 2000), whereas JNK1/2 phosporylation is associated with apoptosis (Davis, 2000). Of 3 known mammalian JNK genes, 2 are expressed in the liver: JNK1 and JNK2 (Czaja, 2003). Both can be activated by death receptor and endoplasmic reticulum stress pathways of apoptosis and may also be the pathway of caspase-independent reactive oxygen species–mediated cell death (Malhi and Gores, 2008).

The MAPK pathways have been shown to be strongly activated after partial hepatectomy and presumably play a key role in regulating hepatocytes proliferation during hepatic regeneration (for review see Fausto, 2000). Interestingly, activation of MAPK pathways also precedes the process of HSC proliferation and activation that is associated with tissue remodeling and leads to hepatic fibrosis (Svegliati-Baroni *et al.*, 2003).

The general function of MAPK cascades is the regulation of gene expression. In this way, MAPKs regulate cell proliferation and cell survival, but also mediate cell death. However, the actual roles of each MAPK cascade are cell-type and context-dependent. Importantly, studies in non-proliferating cells or primary cultures are scarce and rather reveal the physiological role of MAPKs.

Besides MAPKs, other signaling pathways modulate cell death in hepatocytes, thereby influencing the balance between pro- and anti-apoptotic signals. One of them is the NF-κB signaling cascade (Schoemaker and Moshage, 2004).

The transcription factor NF-κB plays a key role during inflammation. In most cells, NF-κB is predominantly composed of a p65:p50 heterodimer. In quiescent cells, NF-κB is maintained in the cytoplasm by binding to its inhibitor IκB. It is believed that NF-κB is activated in response to cytokines. Activation occurs when inhibitory protein IκB, is phosphorylated at specific serine residues. This results in the release of IκB from the p65 subunit of NF-κB which exposes a nuclear localization sequence on the p65 subunit allowing

the translocation of NF-κB to the nucleus. Phosphorylated IκB is consequently ubiquitinated and degraded in the proteasomes. In the nucleus, NF-κB binds to κB binding sites in promoters of target genes and induces transcription of these genes. Many NF-κB-regulated genes are survival or antiapoptotic genes that protect cells against harmful compounds released during inflammation (Schoemaker *et al.*, 2003). NF-κB-inducible anti-apoptotic genes expressed in hepatocytes are prime candidates for novel therapies in liver diseases (Schoemaker *et al.*, 2002).

A coordinate activation of these pathways, ordered in space and time, orchestrates the complex response to injury by inducing genes that regulate cell survival, proliferation, differentiation and tissue specific functions. On this basis, pharmacological or molecular modulation of intracellular kinases and NF-κB have been under consideration as an approach to therapy of neoplastic as well as non-neoplastic conditions (Sebolt-Leopold *et al.*, 1999; Zhu *et al.*, 1999; Sebolt-Leopold, 2000).

1.4. Aims of the thesis

Numerous studies have investigated the mechanisms and pathways of liver damage after exposure to bile acids mimicking a situation of cholestasis. However, the stimulation of these mechanisms associated with hiperbilirrubinemia has not been considered in most of the *in vitro* studies. In addition, the study of hepatotoxic mechanisms seldom considers the fetal/neonatal conditions.

Thus, the main aims of this project are: (a) to evaluate the role of GCDCA alone in human hepatocyte injury; (b) to investigate the effects of the additional presence of UCB and CB on human hepatocyte response and cytotoxicity; (c) to assess the involved intracellular pathways leading to injury in our experimental models; and (d) to explore the reasons behind the different susceptibility of fetal and adult hepatic cells to jaundice, cholestasis or both.

Collectively, with the results obtained in this project, we aim to improve the diagnosis and prognosis of bile acid and bilirubin-associated diseases. Hopefully, the results obtained with this project will be translated back to the community, allowing a more proper therapy and contributing to the well being of the population. Cholestatic liver diseases as a whole are the most frequent hepatic diseases, caused by a range of disturbances that impair bile flow and it is very important to understand the complete picture of the interlaced mechanisms behind those conditions in order to design increasingly accurate and targeted therapies.

2. MATERIALS AND METHODS

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), non-essential aminoacids (100×) and L-glutamine were purchased from Biochrom AG (Berlin, Germany). Glycochenodeoxycholic acid (GCDCA) [*N*-(3α,7α-dihydroxy-5β-cholan-24-oyl) glycine] minimum 96% pure was from Calbiochem (Darmstadt, Germany). Antibiotic antimycotic solution (20×), human serum albumin (HSA) (fraction V, fatty acid free), bovine serum albumin (BSA), trypsin, Hoechst dye 33258, mouse antibody anti-β-actin and goat antibody anti-rabbit labeled with fluorescein isothiocyanate (FITC) were acquired from Sigma Chemical Co (St. Louis, MO, USA). Unconjugated bilirubin (UCB), also from Sigma Chemical Co, was purified according to the method of McDonagh and Assisi (1972). Bilirubin ditaurate [ditaurine amide of bilirubin (disodium salt)] showed in Figure 11, was purchased from Frontier Scientific (Logan, UT, USA) and used as conjugated bilirubin (CB) as described previously by Kajihara *et al.* (2000), Labori *et al.* (2002) and (2009), since it is the only conjugated species of bilirubin that is commercially available.

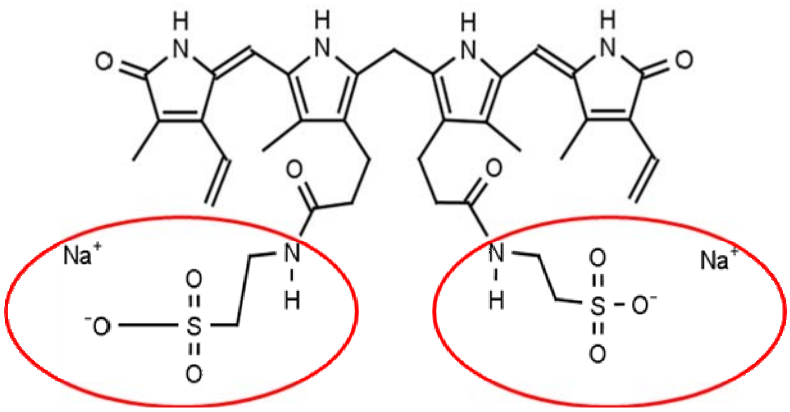

Fig. 11. Bilirubin ditaurate, resulting from the conjugation of one molecule of bilirubin with two molecules of taurine amide sodium salt (red circles). Adapted from http://www.frontiersci.com/detail.php?FSIcat=B850.

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The lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Roche Molecular Biochemicals (Manheim, Germany). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) was obtained from Promega (Madison, WI, USA) and the phenazine methosulfate (PMS) from Sigma Chemical Co. Nitrocellulose membrane and Hyperfilm ECL were from Amersham

Biosciences (Piscataway, NJ, USA). LumiGLO® was acquired from Cell Signalling (Beverly, MA, USA). Sodium dodecyl sulphate (SDS) was acquired from VWR-Prolabo. Acrylamide, bis-acrylamide and Tween-20 were from Merck (Darmstadt, Germany). Primary specific monoclonal antibodies were: rabbit anti-phospho-p38 MAPK (P-p38) from Cell Signaling; mouse anti-phospho-JNK1/2 (P-JNK1/2) and rabbit anti-p65 NF-κB subunit from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Depex-Polystyrene dissolved in xylene (DPX) mountant for microscopy was obtained from BDH, Laboratory Supplies, Poole, UK. Caspase-3 substrate was purchased from Calbiochem (San Diego, CA, USA). Horseradish peroxidase-labelled goat anti-rabbit IgG and anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein assay kit (for protein quantification) was from Bio-Rad Laboratories (Hercules, CA, USA). 75-cm^2 flasks as well as 6-well and 12-well tissue culture plates were from Orange Scientific (Braine-l'Alleud, Belgium).

2.2 Equipment

Axioskop® microscope was obtained from Zeiss, Germany. The phase contrast microscope, model CK2-TR, was from Olympus Optical Co. Ltd. Western blot apparatus and spectrophotometer PR 2100 were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.3. Fetal and adult hepatocyte cell culture

WRL-68, a human epithelial fetal liver cell line, was first deposited in the American Type Culture Collection, Rockville, MD (ATCC accession number CL48) by Apostolov, who also registered its patent (USA patent no. 3 935 066) in 1976. The patent states that WRL-68 cells: (i) form individually separated islands on discrete clumps when cultured in a growth medium; (ii) have a morphology closely resembling that of hepatocytes of the human liver; (iv) show increased production of glycogen in the presence of 1% glucose in the medium; (v) are capable of supporting viruses for the preparation of viral vaccines; and that (vi) their generation time is not more than 24 h.

Morphologically, WRL-68 cells present characteristics of epithelial cell shape compatible with those of liver parenchymal cells, resembling either primary culture of hepatic cells (Miyazaki, 1978; Lescoat *et al.*, 1989) or hepatic cell lines (Darlington *et al.*, 1980; Chessebeuf and Padieu, 1984; Furukawa *et al.*, 1987).

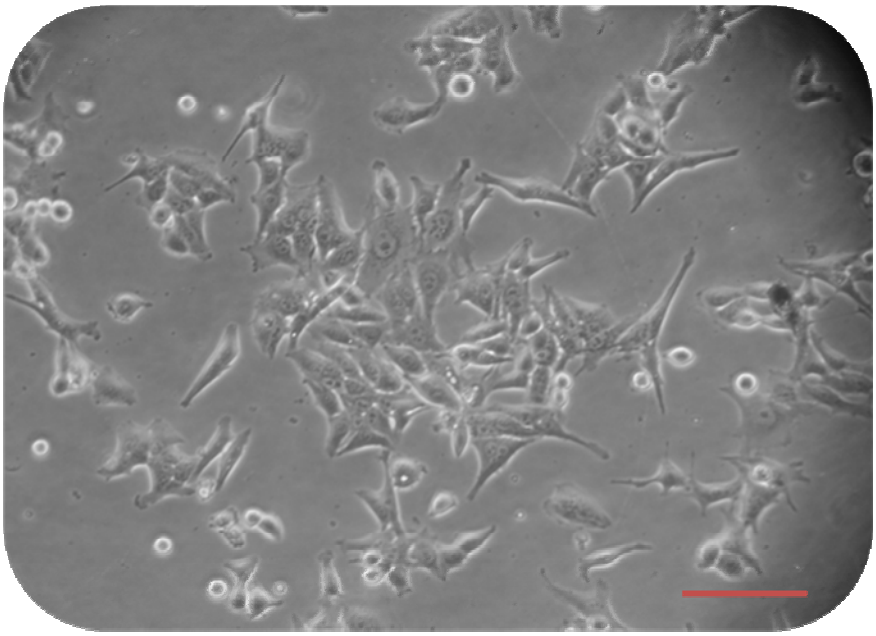

Fig. 12. Typical polygonal arrangement of the epithelial cells WRL-68 observed under a phase contrast microscope. When WRL-68 cells are seeded at low density, polygonal-to-spindle shape and some rounded cells are detected. At higher magnification these cells exhibit prominent, round, or oval-shaped nuclei containing one or more nucleoli. The cytoplasm appears to be granular and dense. They do not grow as a monolayer and began to detach before they cover the entire available surface for the culture plate. Their morphologic characteristics and epithelial cell shape are compatible with those of liver parenchymal cells. Scale bar = 50 μm.

The synthesis of liver-specific serum proteins, particularly albumin and AFP, has been the benchmark for considering whether differentiated functions in hepatocytes cultures had been preserved. Thus, WRL-68 ability to produce albumin and AFP indicates that they retain functions of normal liver parenchymal cells. In addition, the expression of AFP has been described to be also a characteristic of fetal or cancerous liver cells. In this context, WRL-68 cells have been described as a fetal cell line (Gutiérrez-Ruiz *et al.*, 1994). In resume, since WRL-68 cells maintain their fetal hepatic properties, they are considered an useful system for the study of hepatic functions and development *in vitro* (Gutiérrez-Ruiz *et al.*, 1991; Gutiérrez-Ruiz *et al.*, 1992). The WRL-68 cell line used in the present study was purchased from the European Collection of Cell Cultures (ECACC), catalogue no.89121403, lot no.04A010.

The novel non-neoplasic human adult HHL-5 cell line was produced at Dr. Arvind Patel laboratory following immortalization of primary human hepatocytes, isolated from a healthy liver, using a retrovirus vector LXSN16E6E7 based closely on Moloney's mouse leukaemia virus, expressing the human papillomavirus type 16 (HPV16) E6 and E7

oncoproteins, that are known to immortalize human epithelial cells (Clayton *et al.*, 2005). These cells retain primary hepatocyte healthy phenotype, suggesting the maintenance of a large degree of hepatic function without the presence of tumorigenic characteristics (Clayton *et al.*, 2005). In agreement, AFP, a marker associated with a fetal or tumorigenic phenotype, was either absent or expressed at low levels in the HHLs confirming its origin from a non-neoplastic tissue. HHLs also present effective contact inhibition (Clayton *et al.*, 2005), a trait lost in tumour cells. Moreover, the observation that these cells can be maintained in a monolayer status for a considerable period of time (in this case, 7 days) could indicate that they would be of value in repopulating a damaged or depleted liver, without the generation of metastatic tumours (Clayton *et al.*, 2005). We must take into account that immortalized cell lines de-differentiated somewhat in culture after many passages, resulting in expression of AFP and other tumour-related proteins (Woodworth *et al.*, 1988). However, HHLs have not shown the presence of AFP even after 80 passages (Clayton *et al.*, 2005). The HHL-5 cell line used in the present study was kindly provided by Dr. Arvind Patel from Institute of Virology, University of Glasgow, UK.

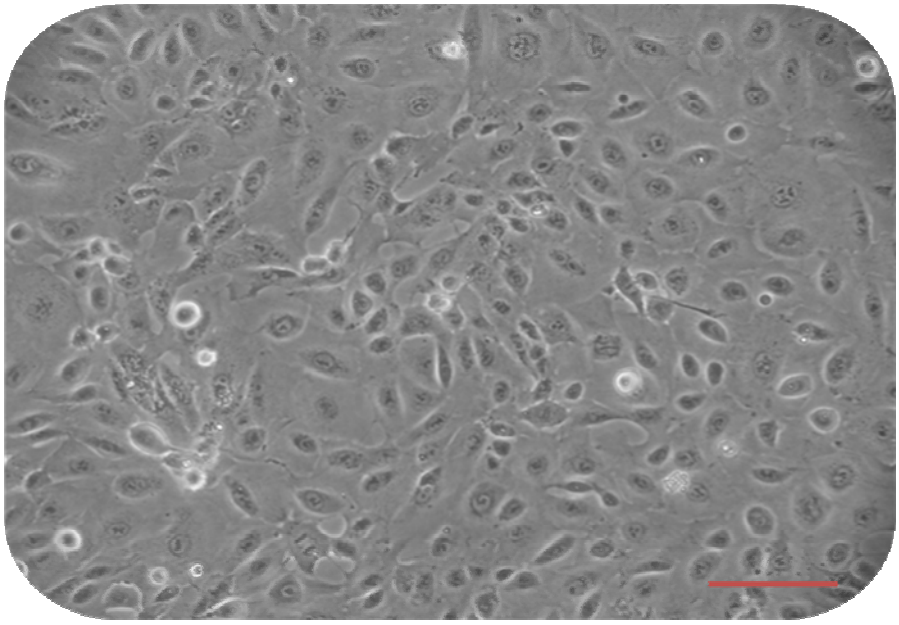

Fig. 13. The arrangement of the HHL-5 cell line was observed using a phase contrast microscope. HHL-5 morphology is compatible with those of liver cells, presenting a typical polygonal arrangement of epithelial cells but, contrary to WRL-68, this cell line grows as a monolayer. Scale bar = 50 μm.

Both cell lines were routinely subcultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% or 5% (for

WRL-68 and HHL-5, respectively) of non-essential aminoacids (NEA) and 1% antibiotic/antimycotic solution. The cells were trypsinized when cultures were sub-confluent (70-80%) using 0.5% trypsin and seeded at a density of 2.0×10^5 cells/mL on 75-cm^2 flasks. For the experimental studies, cells were seeded at a density of 1.0×10^5 cells/mL either on 6-well tissue culture plates or on glass coverslips placed in 12-well tissue culture plates and maintained at 37ºC in a humidified atmosphere of 5% CO^2 for 24 h prior to treatment. All the cells used in this work were between passages 5 and 15.

2.4. Hepatocyte treatment with bilirubin and bile acid species

Hepatocytes were stimulated with 100 μM GCDCA, 100 μM CB, 100 μM UCB, 100 μM GCDCA + 100 μM CB + 100 μM UCB or vehicle alone, in the presence of 100 μM HSA from 1 to 48 h, at 37ºC as described in Figure 14. A concentrated solution of 5 mM GCDCA was prepared in sterile phosphate-buffered saline (PBS) and appropriate dilution in the incubation medium was made. Concentrated solutions of 10 mM CB and 10 mM UCB were prepared in 0.1 M NaOH immediately before use and appropriate dilutions were made in the incubation medium restoring the pH to 7.4 by addition of equal amounts of 0.1 M HCl. All the experiments with CB and UCB were performed with light protection (vials wrapped in tin foil and dim light) to avoid photodegradation.

After treatment, cells were either: a) lysed for immunoblotting studies and caspase-3 activity assay; b) fixed for 30 min with freshly prepared 4% (w/v) paraformaldehyde in PBS for immunocytochemical studies and evaluation of apoptosis; or c) used for assessment of cell viability using the MTS test. The cell-free medium was also collected for LDH measurements.

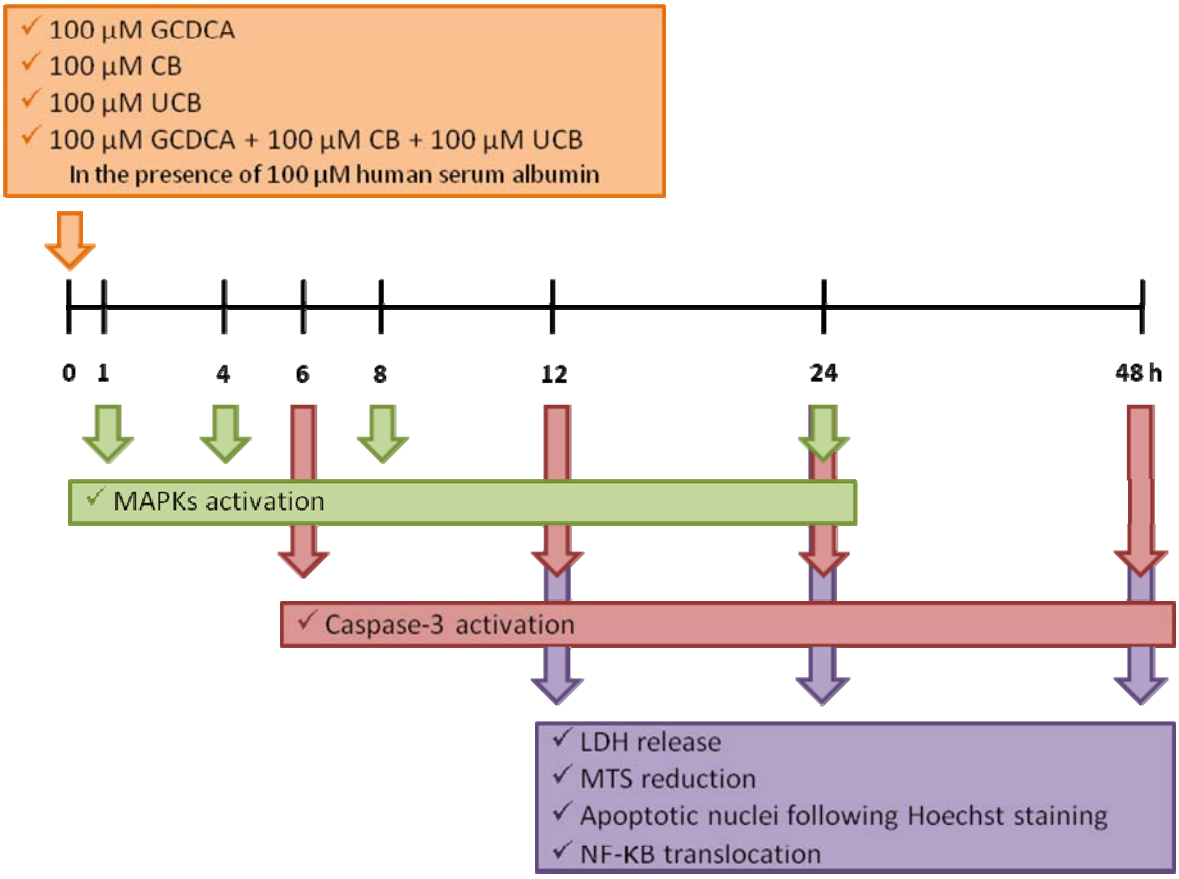

Fig. 14. Schematic representation of the experimental design. Cultured hepatocytes were incubated with 100 µM GCDCA, 100 µM CB, 100 µM UCB, 100 µM GCDCA + 100 µM CB + 100 µM UCB (all in the presence of 100 µM HSA) or vehicle alone (100 µM HSA) from 1 to 48 h, at 37ºC. After 1, 4, 8 and 24 h of treatment, MAPKs activation was evaluated by western blot assay. At 6, 12, 24 and 48 h after treatment we assessed caspase-3 activity using a specific substrate. At 12, 24 and 48 h after incubation we appraised cellular parameters as LDH release and MTS reduction. The estimation of apoptotic nuclei by Hoescht staining and evaluation of NF-κB translocation to the nucleus by immunocytochemistry was also made after 12, 24 and 48 h incubation.

2.5. Cytotoxicity evaluation

Standard evaluation of cytotoxicity was performed by measuring: (i) the lactate dehydrogenase (LDH) released by nonviable cells; (ii) the apoptotic cell death either by determining the activity of caspase-3, a known effector caspase (Samali *et al.*, 1999) or the number of apoptotic nuclei; and (iii) the ability of viable cells to reduce the MTS compound.

2.5.1. LDH release

The presence of LDH was determined in the incubation medium using the Cytotoxicity Detection kit as usual in our laboratory (Silva *et al.*, 2006). In brief, 100 µL of

incubation medium was transferred into corresponding wells of a 96-well microplate. Then 100 µL of the reaction mixture [catalyst solution (Diaphorase/NAD+ mixture) plus dye solution (iodotetrazolium chloride and sodium lactate), in a proportion of 1:45] was added to each well and the plate was incubated for 10 min at 15-25°C. At the end of the incubation the reaction was stopped by adding 50 µL per well of 1 N HCl and the absorbance measured at 490 nm with a reference wavelength of 620 nm. All readings were corrected for the possible interference of UCB and CB absorption and the results expressed as percent of LDH release, obtained by treating nonincubated cells with 2% Triton X-100 in DMEM for 30 min.

2.5.2. Apoptosis assessment

The caspase-3 activity was assayed using the caspase-3 substrate, according to the manufacturer´s instructions. In brief, following incubation, the medium was discarded and adherent cells were harvested in chilled cell lysis buffer [50 mM HEPES (pH 7.4); 100 mM NaCl; 0.1% (w/v) CHAPS; 1 mM DTT; 0.1 mM EDTA] following a 30 min incubation on ice. Cell lysate was centrifuged at 10,000×*g* for 10 min and supernatant transferred to a new eppendorf. Then, 20 µL of sample supernatant was transferred into corresponding wells of a 96 well assay plate and 80 µL of the reaction mixture containing 0.2 mM of Ac-DEVD-pNA, a specific substrate of caspase-3, in protease buffer assay [50 mM HEPES (pH 7.4); 100 mM NaCl; 0.1% (w/v) CHAPS; 10 mM DTT; 0.1 mM EDTA; 10% (v/v) glycerol] was added, and incubated at 37°C. The amount of pNA released by enzyme reaction was measured at 405 nm every 30 min until 2 h. The absorbance results obtained for each sample were normalized to protein concentration measured in cell lysate supernatant using the protein assay kit according to the manufacturers' instructions, and presented as fold change versus control.

Evaluation of hepatocytes' nuclear morphology following Hoechst staining was performed as described previously (Silva *et al*., 2001). In brief, fixed cells were incubated with Hoechst dye 33258 (5 µg/ml in PBS) for 2 min at room temperature, washed with PBS, and mounted using DPX mountant. Fluorescence was visualized using an Axioskop® microscope. Images were acquired using a digital camera, attached to the tri-ocular tube of the microscope. A minimum of 10 random microscopic fields (400×) per sample were counted (>500 cells per sample) and mean values expressed as percentage of apoptotic nuclei. Hepatocytes under apoptosis were identified by: a) marked condensation of chromatin and cytoplasm (apoptotic cells); (b) cytoplasmic fragments with or without condensed chromatin (apoptotic bodies); and (c) intra- and extracellular chromatin fragments, as previously

described by Kerr and collaborators (Kerr *et al*., 1972). Results are expressed as the percentage of apoptotic cells.

2.5.3. MTS reduction

The ability of viable cells to reduce the MTS compound was evaluated as previously described (Riss and Moravec, 1992). In brief, after the treatment, incubation media were removed and cells were incubated for 1 h, at 37°C, with 500 µL of the reaction mixture containing 0.2 mg/mL MTS plus 45 µg/mL PMS in DMEM. At the end of incubation, 100 µL of media was transferred into corresponding wells of a 96 well assay plate and absorbance measured at 490 nm. Results were expressed as percentage of control.

2.6. Western blot assay

Phosphorylation of p38 and JNK1⁄2 was analyzed following 1, 4, 8 and 24 h treatment. Total cell extracts were obtained by lysing cells in ice-cold cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na3VO4, 1 µg/mL Leupeptin, 1 mM PMSF] for 5 min on ice followed by sonication. The lysate was centrifuged at 14 000 g for 10 min at 4ºC, and the supernatants were collected and stored at -80ºC. Protein concentrations were determined using a protein assay kit, according to the manufacturer's specifications. Equal amounts of protein (50 µg) were subjected to SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were washed with Tris-buffered saline containing Tween 20 (T-TBS; 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) and blocked for 1 h at room temperature (22–25ºC) in blocking buffer [T-TBS plus 5% (w/v) non-fat dried milk]. Membranes were incubated with primary antibody overnight at 4ºC [rabbit anti-P-p38 MAPK (1:1000), mouse anti-P-JNK1/2 (1:500), or mouse anti-β-actin (1:10 000) in 5% (w/v) bovine serum albumin]. After repeated washes in T-TBS, the membranes were incubated with horseradish peroxidase-labelled secondary antibody [anti-rabbit (1:5000) and anti-mouse (1:5000) in 5% (w/v) non-fat milk], for 1 h at room temperature. Protein bands were detected by LumiGLO® and visualized by autoradiography with Hyperfilm ECL. The relative intensities of protein bands were analyzed using the Quantity One® 1-D densitometric analysis software (Bio-Rad) and results expressed as fold-change versus respective control.

2.7. Immunocytochemistry

Nuclear translocation of NF-κB was assessed following 12, 24 and 48 h treatment by NF-κB immunostaining performed as usual in our laboratory (Fernandes *et al.*, 2006). In brief, fixed cells on coverslips were permeabilized using blocking buffer [1% (w/v) BSA and 0.4% (v/v) Triton x-100 in PBS] for 1 h at room temperature and primary antibody (polyclonal rabbit anti-p65 NF-κB subunit (1:200) in blocking buffer] incubated overnight at 4°C. Cells were then incubated with FITC-labeled goat anti-rabbit antibody (1:160) as the secondary antibody for 1 h at room temperature, washed with PBS, and mounted as previously described (2.4.3). To identify the total number of cells, hepatocyte nuclei were stained with Hoechst dye 33258 as above mentioned. Fluorescence was visualized using a Leica DC 100 camera adapted to an Axioskop® microscope. Pairs of U.V. and green-fluorescence images of 10 random microscopic fields (original magnification: 400×) were acquired per sample. NF-κB-positive nuclei, was identified by localization of the NF-κB p65 subunit staining exclusively at the nucleus, and total cells were counted (~400 cells per sample) to determine the percentage of NF-κB-positive nuclei.

2.8. Statistical Analysis

Results were expressed as mean ± SEM of at least three experiments. Statistical analysis was performed by Student's *t*-test for unpaired data and the differences were considered statistically significant when $P < 0.05$.

3. RESULTS

3.1. Decreased viability of hepatocytes is induced by bilirubin and bile acid species, mainly at 48 h of incubation

As previously reported, GCDCA induce cytolysis of rat hepatocytes, releasing the lactate dehydrogenase enzyme from the cytoplasm to the surrounding media (Spivey *et al.*, 1993; Benz *et al.*, 1998; Yerushalmi *et al.*, 2001). To investigate the ability of GCDCA, CB, UCB and GCDCA+CB+UCB to induce membrane disruption in our culture of fetal (WRL-68) and adult (HHL-5) hepatocyte cell lines , cells were treated for periods of 12, 24 and 48 h, and the levels of LDH activity in the incubation media were determined. As demonstrated in Figure 15, the release of LDH by HHL-5 cells remained unchanged after 12 h and 24 h incubations, while it significantly increased after 48 h incubation but only for GCDCA+CB+UCB treatment (P<0.01). Interestingly, this value was markedly different when compared to GCDCA, CB or UCB incubation alone (P<0.05). Relatively to WRL-68 cells (Fig. 16), the release of LDH also increased significantly after 48 h incubation with GCDCA+CB+UCB when compared to control (P<0.05). Once again, these values were significant when compared to GCDCA (P<0.05) and UCB (P<0.01) incubations.

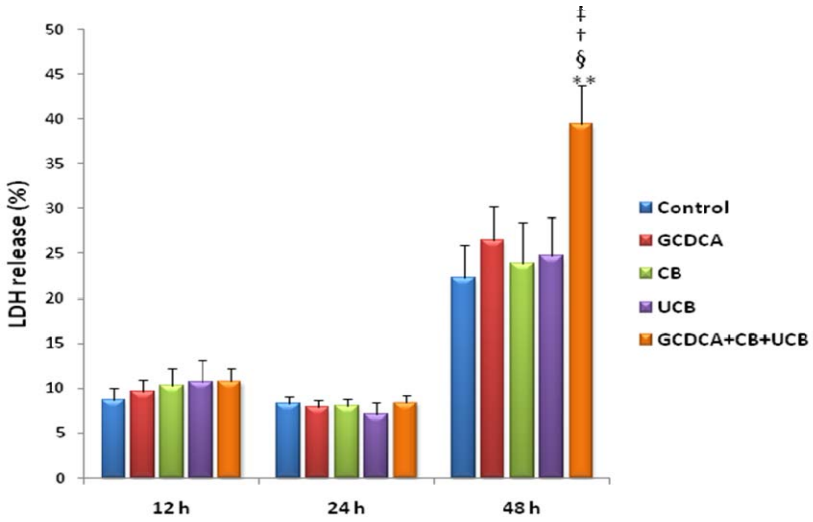

Fig. 15. Incubation of HHL-5 cells with glycochenodeoxycholic acid (GCDCA) + conjugated bilirubin (CB) + unconjugated bilirubin (UCB) induces cytolysis at 48 h. Hepatocytes were treated with 100 µM GCDCA, 100 µM CB, 100 µM UCB, 100 µM GCDCA+CB+UCB, in the presence of 100 µM human serum albumin, or vehicle alone (Control) for the indicated time periods. The incubation medium was collected for determination of released lactate dehydrogenase (LDH). Data are means ± SEM from four independent experiments. **P<0.01 *vs*. control, $^{\S}P$<0.05 *vs*. GCDCA, $^{\dagger}P$<0.05 *vs*. CB, $^{\ddagger}P$<0.05 *vs*. UCB.

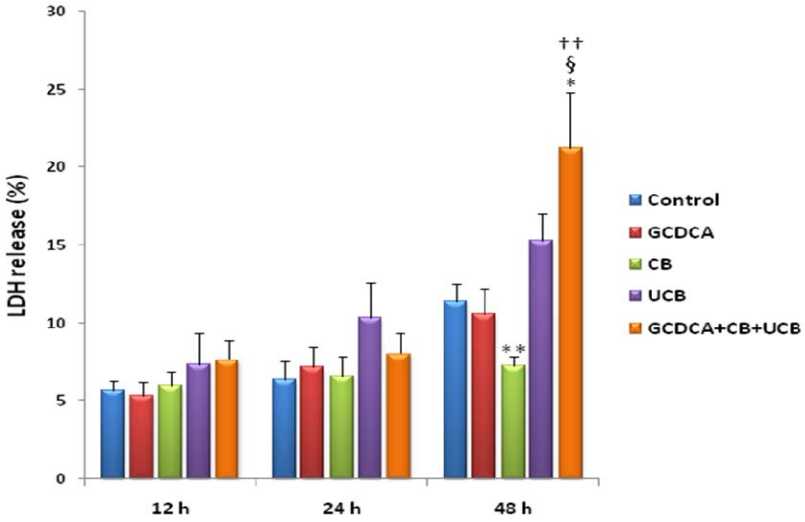

Fig. 16. Incubation of WRL-68 cells with glycochenodeoxycholic acid (GCDCA) + conjugated bilirubin (CB) + unconjugated bilirubin (UCB) induces cytolysis at 48 h. Hepatocytes were treated with 100 µM GCDCA, 100 µM CB, 100 µM UCB, 100 µM GCDCA+CB+UCB, in the presence of 100 µM human serum albumin, or vehicle alone (Control) for the indicated time periods. The incubation medium was collected for determination of released lactate dehydrogenase (LDH). Data are means ± SEM from four independent experiments. $^{**}P<0.01$ *vs*. control, $^{*}P<0.05$ *vs*. control, $^{\S}P<0.05$ *vs*. GCDCA, $^{\dagger\dagger}P<0.01$ *vs*. CB.

Comparing the two cell lines at 48 h (controls, Fig 15 and 16), HHL-5 showed an increased susceptibility to cytolysis. In addition, all treatments induced a significantly higher LDH leakage to the media in HHL-5 cell line. By evaluating the relative answer of the two cell lines (normalizing to the controls) at 48 h upon bilirubin and bile acid species treatment (Table 2), WRL-68 showed less respond to GCDCA and CB, while slightly more vulnerable than HHL-5 to UCB and GCDCA+CB+UCB. These findings indicate that HHL-5 may be more prone to cytolysis in an obstructive jaundice and that WRL-68 may be, to some extent, more sensitive to a condition of unconjugated hyperbilirubinemia.

Table 2 – Relative answer of the two cell lines (WRL-68/HHL-5) in terms of released lactate dehydrogenase (LDH) upon 48 h treatment with bilirubin and bile acid species, alone or in association.

	WRL-68/HHL-5
GCDCA	0.78
CB	0.59
UCB	1.2
GCDCA+CB+UCB	1.1

GCDCA, glycochenodeoxycholic acid; CB, conjugated bilirubin; UCB, unconjugated bilirubin.

3.2. Apoptosis is enhanced in hepatocytes treated with bilirubin and bile acid species

3.2.1. Increase of caspase-3 activity

Based on previous studies indicating that GCDCA induces mixed features of cell death in primary cultures of rat hepatocytes (Benz *et al.*, 1998; Yerushalmi *et al.*, 2001), we next examined the occurrence of apoptosis by assessing caspase-3 activity. We investigated if GCDCA, CB, UCB or GCDCA+CB+UCB induced caspase-3 activity in our study model at periods of 6, 12, 24 and 48 h. Regarding HHL-5 cells (Fig. 17), although no increase was evident for individual treatments with GCDCA, CB or UCB, caspase-3 activity increased ~1.5-fold after GCDCA+CB+UCB exposure at 6 h (P<0.01 *vs.* control, P<0.01 *vs.* GCDCA, P<0.05 *vs.* UCB). Interestingly, this increment was magnified to ~3.5-fold at 12 h, and remained significantly elevated until 48 h. For this later time-point, it was already observed some caspase-3 activation following GCDCA, CB or UCB incubation, although without statistical significance.

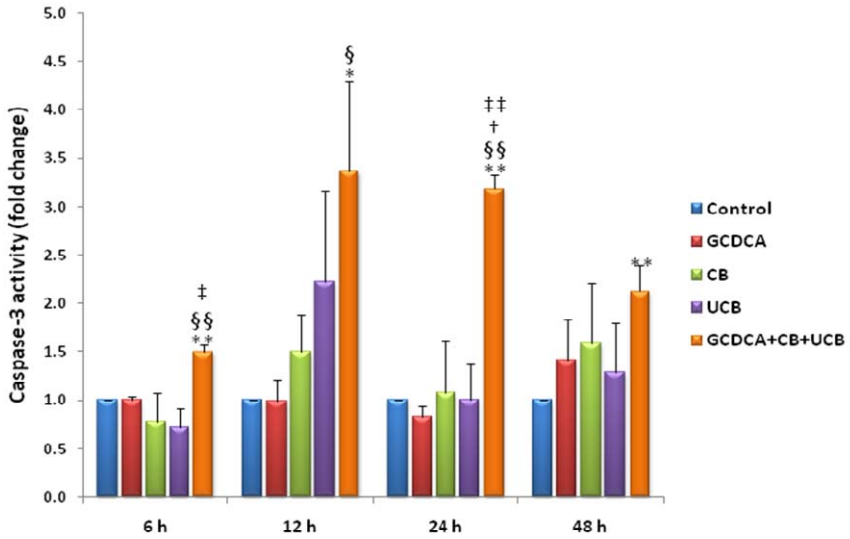

Fig. 17. Incubation of HHL-5 cells with glycochenodeoxycholic acid (GCDCA) + conjugated bilirubin (CB) + unconjugated bilirubin (UCB) increases caspase-3 activity. Hepatocytes were treated with 100 µM GCDCA, 100 µM CB, 100 µM UCB, 100 µM GCDCA+CB+UCB, plus 100 µM human serum albumin or vehicle alone (Control) for the indicated time periods. Total cell lysates were used to detect caspase-3 activity. Data are means ± SEM from five independent experiments. $^{**}P$<0.01 *vs.* control, $^{*}P$<0.05 *vs.* control, $^{\S\S}P$<0.01 *vs.* GCDCA, $^{\S}P$<0.05 *vs.* GCDCA, $^{\dagger}P$<0.05 *vs.* CB, $^{\ddagger\ddagger}P$<0.01 *vs.* UCB, $^{\ddagger}P$<0.05 *vs.* UCB.

Concerning WRL-68 cells (Fig. 18), caspase-3 activity increased 4-fold after GCDCA+CB+UCB at 6 h incubation ($P<0.05$) and this activation was sustained until 12 h incubation ($P<0.01$). In these cells, GCDCA also enhanced caspase-3 activity after 6 and 12 h after treatment, though not in a statistical significant manner. With CB and UCB incubations, caspase-3 activity also increased significantly after 12 h ($P<0.05$ and $P<0.01$, respectively). At 24 h, caspase-3 activity remained significantly high only with GCDCA-CB+UCB treatment. After 48 h of incubation, caspase-3 activity decreased in each condition.

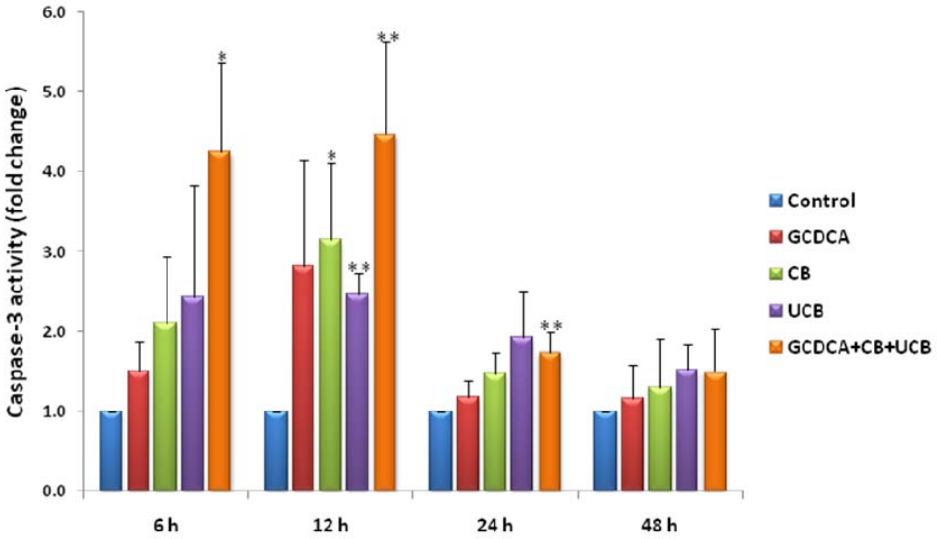

Fig. 18. Incubation of WRL-68 cells with glycochenodeoxycholic acid (GCDCA) + conjugated bilirubin (CB) + unconjugated bilirubin (UCB) increases caspase-3 activity. GCDCA+CB+UCB increases caspase-3 activity in the WRL-68 cell line, from 6 to 24 h. Hepatocytes were treated with 100 µM GCDCA, 100 µM CB, 100 µM UCB, 100 µM GCDCA+CB+UCB, plus 100 µM human serum albumin or vehicle alone (Control) for the indicated time periods. Total cell lysates were used to detect caspase-3 activity. Data are means ± SEM from five independent experiments. **$P<0.01$ *vs*. control, *$P<0.05$ *vs*. control.

Altogether, in both cell lines, co-incubation of GCDCA with CB and UCB after 6, 12 and 24 h showed a significant increase in caspase-3 activity. However, WRL-68 cell line showed globally higher answer to treatments in terms of caspase-3 activity. As shown in Table 3, WRL-68 cells demonstrated an earlier increase in caspase-3 activity namely by both bilirubin species followed by that of GCDCA+CB+UCB treatment with a peak at 6 h, and lasting at least 12 h.

Table 3 –Relative answer of the two cell lines (WRL-68/HHL-5) in terms of caspase-3 activity upon treatment with bilirubin and bile acid species alone or in association.

	WRL-68/HHL-5			
	6 h	12 h	24 h	48 h
GCDCA	1.5	2.8	1.4	0.8
CB	3.5	2.1	1.4	0.8
UCB	4.8	1.1	1.9	1.2
GCDCA+CB+UCB	1.9	1.3	0.5	0.7

GCDCA, glycochenodeoxycholic acid; CB, conjugated bilirubin; UCB, unconjugated bilirubin.

3.2.2. Increase of apoptotic features

Having verified that caspase-3 was activated, we decided to evaluate if the cells exhibited the morphologic characteristics of apoptosis. In previous studies, rat hepatocytes exposed to GCDCA underwent characteristic nuclear fragmentation and demonstrated condensed chromatin (Benz *et al.*, 1998), typical features of apoptosis. Therefore, we next examined whether CB, UCB or GCDCA+CB+UCB also exert this effect in our culture model. Fixed cells were assayed for the characteristic nuclear morphology of apoptosis using Hoechst staining. As shown in Figure 19 and 20, in both cell lines, hepatocytes incubated with GCDCA+CB+UCB exhibited profound changes in nuclear morphology, consistent with apoptosis, in a more marked way than when incubated with GCDCA, CB or UCB alone.

Quantification of apoptotic nuclei showed that, in HHL-5 cell line (Fig.19), treatment with CB and UCB increased the number of apoptotic nuclei along time (P <0.05) with a most evident effect at 12 h. Although incubation with GCDCA alone only slightly increased the percentage of apoptotic nuclei, co-treatment with CB+UCB led to ~40 % apoptotic cell death at 12 h (P<0.01). This value slightly decreased with time but remained statistically significant at 24 and 48 h (P<0.01). Interestingly, these results were also significantly higher when compared to the values of GCDCA alone (P<0.05 for 12 h and P<0.01 for 24 and 48 h), indicating an increased toxicity due to the presence of CB and UCB.

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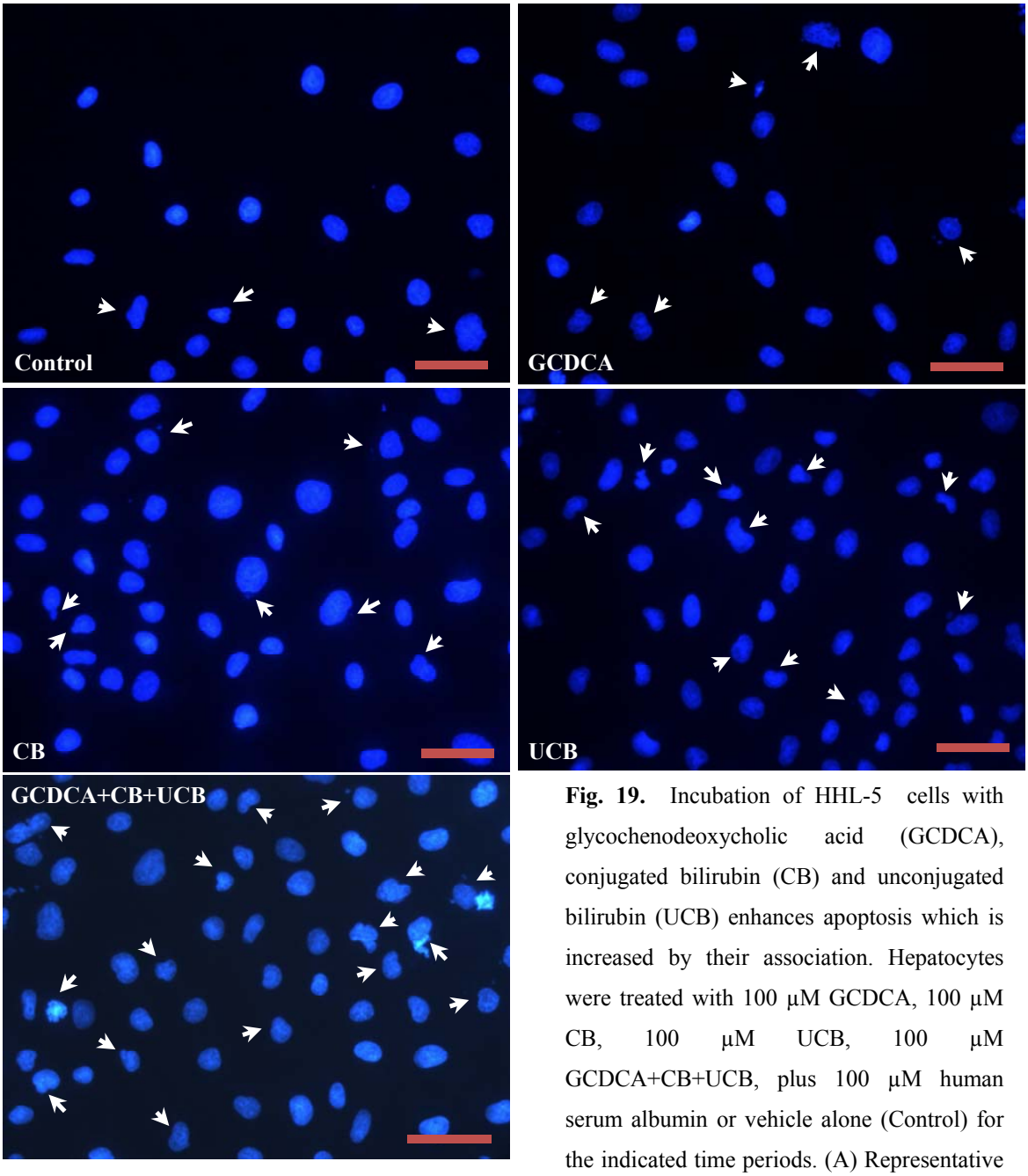

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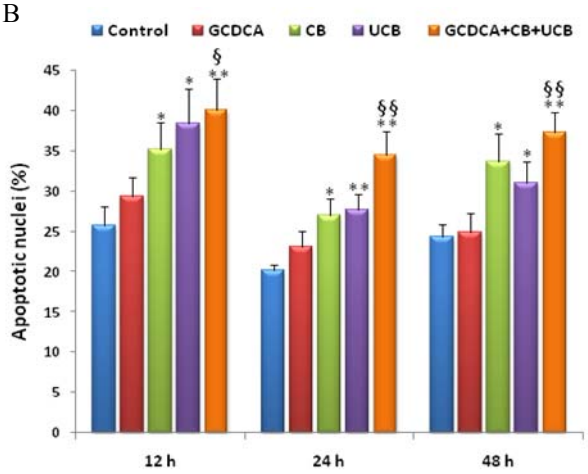

Fig. 19. Incubation of HHL-5 cells with glycochenodeoxycholic acid (GCDCA), conjugated bilirubin (CB) and unconjugated bilirubin (UCB) enhances apoptosis which is increased by their association. Hepatocytes were treated with 100 µM GCDCA, 100 µM CB, 100 µM UCB, 100 µM GCDCA+CB+UCB, plus 100 µM human serum albumin or vehicle alone (Control) for the indicated time periods. (A) Representative results of one experiment at 12 h. Hepatocyte nuclei are shown in blue following Hoechst staining and apoptotic nuclei presenting chromatin condensation and blebs are indicated (arrows). Scale bar = 50 µm. (B) The number of apoptotic nuclei was counted and expressed as percentage of the total number of nuclei. Data are means ± SEM from three independent experiments. $^{**}P<0.01$ *vs.* control, $^{*}P<0.05$ *vs.* control; $^{§§}P<0.01$ *vs.* GCDCA, $^{§}P<0.05$ *vs.* GCDCA.

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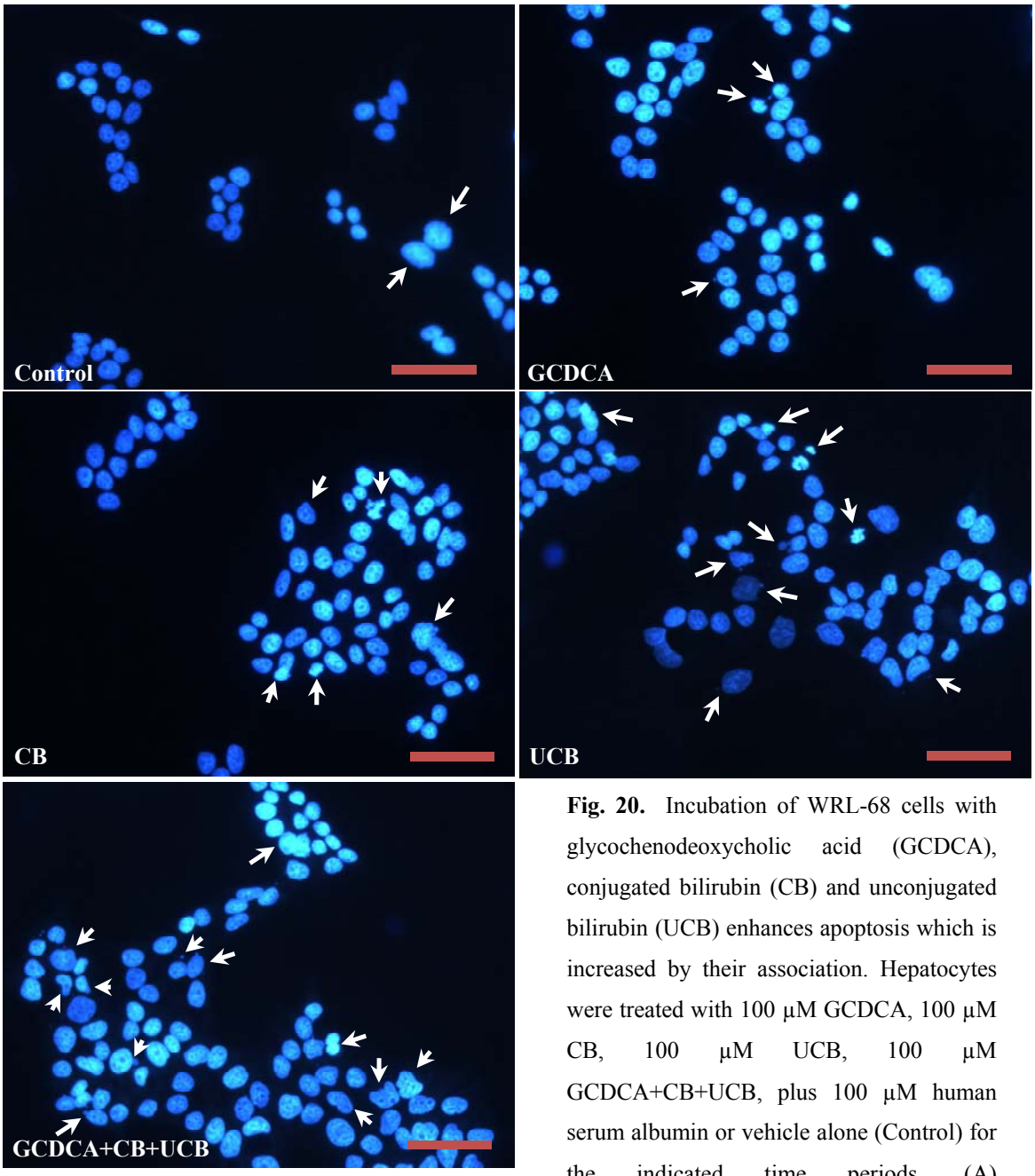

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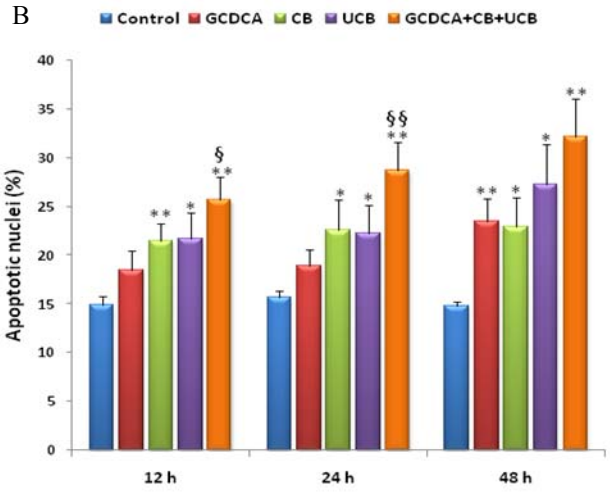

Fig. 20. Incubation of WRL-68 cells with glycochenodeoxycholic acid (GCDCA), conjugated bilirubin (CB) and unconjugated bilirubin (UCB) enhances apoptosis which is increased by their association. Hepatocytes were treated with 100 µM GCDCA, 100 µM CB, 100 µM UCB, 100 µM GCDCA+CB+UCB, plus 100 µM human serum albumin or vehicle alone (Control) for the indicated time periods. (A) Representative results of one experiment at 12 h. Hepatocyte nuclei are shown in blue following Hoechst staining and apoptotic nuclei presenting chromatin condensation and blebs are indicated (arrows). Scale bar = 50 µm. (B) The number of apoptotic nuclei was counted and expressed as percentage of the total number of nuclei. Data are means ± SEM from three independent experiments. $^{**}P<0.01$ *vs*. control, $^{*}P<0.05$ *vs*. control; $^{\S\S}P<0.01$ *vs*. GCDCA, $^{\S}P<0.05$ *vs*. GCDCA.

In WRL-68 cell line (Fig. 20), incubation with CB induced ~ 22% of apoptotic nuclei along the time (P <0.05), while UCB incubation led to ~22% (P <0.05) of apoptosis after 12 and 24 h but increased to ~27 % (P<0.05) after 48 h. Regarding GCDCA incubation, the percentage of apoptotic nuclei only increased significantly at 48 h to ~23% (P<0.01). In addition, co-treatment with GCDCA+CB+UCB induced a significant increase in the extent of apoptosis when compared to control (P <0.01) or GCDCA-treated cells (P <0.05 for 12 h and P <0.01 for 24 h), indicating once again that CB and UCB may increase the apoptosis elicited by GCDCA alone.

Comparing the two cell lines and normalizing to the controls, we observed that values for apoptotic nuclei were similar, with the exception of those for 48 h which showed to be more elevated in WRL-68 cells (Table 4).

Table 4 – Relative answer of the two cell lines (WRL-68/HHL-5) in terms of apoptotic nuclei upon 48 h treatment with bilirubin and bile acid species, alone or in association.

	WRL-68/ HHL-5
GCDCA	1.6
CB	1.1
UCB	1.5
GCDCA+CB+UCB	1.4

GCDCA, glycochenodeoxycholic acid; CB, conjugated bilirubin; UCB, unconjugated bilirubin.

Altogether, these results of cytotoxicity point out that GCDCA+CB+UCB significantly increases different types of cell death, including cytolysis and apoptosis, the last by an early caspase-3 activation followed by the presence of characteristic apoptotic nuclei. In addition, exposure to each one of these toxicants alone appears to preferentially induce apoptosis.

3.3. MTS reduction is enhanced in hepatocytes treated with bilirubin and bile acid species

We next assessed the effect of GCDCA, CB, UCB or GCDCA+CB+UCB in the hepatocyte function by using the MTS test. This test may either be used to assess cellular toxicity or cellular proliferation upon a stimulus.

In HHL-5 cell line (Fig. 21), after 12 h, either GCDCA, CB or UCB treatment increased MTS reduction (P<0.05), which was more markedly elevated following GCDCA+CB+UCB exposure (P<0.01). This effect was continuously maintained for 24 and 48 h, though less pronounced.

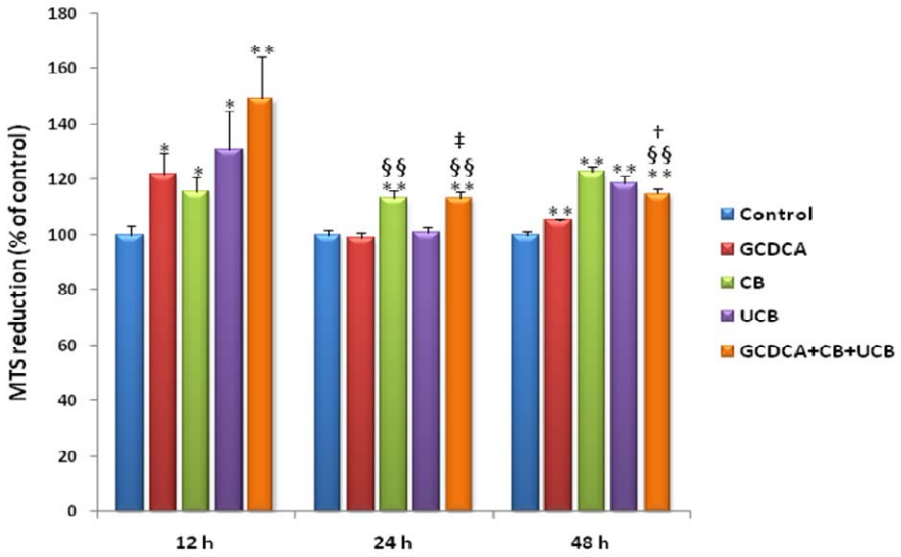

Fig. 21. Bile compounds enhance proliferation in HHL-5 cell line. Hepatocytes were treated with 100 µM glycochenodeoxycholic acid (GCDCA), 100 µM conjugated bilirubin (CB), 100 µM unconjugated bilirubin (UCB), 100 µM GCDCA+CB+UCB, plus 100 µM human serum albumin (HSA) or vehicle alone (Control) for the indicated time periods. Attached cells were used to evaluate MTS metabolism, as described in Materials and Methods. The results were expressed as percentage from control. **P<0.01 *vs*. control, *P<0.05 *vs*. control; $^{§§}P$<0.01 *vs*. GCDCA, $^{†}P$<0.05 *vs*. CB, $^{‡}P$<0.05 *vs*. UCB.

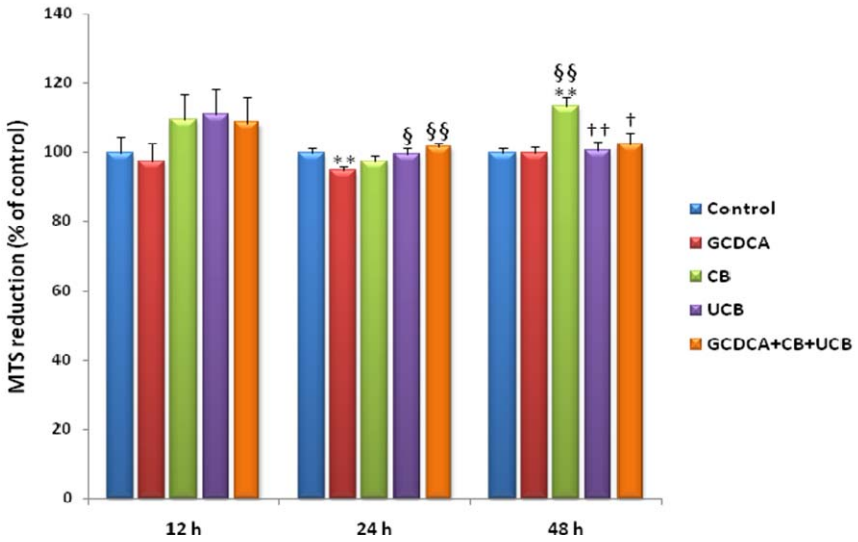

Fig. 22. Bile compounds enhance proliferation in WRL-68 cell line. Hepatocytes were treated with 100 μM glycochenodeoxycholic acid (GCDCA), 100 μM conjugated bilirubin (CB), 100 μM unconjugated bilirubin (UCB), 100 μM GCDCA+CB+UCB, plus 100 μM human serum albumin (HSA) or vehicle alone (Control) for the indicated time periods. Attached cells were used to evaluate MTS metabolism, as described in Materials and Methods. The results were expressed as percentage from control. $^{**}P<0.01$ *vs*. control, $^{§§}P<0.01$ *vs*. GCDCA, $^{§}P<0.05$ *vs*. GCDCA, $^{††}P<0.01$ *vs*. CB, $^{†}P<0.05$ *vs*. CB.

In WRL-68 cell line (Fig. 22), the effects on MTS reduction were less evident, showing only a slight increase for CB, UCB and GCDCA+CB+UCB at 12 h; and for CB at 48 h.

Collectively, it seems that HHL-5 are more susceptible than WRL-68 to GCDCA, CB or UCB-induced proliferation. It deserves to be noted that WRL-68 cells already have an increased rate of cellular proliferation in culture what may account for the results obtained. Further studies using a specific marker of cellular proliferation such as BrdU (5-bromo-2-deoxyuridine) should be used to confirm the present data.

3.4. JNK1/2 signaling pathway is activated in hepatocytes treated with bilirubin and bile acid species while p38 signaling pathway is decreased

Several studies have shown that MAPK signaling pathways play a key role in mediating the hepatocyte response during cholestasis-induced inflammation (Simpson *et al.*, 1997; Paumgartner and Beuers, 2002). Therefore, activation of MAPK cascades was assessed

in total hepatocyte lysates by western blotting using antibodies specific for the phosphorylated (activated) forms of the kinases, i.e. P-p38 and P-JNK1/2.

Surprisingly, we did not observe activation of p38 MAP kinase in HHL-5 cells, but rather a high basal activity which is rapidly inactivated after CB or UCB treatment. As shown in Fig. 23, HHL-5 hepatocytes treated with GCDCA exhibited a slight activation of p38, from 1 to 24 h. However, p38 activation was sustainably decreased with CB and UCB treatments, in every time points. With GCDCA+CB+UCB incubation, p38 activation was enhanced only after 24 h (~1.30 fold). On the other hand, GCDCA treatment did not influence JNK1/2 activation, while GCDCA+CB+UCB incubation stimulated a significant activation of JNK1/2 from 1 to 8 h, with a peak at 4 h (~1.4 fold, $P<0.05$), CB and UCB-induced activation of JNK1/2 was less pronounced. It should be noted that untreated hepatocytes already exhibited a robust activation of p38 at 4 and 8 h, whereas basal activation of JNK1/2 was barely detectable (Fig. 23.A, lane 2 and 3).

Regarding WRL-68 cell line (Fig. 24), p38 was activated, although not in a significant manner, by GCDCA after 4 h. Both CB and UCB incubation diminished the p38 activation, being this reduction statistically significant at 4 h ($P<0.05$) and 1 h ($P<0.01$) respectively. For GCDCA+CB+UCB treatment it was also observed a reduction of p38 activation, especially at 4 h ($P<0.05$). Regarding JNK1/2, while GCDCA seemed to have no major effect, CB induced JNK1/2 activation through all the time points, being this activation statiscally significant after 1h ($P<0.01$). Fetal cells also showed a sustained activation of JNK1/2 after UCB treatment, especially at 1 h ($P<0.01$). In addition, GCDCA+CB+UCB only exerted an effect in JNK1/2 activation after 24 h ($P<0.01$).

Summarizing, it seems that UCB treatment is the one element tested which make the most use of MAPKs pathways, in both cell lines and in the time points studied. In fact, maximal JNK1/2 activation occurs in the same conditions as p38 repression occurs. When UCB is highly decreasing P-p38, activation of JNK1/2 pathway is at it upmost.

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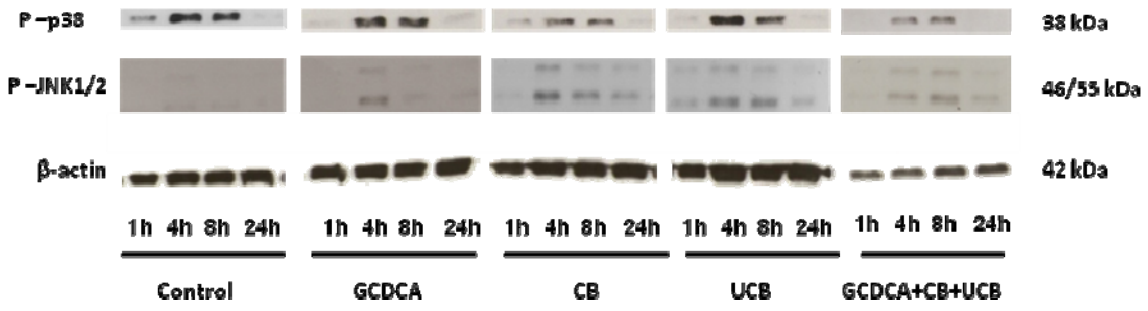

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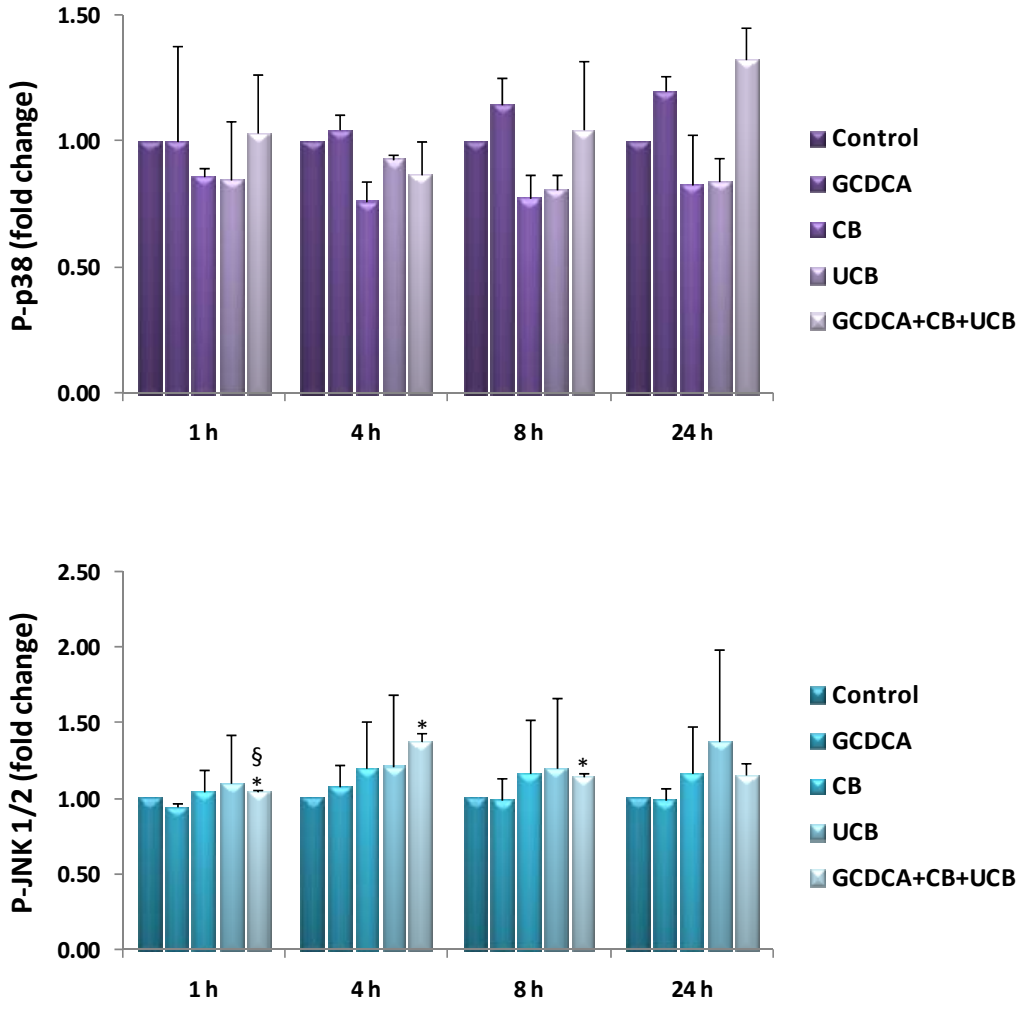

Fig. 23. MAPKs activation in HHL-5 cells after incubations. Hepatocytes were treated with 100 μM glycochenodeoxycholic acid (GCDCA), 100 μM conjugated bilirubin (CB), 100 μM unconjugated bilirubin (UCB), 100 μM GCDCA+CB+UCB, plus 100 μM human serum albumin (HSA) or vehicle alone (Control) for the indicated time periods. Total cell lysates were analysed by western blotting with antibodies specific for the phosphorylated forms of the two MAPKs, P-p38 and P-JNK1/2. (A) Representative results of one experiment are shown. Similar results were obtained in two independent experiments. (B) The intensity of the bands was quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as mean ± SEM fold change compared with untreated cells. *$P<0.05$ *vs.*control, $^{§}P<0.05$ *vs.* GCDCA.

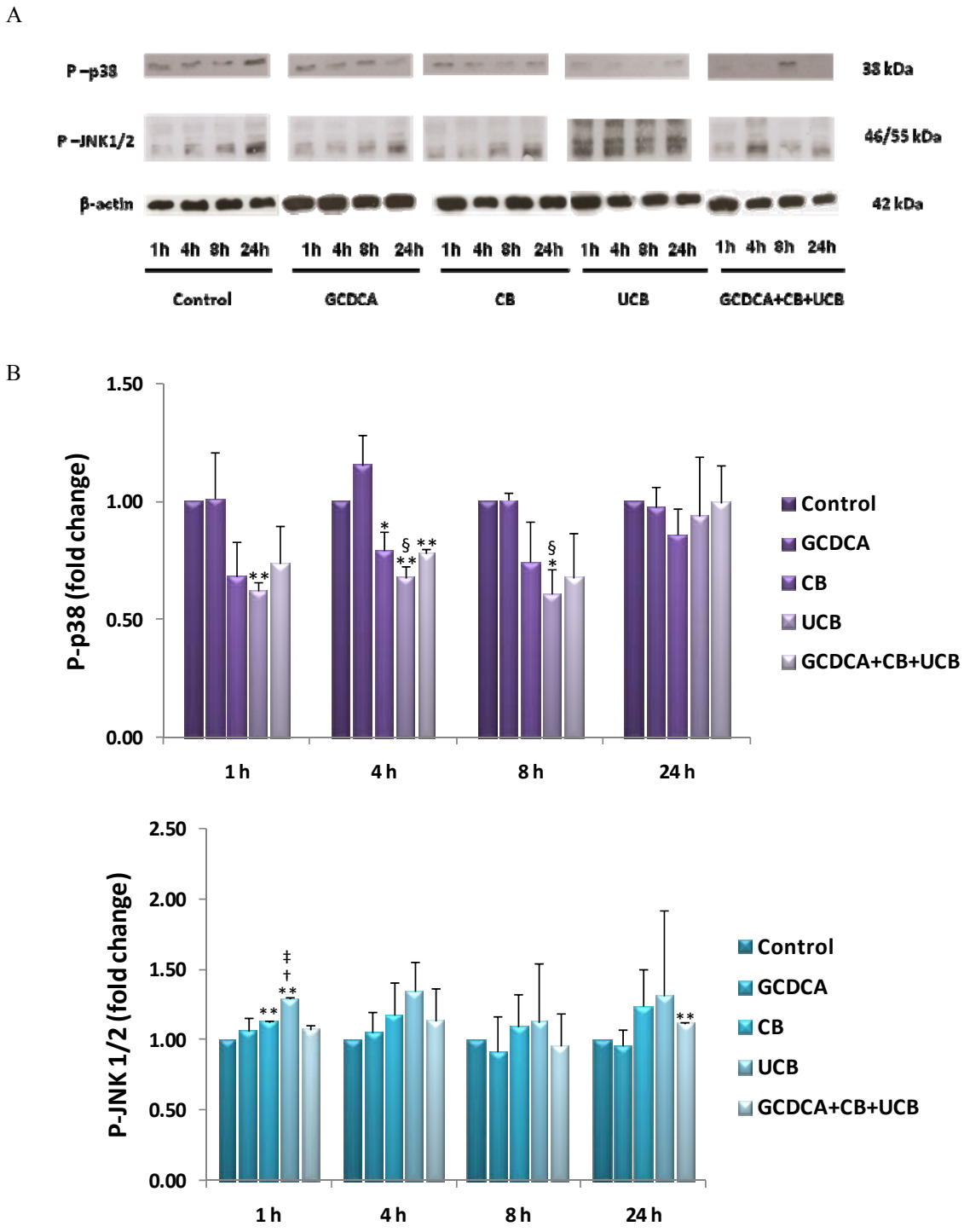

Fig. 24. MAPKs activation in WRL-68 cells after incubations. Hepatocytes were treated with 100 µM glycochenodeoxycholic acid (GCDCA), 100 µM conjugated bilirubin (CB), 100 µM unconjugated bilirubin (UCB), 100 µM GCDCA+CB+UCB, plus 100 µM human serum albumin (HSA) or vehicle alone (Control) for the indicated time periods. Total cell lysates were analysed by western blotting with antibodies specific for the phosphorylated forms of the two MAPKs, P-p38 and P-JNK1/2. (A) Representative results of one experiment are shown. Similar results were obtained in two independent experiments. (B) The intensity of the bands was quantified by scanning densitometry, standardized with respect to β-actin protein and expressed as mean ± SEM fold change compared with untreated cells. $^{**}P<0.01$ *vs.*control, $^{*}P<0.05$ *vs.*control, $^{\S}P<0.05$ *vs.* GCDCA, $^{\dagger}P<0.05$ *vs.* CB, $^{\ddagger}P<0.05$ *vs.* UCB.

3.5. NF-κB signaling pathway is activated in hepatocytes treated with bilirubin and bile acid species

There have been studies in primary rat hepatocytes demonstrating that some bile acids like GCDCA and TCDCA do not activate NF-κB (Schoemaker *et al.*, 2003), although it has been reported that NF-κB pathway is induced in cholestatic livers (Miyoshi *et al.*, 2001). Therefore, we investigated whether NF-κB is activated after GCDCA, CB, UCB or GCDCA+CB+UCB treatment in our culture model. To determine if the NF-κB signaling pathway was activated, we investigated NF-κB nuclear translocation by immunocytochemistry (Figs. 25A and 26A). In both cell lines, nuclear localization of the p65 NF-κB subunit was barely detectable in vehicle-treated cells, being located mainly in the cytoplasm.

In HHL-5 cell line (Fig. 25), the maximum nuclear detection of p65 NF-κB subunit occurred for CB incubation at 12 h (~3.5 %, $P<0.05$) decreasing thereafter (~2%, $P<0.01$ for 24 h and ~1.5%, not significant for 48 h). This profile was also observed for GCDCA+CB+UCB treatment, although with less magnitude (~3 %, $P<0.01$ for 12 h, ~1.5%, $P<0.05$ for 24 h and ~1.3%, $P<0.05$ for 48 h). In addition, also GCDCA and UCB showed to promote NF-κB activation, but presenting maximum levels at 24 h (~2%, $P<0.05$ for GCDCA and $P<0.01$ for UCB).

In WRL-68 cell line (Fig. 26), the maximum nuclear detection of p65 NF-κB subunit occurred for CB and GCDCA+CB+UCB incubations, mainly at 12 h (~2%, $P<0.05$) and 24 h (~1.7%, $P<0.05$). UCB also increased NF-κB translocation to the nucleus (~1.5 %) at 12 h decreasing thereafter. On the contrary, GCDCA decreased NF-κB positive nuclei at 12 h, although not significantly.

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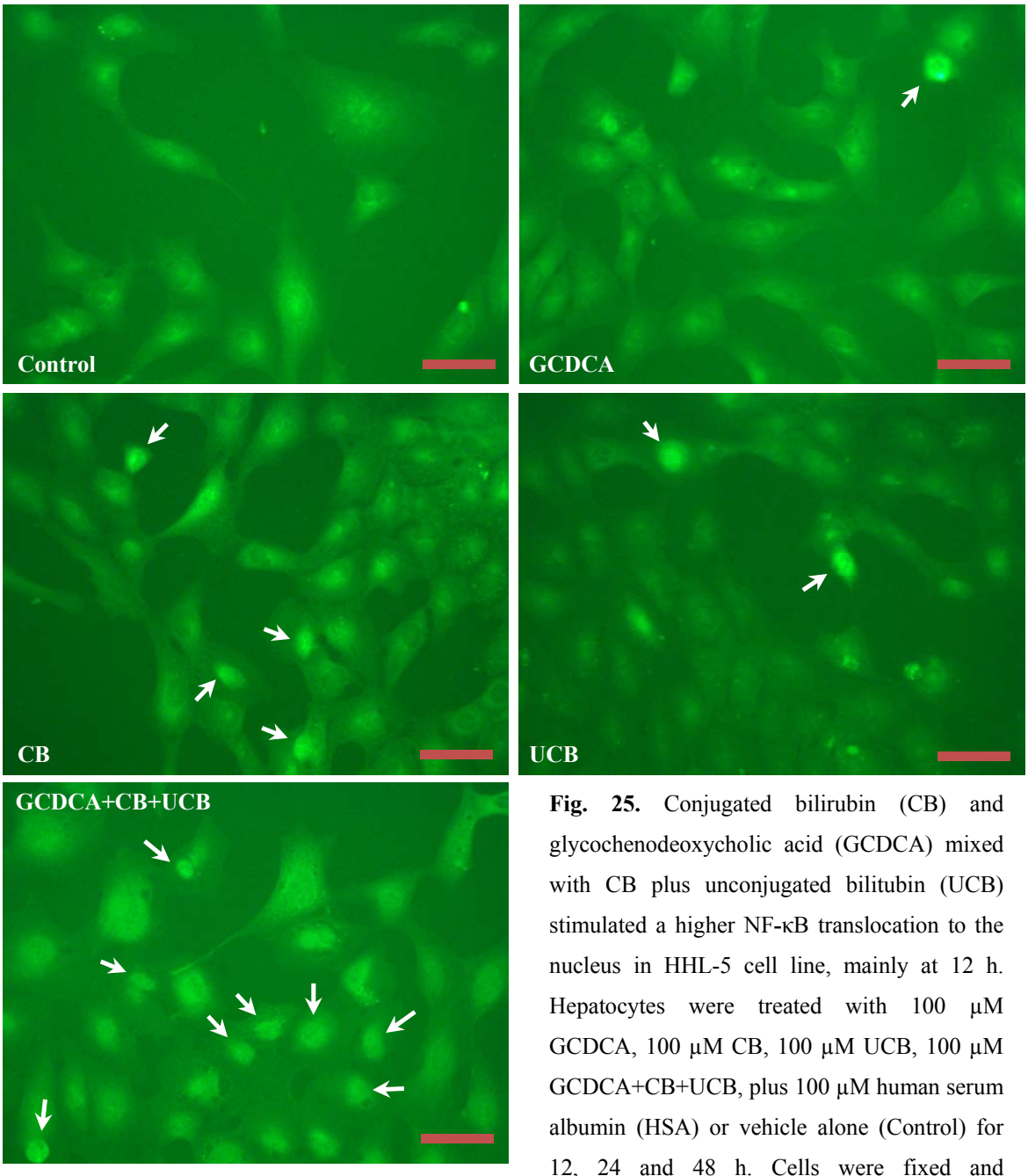

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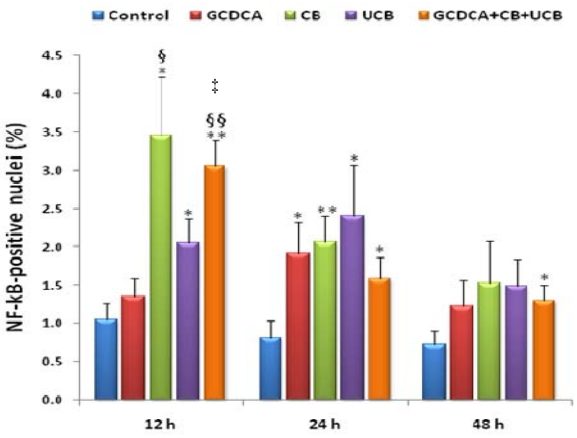

Fig. 25. Conjugated bilirubin (CB) and glycochenodeoxycholic acid (GCDCA) mixed with CB plus unconjugated bilitubin (UCB) stimulated a higher NF-κB translocation to the nucleus in HHL-5 cell line, mainly at 12 h. Hepatocytes were treated with 100 μM GCDCA, 100 μM CB, 100 μM UCB, 100 μM GCDCA+CB+UCB, plus 100 μM human serum albumin (HSA) or vehicle alone (Control) for 12, 24 and 48 h. Cells were fixed and immunostained with an antibody directed against the p65 NF-κB subunit. (A) Representative results of one experiment at 12 h are shown. (B) The number of NF-κB-positive nuclei was counted and expressed as a percentage of total nuclei. Data are means (± SEM) of two independent experiments performed in triplicate. $^{**}P<0.01$ *vs*. control, $^{*}P<0.05$ *vs*. control; $^{\S\S}P<0.01$ *vs*. GCDCA, $^{\S}P<0.05$ *vs*. GCDCA, $^{\ddagger}P<0.05$ *vs*. UCB.

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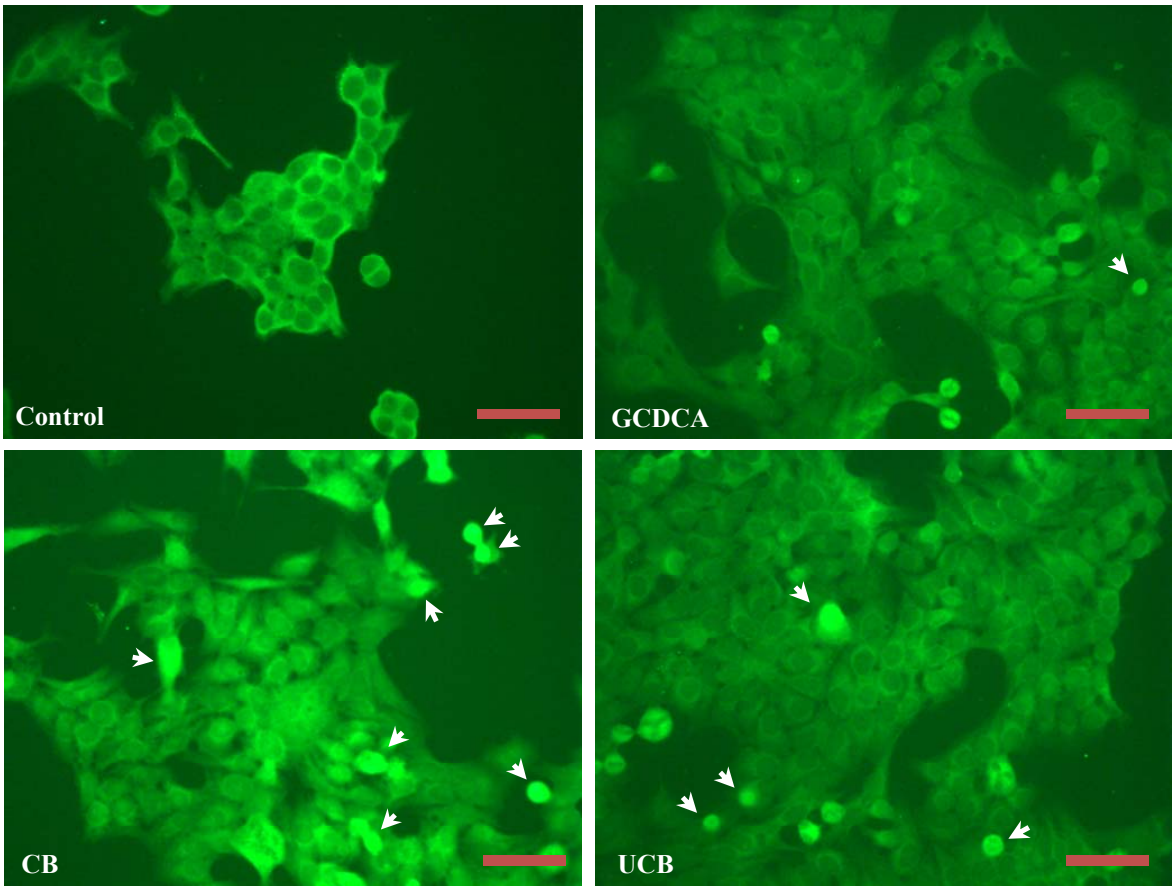

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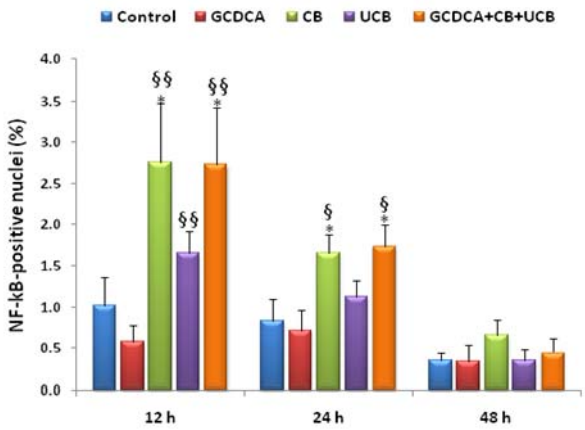

Fig. 26. Conjugated bilirubin (CB) and glycochenodeoxycholic acid (GCDCA) mixed with CB plus unconjugated bilitubin (UCB) stimulated a higher NF-κB translocation to the nucleus in WRL-68 cell line, mainly at 12 h. Hepatocytes were treated with 100 μM GCDCA, 100 μM CB, 100 μM UCB, 100 μM GCDCA+CB+UCB, plus 100 μM human serum albumin (HSA) or vehicle alone (Control) for 12, 24 and 48 h. Cells were fixed and immunostained with an antibody directed against the p65 NF-κB subunit. (A) Representative results of one experiment are shown. Scale bar = 50 μm. (B) The number of NF-κB-positive nuclei was counted and expressed as a percentage of total nuclei. Data are means (± SEM) of two independent experiments performed in triplicate. **P*<0.05 *vs*. control; §§*P*<0.01 *vs*. GCDCA, §*P*<0.05 *vs*. GCDCA.

Table 5 –Relative answer of the two cell lines (WRL-68/HHL-5) in terms of NF-κB activation upon treatment with bilirubin and bile acid species alone or in association.

	WRL-68/HHL-5		
	12 h	**24 h**	**48 h**
GCDCA	0.5	0.4	0.6
CB	0.8	0.8	0.9
UCB	0.8	0.5	0.5
GCDCA+CB+UCB	0.9	1.1	0.8

GCDCA, glycochenodeoxycholic acid; CB, conjugated bilirubin; UCB, unconjugated bilirubin.

In general, it seems that CB is the main activator of NF-κB in our study model for both HHL-5 and WRL-68 cell lines, increasing the activation of this transcription factor when cells are co-treated with GCDCA+CB+UCB. In addition, this effect appears to be more pronounced in HHL-5 cells as well as more sustained in time when compared to WRL-68 cells (Table 5).

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4. DISCUSSION

During cholestasis, the secretion of bile is reduced and the accumulation of toxic bile compounds may initiate or aggravate liver damage (Greim *et al.*, 1972). In this thesis we investigated the reactivity of hepatocytes to a condition of cholestasis associated with hyperbilirubinemia.

It is well known that the accumulation of hydrophobic bile acids, namely the GCDCA, plays a role in the induction of necrosis and apoptosis of hepatocytes in cholestatic conditions (Spivey *et al.*, 1993; Benz *et al.*, 1998; Yerushalmi *et al.*, 2001). However, nothing was known about the role of bilirubin as initiator or intensifier of liver injury during human chronic cholestatic liver diseases. Moreover, it was never explored whether fetal hepatocytes respond differently from adult ones to bilirubin and GCDCA-induced toxicity.

The present study is the first to demonstrate that bilirubin ditaurate (used as CB as previous described in Kajihara *et al.,* 2000; Labori *et al.,* 2002 and 2009), plus UCB mixed together with GCDCA have a key role in liver damage during human chronic cholestatic conditions associated with jaundice. We showed that this insult aggravates membrane disruption after 48 h in a marked manner and promotes hepatocyte apoptosis in every time point. We also verified an increased activation of JNK1/2 pathway and a decreased activation of p38 pathway, important regulatory pathways linking extracellular signals to the intracellular machinery responsible for a plethora of cellular processes. Moreover, NF-κB, a typical effector of inflammatory signaling cascades, was also triggered. Interestingly, we verified that the fetal cell line (WRL-68) had different responses to stimulation of GCDCA+CB+UCB when compared to the adult cell line (HHL-5). Indeed, WRL-68 showed less percentage of cytolysis but a higher level of caspase-3 activity when exposed to the same toxic stimuli. This cell line also demonstrated a more marked decrease in p38 pathway activity and a higher activation of JNK1/2 pathway compared to HHL-5. Interestingly, the fetal cells showed lower and less sustained NF-κB translocation to the nucleus. In general, all the stimuli resulted in an earlier reactivity of the human fetal cell line compared with the human adult one.

In severe cholestasis, serum bile acid concentrations may reach values up to 500 μM being the hepatocellular injury attributed to the direct membrane-damaging action of these compounds (Greim *et al.*, 1972). However, in most patients with cholestatic liver disease, such high bile acid concentrations are not observed and, therefore, hepatocellular damage at

lower bile acid concentrations is considered of special interest (Benz *et al.*, 1998). For that reason, we used concentrations of 100 μM GCDCA, 100 μM CB, 100 μM UCB or 100 μM GCDCA+100 μM CB+100 μM UCB, in the presence of 100 μM HSA, to mimic pathophysiological conditions in order to assess the effects of these compounds in our two models of hepatocytes.

At 48 h, the co-incubation with GCDCA+CB+UCB in equimolar concentrations significantly increased LDH release, indicating a higher percentage of cytolysis and demonstrating that the GCDCA hepatotoxic effect is highly enhanced in presence of both bilirubin species. This finding suggests that CB+UCB may contribute to hepatodegeneration caused by hydrophobic bile acids such as GCDCA, thus playing an important role in mediating liver injury during cholestatic-associated hyperbilirubinemia. The hepatocellular injury caused by GCDCA has been attributed to the direct membrane-damaging action of bile acids by acting as detergents on cell membranes (Greim *et al.*, 1972). In addition, it is possible that basic cellular mechanisms of hepatocyte injury may be primarily involved, ultimately causing cell death and if that is the case, it is likely that CB+UCB are exacerbating this effect. Curiously, LDH release was higher in the adult cell line suggesting that the fetal cell line may be less prone to cytolysis, namely by GCDCA and CB. In accordance, experiments with WRL-68 cell line showed that these cells have higher cellular viability in the presence of some toxic stimuli (*Solanum nigrum* L.) than other cell lines such as the HepG2 liver cancer cell line (Lin *et al.*, 2007).

In the present study, caspase-3, a final effector in apoptotic cell death (Samali *et al.*, 1999), showed to be increasingly activated following treatments at times far below from those at which necrosis is observed. It has been postulated that apoptosis may be an important mechanism for liver injury during cholestasis (Miyoshi *et al.*, 1999). Indeed, other studies in primary cultured rat hepatocytes confirm that short exposure to low concentrations of GCDCA is enough to induce apoptosis (Patel *et al.*, 1994; Benz *et al.*, 1998). Besides cultured rat hepatocytes, apoptosis has also been shown in freshly isolated rat hepatocytes (Gumpricht *et al.*, 2000) and hepatoma cell lines (Jones *et al.*, 1997) exposed to low concentrations of hydrophobic bile acids such as DCA (*e.g.,* 50 μM). CB+UCB showed to enhance caspase-3 activity when co-incubated with GCDCA demonstrating, once again, their additive effect in hepatotoxicity. In addition, the fetal cell line WRL-68 demonstrated a higher caspase-3 activity for shorter time points suggesting an increased vulnerability for the presence of GCDCA+CB+UCB. In fact, apoptosis is a more common process in fetal liver cells rather

than in the adult ones. As the liver differentiates from embryonic liver progenitor cells into a mature organ containing hepatocytes, cholangiocytes and immune cells, apoptosis is one important mechanism to take place and even to play a pro-survival role (Shafritz and Dabeva, 2002; Beath, 2003). In this context, apoptosis is considered an important regulatory component of development, limiting organ growth, helping in morphogenesis and directing organ shape and lobe formation (Monga *et al.*, 2003). However, excessive apoptosis can lead to hepatocellular degeneration (Daniel, 2000) and it is implicated in the pathogenesis of a number of hepatic disorders, including cholestasis (Patel and Gores, 1995). Moreover, caspase-3 activation in hepatocytes treated with GCDCA, CB or UCB, as well as GCDCA+CB+UCB decreased with prolonged incubation. These findings are similar to those of Utanohara *et al.* (2005) with GCDCA stimulation in isolated rat hepatocytes, showing that apoptosis plays a major role in the early effects of cholestasis rather than the extended ones.

Caspase-3 activity was accompanied by nuclei ultrastructural alterations, such as the formation of voluminous buds and the fragmentation of the nucleus. These modifications are viewed as the characteristic end stage of apoptotic cell death (Kwo *et al.*, 1995). GCDCA+CB+UCB induced a higher percentage of apoptotic nuclei than GCDCA, CB or UCB alone, at all times, in both cell lines. These results corroborate the data obtained from caspase-3 activity evaluation. In fact, Benz *et al.* (1998) also observed nuclear fragmentation with 100 μM GCDCA in primary rat hepatocytes. Apoptotic DNA fragmentation and morphologic signs of apoptosis were not completely absent in control cells. This might be due to a weak presence of spontaneous apoptosis, also observed in rat primary cell cultures (Benz *et al.*, 1998).

These achieved results question those obtained by Granato *et al.* (2003) reporting that, after 4 h incubation, the mixture CB+UCB seemed to protect the GCDCA-induced apoptosis. One reason for this incongruity might be the short exposure time. Indeed, even the beneficial role of UDCA on GCDCA-induced apoptosis appear to depend on the exposure time, showing to be advantageous on short exposure periods but damaging for prolonged exposure times (Utanohara *et al.*, 2005). In addition, these studies used rat primary hepatocytes which can react differently from human hepatocytes to bilirubin, since differences in susceptibility have been referred in several studies (Leung *et al*., 2005; Jo *et al*., 2001).

The data indicate that the hydrophobic bile acid GCDCA plus both species of bilirubin induce cytolysis as well as apoptosis. After exposure of HHL-5 and WRL-68 hepatocytes to toxic stimuli, the resulting type of cell damage occurs in a time-dependent manner. Long

exposure to the toxic stimuli induced significant cytolysis, whereas short exposure to the same stimuli was followed by apoptotic cell damage. It seems likely that in extended cholestasis, injury of hepatocytes is due mainly to cytolysis, whereas in acute condition apoptosis represents the predominant mechanism of cytoxicity by GCDCA+CB+UCB. This time-course of damage may represent a first attempt of the liver to repair the injury with the removal of demised cells by apoptosis without pronounced inflammation, followed by a period of persistent injury with energy depletion and consequent cytolysis (Fig. 27).

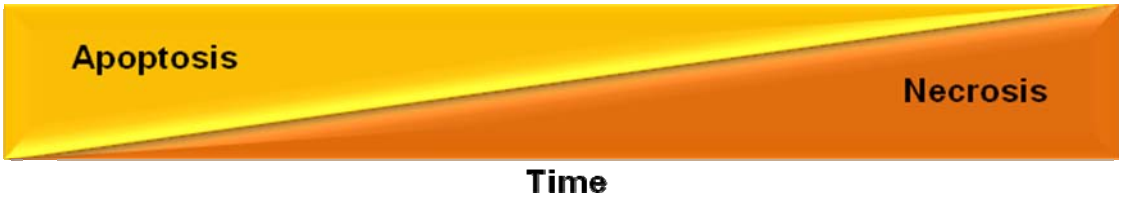

Fig. 27. Schematic representation of the cell death balance obtained in our investigation. Apoptosis prevails in an early time as the most important type of cell death in conditions of jaundice and cholestasis. When injury persists, apoptosis is followed by hepatic necrosis, which appears as the main cellular death mechanism at 48 h.

We observed that GCDCA, CB, UCB and GCDCA+CB+UCB stimulated hepatocyte proliferation, especially at 12 h. In fact, although bile acids appear to increase cell proliferation in the intestinal tract and liver (Bayerdorffer *et al.*, 1993), other studies have described a mechanism involving damage-induced regenerative response (Hofmann, 2002). Additionally, it has been shown that cytokines, namely TNF-α and IL-6 (Cressman *et al.*, 1996; Yamada *et al.*, 1997), significantly increase cell proliferation in HepG2 cell line as well as in primary hepatocytes (Brand *et al.*, 2007). Knowing that cytokines are elevated in cholestasis (Bird *et al.*, 1990; Khoruts *et al.*, 1991; Simpson *et al.*, 1997), and that preliminary data indicate an increased production of IL-6 in our study model (data not shown), we may suggest that this biomolecule may be involved in the increased proliferation observed. In addition, it is also plausible to speculate that GCDCA may be inducing an increase of the enzymatic activity of the enzymes responsible for the reduction of the MTS, and therefore an increment of MTS reduction is observed.

Regarding the MAPKs pathways, our results showed a sustained activation of JNK1/2. Indeed, previous studies have argued that toxic stress use the JNK1/2 pathway in the hepatocyte cell death process (Qiao *et al.*, 2003). The fetal cell line seemed to have a higher activation of this MAPK. This fact may be due to JNK1/2 pathway important role in

proliferation (Hui *et al.*, 2007). However, other studies have linked JNK1/2 pathway signaling to increase apoptosis, which in our model is also greater in the fetal cell line, although more recent studies have suggested that JNK1/2 signaling may have both pro- and anti-apoptotic signaling effects (Brenner, 1998; Roulston *et al.*, 1998; Liedtke *et al.*, 2002). In fact, studies with DCA stimulation in primary rodent hepatocytes demonstrated that loss of JNK2 function enhanced DCA-induced apoptosis while loss of JNK1 function suppressed DCA-induced apoptosis (Qiao *et al.*, 2003). Therefore, the pathway of JNK1/2 may have dissimilar roles in hepatocytes.

Interestingly, p38 presented diminished activation after bilirubin and bile acid species treatment in our study model. Actually, in other investigations, p38 activity was reported to be constitutively active in the liver and down-regulated following oxidative stress (Mendelson *et al.*, 1996). It has been demonstrated that oxidative stress plays an important role in cholestasis (Poli, 2000; Jaeschke *et al.*, 2002; Sokol *et al.*, 2006; Perez *et al.*, 2008; Perez and Briz, 2009). Reinehr *et al.* (2004) have demonstrated that bile acids induce an almost instantaneous oxidative stress response, which triggers JNK1/2 activation and may in turn activate apoptosis. Additionally, as UCB also induces oxidative stress in other cellular models (Brito *et al.*, 2004), it is reasonable to hypothesize that UCB may also be inducing oxidative stress in this situation. P-p38 repression appeared more marked in the fetal cell line. In fact, studies performed in human placenta from pregnancies complicated by HELLP (a gestational illness that stands for hemolysis, elevated liver enzymes and low platelets count and is frequently mistaken by hepatitis) showed that the expression of MAPK p38α (an isomer of p38) was significantly decreased compared to the group with normal pregnancies (Corradetti *et al.*, 2009). Enhanced or persistent p38 activity may result in the high levels of liver cell apoptosis observed in double mutant embryos (Hui *et al.*, 2007). If this is the case, since we observed a decreased phosphorylated p38, we may speculate that a cell decision survival was undertaken.

Crosstalk between different signaling pathways may also influence the kinetics of MAPKs cascade and, consequently, its effect on cell fate (Whitmarsh *et al.*, 1995; Raingeaud *et al.*, 1996). Investigations from other groups proposed that the p38 pathway may work as a negative regulator of the JNK pathway activity (Hui *et al.*, 2007). Hence, it is tempting to speculate that since in the present study P-p38 is reduced, its inhibitory effect over JNK1/2 activation is overwhelmed and an increment of P-JNK1/2 may occur as it was found.

It has been described that exposure of HepG2 hepatoma cell line to bile acids enhances both p38 and JNK1/2 (Nonaka *et al.*, 2008). However, the role of MAPKs in proliferating and

transformed cancer cell lines is certainly different from that in healthy, non-neoplasic cell lines, as it is different in non-transformed primary human hepatocytes (Dhillon *et al.*, 2007). Hence, our findings have totally different implications than the results in HepG2 cell line such as the pathophysiologic role of MAPKs in non-neoplasic human cell lines.

Interestingly, it has been shown that phosphorylated kinases mostly localize in proliferating cells involved in tissue repair (hepatocytes and HSC), and have been associated with tissue remodeling (Svegliati-Baroni *et al.*, 2003). Most attractive is the fact that activation of these pathways in HSC precedes their transformation in myofibroblast-like cells and collagen deposition, with consequent hepatic fibrosis. Therefore, we may hypothesize that the early activation of JNK1/2 by bilirubin and bile acid species may represent in an acute condition a reaction of the hepatocyte following cell death to initiate tissue reparation. Moreover, the selective targeting of kinase inhibitors may be considered a good approach for the prevention of the excessive deposition of extracellular matrix components and liver fibrosis.

MAPK pathways mediate inflammatory responses possibly through the downstream activation of transcription factors such as NF-κB (Kyriakis and Avruch, 2001) that translocate to the nucleus and positively regulate the induction of inflammatory genes (Tak and Firestein, 2001). We observed that CB is the main inducer of NF-κB translocation to the nucleus. It has been demonstrated that NF-κB activation in cholestasis may operate not only to reduce hepatocyte apoptosis, but also to reduce overall liver injury (Miyoshi *et al.*, 2001). Contradictory results were obtained by others for NF-κB translocation to the nucleus in two different studies, one pointing out no significant activation in rat primary hepatocytes (Schoemaker *et al.*, 2003), and the other revealing an activation of NF-κB in mice with induced cholestatic liver disease (Miyoshi *et al.*, 2001). Therefore, we can presume that, in our two human cell line models, CB does not activate apoptosis in the same extent as UCB possibly due to the activation of NF-κB that potentially induces transcription of survival genes and protect hepatocytes. Moreover, higher NF-κB activation in the adult cell line may also indicate less susceptibility to cell death by apoptosis.

Labori *et al.* (2009) demonstrated that, in pig liver, bilirubin ditaurate does not induce canalicular membrane damage but UCB does, showing that UCB may be more toxic than CB. In our study, caspase-3 activation was higher with UCB incubation than with CB alone, mainly in the fetal cell line. In general, apoptotic features were also increased with UCB treatment compared to the CB incubation. Additionally, NF-κB was more present in the

nucleus with the CB incubation rather than the UCB treatment, which can be exerting some pro-survival effect.

Nishimura *et al.* (1985) refers to bilirubins as the major factor of harm on the hepatic function during biliary obstruction. In fact, our results indicate that hepatocytes are more susceptible to injury when GCDCA is associated with bilirubin species, revealing higher cell death and MAPKs activation.

Most attractive is the future use of human cell lines as a gold standard to evaluate toxic compounds and drug therapeutical efficacy, as well as for the temporary metabolic support of patients awaiting liver transplantation or spontaneous reversion of their liver disease. In fact, hepatocyte transplantation has already been used to correct metabolic defects and to provide metabolic support in experimental animal models of hepatic failure (Demetriou *et al.*, 1986a; Demetriou *et al.*, 1986b; Arkadopoulos *et al.*, 1998a; Arkadopoulos *et al.*, 1998b). In addition, hyperbilirubinemia of rats genetically deficient in UDP-glucuronosyl transferase, the enzyme responsible for UCB conjugation, was corrected by hepatocyte transplantation (Demetriou *et al.*, 1986b). Therefore, all the data obtained in cell lines, namely the fetal cell lines with stem cell potential (Kakinuma *et al.*, 2009a), may give us important knowledge for the use of this simple technique in the treatment of liver failure in humans.

Collectively, the present data evidence that jaundice in conditions that mimic chronic cholestasis increases hepatocyte reactivity, apoptosis and necrosis. In addition, it also demonstrates that the fetal cell line response is similar to the adult one, although it presents a faster and more obvious cellular response, reflecting an increased reactivity. This study provides an insight into possible routes of hepatoxicity activated upon exposure to jaundice associated with cholestasis, and may ultimately prove useful in the development of new therapeutic strategies to prevent the adverse outcomes observed following this condition, which have decisive impact on the quality of life of the patients and their families.

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