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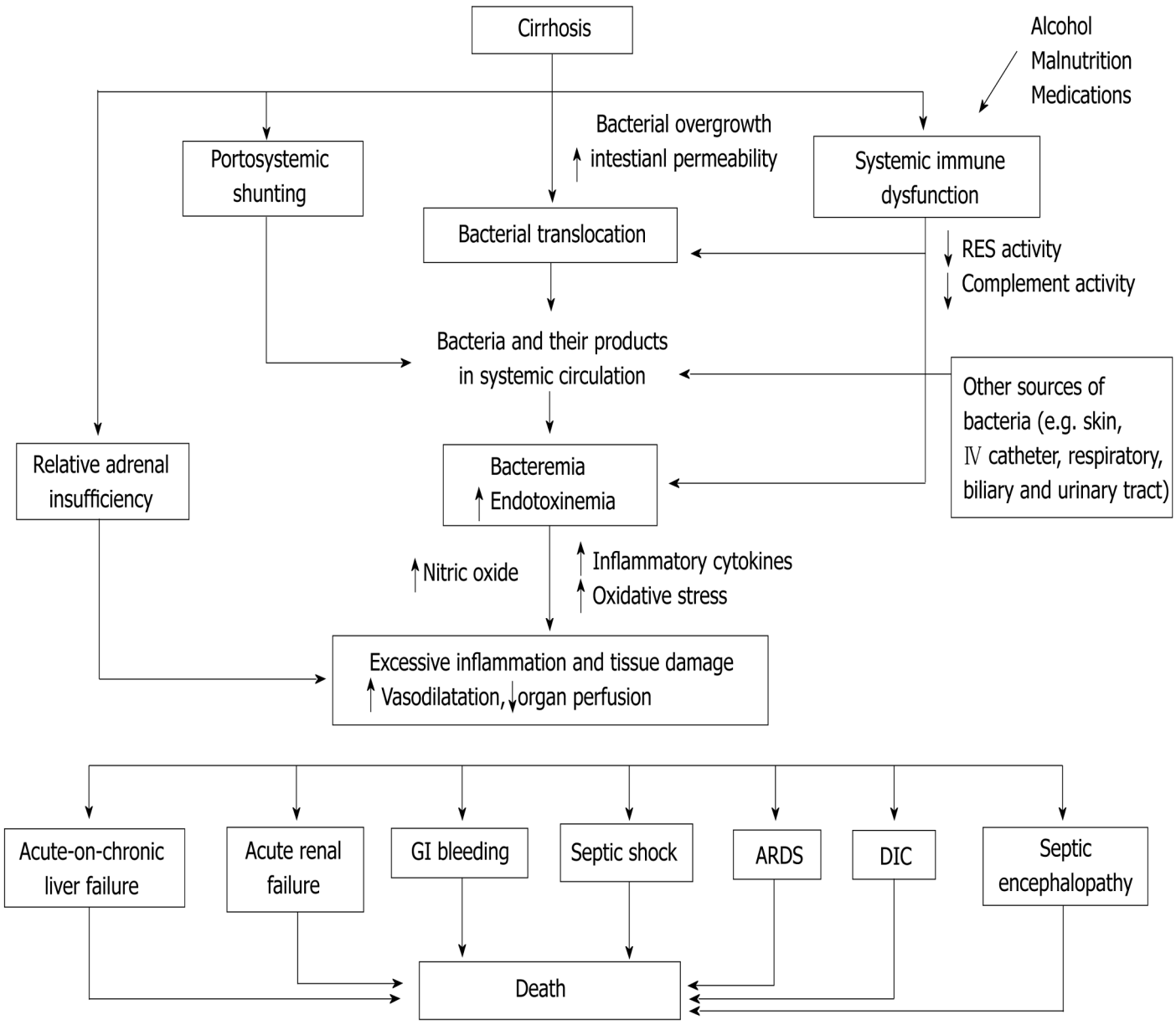

Short-term motality: about 20% without organ failure;
30%-50% with 1 organ failure; 55%-100% with > 1 organ failure.

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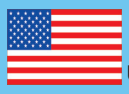

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REVIEW

Bacterial infections other than spontaneous bacterial peritonitis in cirrhosis

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Abstract

Cirrhotic patients are immunocompromised with a high risk of infection. Proinflammatory cytokines and hemodynamic circulation derangement further facilitate the development of serious consequences of infections. Other than spontaneous bacterial peritonitis, bacteremia and bacterial infections of other organ systems are frequently observed. Gram-negative enteric bacteria are the most common causative organism. Other bacterial infections, such as *enterococci*, *Vibrio spp.*, *Aeromonas spp.*, *Clostridium spp.*, *Listeria monocytogenes*, *Plesiomonas shigelloides* and *Mycobacterium tuberculosis* are more prevalent and more virulent. Generally, intravenous third generation cephalosporins are recommended as empirical antibiotic therapy. Increased incidences of gram-positive and drug-resistant organisms have been reported, particularly in hospital-acquired infections and in patients receiving quinolones prophylaxis. This review focuses upon epidemiology, microbiology, clinical features and treatment of infections in cirrhosis other than spontaneous bacterial peritonitis, including pathogen-specific and liver disease-specific issues.

Key words: Bacterial infection; Cirrhosis

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INTRODUCTION

Despite the advancement in medical care for patients with advanced liver disease in the past decades, bacterial infections remain very common and account for significant morbidity and mortality (approximately 30%) in these patients[1,2]. Cirrhosis is an immunocompromised state which predisposes the patient to a variety of infections[3]. Once infection occurs, the proinflammatory cytokines and hemodynamic circulation derangement further facilitate the development of serious consequences of infections such as septic shock, multiple organ failure and death[3]. Bacterial infections are commonly caused by gram-negative enteric bacteria; however, a number of uncommon pathogens are also more frequently observed and more virulent in cirrhotic patients. Moreover, these pathogens can present with various clinical syndromes which may be difficult to recognize. Appropriate preventive measurements have been shown to reduce the risk of overall bacterial infections. Early recognition and prompt

management are warranted in order to minimize their complications. The outline of bacterial infection in cirrhotic patients is shown in Figure 1.

EPIDEMIOLOGY

Bacterial infection is responsible for approximately 30%-50% of deaths in cirrhotic patients[1,3-5]. Compared to a 5%-7% infection rate reported in hospitalized patients in general, those hospitalized with cirrhosis have an infection rate of 32%-34%[6,7] and which may be up to 45% in those with gastrointestinal bleeding[8]. The most common bacterial infections are spontaneous bacterial peritonitis (SBP) (25%-31%), urinary tract infection (UTI) (20%-25%), pneumonia (15%-21%), bacteremia (12%) and soft tissue infection (11%)[7,9]. Approximately 75% of bacterial infections in patients with cirrhosis are caused by gram-negative bacteria, e.g. *Escherichia coli, Klebsiella spp.*, *Enterobacter spp.*, *P. aeruginosa, Vibrio spp.*, *Aeromonas spp.*, whereas gram positive comprise 20.2% and anaerobes only 3.2%[10]. However, in cirrhotic patients who had been hospitalized, received quinolones prophylaxis and had invasive procedures, the risk of gram-positive organisms is more frequently encountered (38%-70%)[7,11,12]. In addition, resistant organisms are isolated in up to 64% of hospital-acquired infection and are associated with poor outcomes[12].

PATHOGENESIS AND CONSEQUENCES OF INFECTION IN CIRRHOSIS

State of immune dysfunction in cirrhosis

Cirrhotic patients are in a multifactorial state of local and systemic immune dysfunction[3]. Porto-systemic shunting allows less bacteria and endotoxins to be cleared by the liver from the portal circulation[1]. Systemic reticuloendothelial system function is also significantly impaired[1,3,13]. Cirrhosis is associated with a decrease in bactericidal activity of phagocytic cells, an impaired opsonic activity and a reduction in complement and protein C levels[1,3,13]. In addition, immunodeficiency state can be further complicated by compelling factors such as skin/mucosal problems, malnutrition, alcohol intake and immunomodulatory therapy (Table 1).

Bacterial translocation

Bacterial translocation is defined as the migration of bacteria or bacterial products from the intestinal lumen to mesenteric lymph nodes and other extra-intestinal sites. It has been implicated as the key step in the pathogenesis of SBP and spontaneous bacteremia in cirrhotic patients. The mechanisms of bacterial translocation are complex and not yet completely understood. Immune dysfunction, intestinal bacterial overgrowth and altered intestinal permeability are hypothesized to contribute to the development of bacterial translocation[14]. Gram-negative enteric bacteria, enterococci and other streptococci have been reported to be the most adept at translocating to mesenteric lymph nodes. More recently, it has been linked to the development of the hyperdynamic circulatory state in cirrhosis, characterized by splanchnic and systemic vasodilatation, increased cardiac output and decreased arterial blood pressure[1]. An amplification of bacteria and their products can lead to activation of monocytes, lymphocytes and pro-inflammatory cytokines, which exacerbate the pre-existing hyperdynamic circulation in cirrhosis[1,14].

Systemic inflammatory response syndrome and sepsis in cirrhosis

Cirrhotic patients are prone to develop sepsis, septic shock, sepsis-induced organ failure and death[3,15]. In cirrhosis, bacterial infection is accompanied by an imbalanced cytokine response, which converts responses that are normally helpful against infections into excessive, detrimental inflammation[3,15]. The pathophysiology of the exaggerating inflammatory response in cirrhotic patients has been postulated. In the early stage of sepsis, bacteria and their products, particularly lipopolysaccharides, activate toll-like receptor-4, which induces the release of pro-inflammatory cytokines[5,15]. Nitric oxide (NO), a key mediator contributing to a circulation compromised in septic patients, is markedly released in infected cirrhotic patients[5,15].

A pre-existing hyperdynamic circulatory state predisposes devastating consequences from a sepsis-induced NO and cytokine storm which eventually leads to refractory hypotension, inadequate tissue perfusion, multiorgan failure and death[1,5,15]. Additional factors, such as relative adrenal insufficiency[16], beta-blockers[17], low levels of protein C and high-density lipoprotein, may further adversely complicate the course of sepsis in cirrhosis[3].

Clinical consequences and prognosis of infections in cirrhosis

Bacterial infection in cirrhotic patients is associated with poor clinical outcomes (up to 4-fold mortality)[2]. The mortality rate of sepsis in cirrhotic patients is approximately 26%-44%[2,13].

A recent analytical review of 11 987 cirrhotic patients suggested several clinical predictors of death after infection, such as advanced liver disease, presence of shock and/or organ failure (particularly kidneys), gastrointestinal bleeding, encephalopathy, hepatocellular carcinoma and nosocomial acquisition[2]. Patients who survived a significant episode of infection are still at high risk of death (up to 30%) within 1 year[2].

Acute renal dysfunction following infections has been observed in 27%-34% of patients with advanced cirrhosis[18-20]. Thus, it is a strong independent risk of death in these patients with a 40%-50% mortality rate[2,19,20]. Several risk factors for the development of renal failure in cirrhotic patients with bacterial infections include advanced liver disease[19-21], pre-existing renal insufficiency[21], inadequate circulatory volume[19], low baseline cardiac output[22], lack of resolution of infection[20] and not receiving early albumin infusion[18]. Renal failure that does not respond to albumin infusion in the setting of

Table 1 Immune dysfunction in cirrhotic patients

Natural barriers	Fragile, thin and/or edematous skin
	Alteration of gastrointestinal motility, mucosal permeability and bacterial flora
	↑ Gastrointestinal mucosal ulcerations
Hepatic RES activity	Portosystemic shunting
	Kupffer cells – ↓ number, impaired function
Cellular defense mechanisms	RES – ↓ activation, ↓ chemotaxis, ↓ phagocytosis, ↓ production of pro-inflammatory cytokines
	PMN – ↓ lifespan, ↓ intracellular killing activity, ↓ phagocytosis, ↓ chemotaxis
Serum factors	↓ Complement levels (C3, C4, CH50)
	↓ Opsonic activity
	↓ Protein C activity
Iatrogenic and treatment-related factors	Invasive procedure and catheters
	Medications: immunosuppressive agents, proton pump inhibitors
Other compelling factors	Malnutrition
	Alcohol drinking

RES: Reticuloendothelial system; PMN: Polymorphonuclear neutrophil.

bacterial infection without septic shock was recently considered hepatorenal syndrome (HRS)[23]. Sepsis-related renal failure and HRS can persistently progress despite the resolution of infection, thus needing further special interventions[18].

Bacterial infections can precipitate a rapid deterioration of liver functions and encephalopathy which is associated with poor short-term prognosis[1,15]. Pulmonary complications are increasingly common in cirrhotic patients. Acute respiratory distress syndrome may develop as a result of exaggerated systemic inflammatory response syndrome (SIRS) in severe sepsis which leads to higher mortality[24]. Aspiration is common in encephalopathic patients. Prognosis of cirrhotic patients who were intubated were dismal, with a 33%-60% mortality rate[25].

The effects of sepsis on coagulation cascades are more complex in cirrhosis. Patients with advanced cirrhosis are associated with thrombocytopenia and low clotting factors (e.g. factor V, Ⅶ, X and prothrombin). The consumption of coagulation factors and the enhanced fibrinolytic activity by sepsis-induced inflammatory cytokines leads to a further worsening of pre-existing coagulation and platelet abnormalities[5,15,26]. Presence of bacterial infection in patients with variceal bleeding is independently associated with failure to control and early recurrent bleeding[27]. Antibiotic prophylaxis in cirrhotic patients with variceal hemorrhage decreases infections, rebleeding and mortality[28] (Figure 2).

ORGAN-SPECIFIC INFECTIONS

Urinary tract infection

UTI is the second most common bacterial infection in cirrhosis after SBP[7]. In cirrhotic patients, the prevalence of bacterinuria is 16%-18%, which is twice as frequent as matched controls[29,30] and which may be attributed to increased bladder post-void residual volume in cirrhotic patients[31]. Notably, bacterinuria is not consistently associated with an increased risk of sepsis[29]. Several predisposing factors for UTI have been suggested, including advanced liver disease, urinary catheter and female sex[13,29,30]. As in non-cirrhotic individuals, the common pathogens are gram-negative bacilli and coagulase-negative staphylococcus[29,30]. Treatment with cephalosporins or quinolones is generally effective. Notably, a high prevalence of resistant bacteria has recently been reported, not only in hospital-acquired (69%), but also community-acquired UTI (22%)[32].

Pneumonia

Pulmonary infections are common in cirrhotic patients. The causative organisms of community-acquired pneumonia appear to be the same as in general adults[33]. Compared to non-cirrhotic, cirrhotic patients with community-acquired pneumonia are more frequently associated with bacteremia, multi-lobar involvement, impaired consciousness, renal failure, septic shock and death (overall mortality 7.4% *vs* 14.4%, $P < 0.024$)[34]. Excessive alcohol intake can further impair pulmonary host defense and increase the risk of oropharyngeal aspiration[35]. Careful monitoring and empirical treatment with intravenous beta-lactams plus macrolides or intravenous antipneumococcal quinolones is recommended[33]. The risk of hospital-acquired pneumonia is increased in cirrhosis, particularly in the setting of gastrointestinal hemorrhage, tracheal intubation and encephalopathy. Thus, it is associated with resistant organisms and dreadful outcomes[7,12]. Empirical antibiotics for cirrhotic patients with hospital-acquired pneumonia should include intravenous antipseudomonal cephalosporins, carbapenams or piperacillin-tazobactam, plus ciprofloxacin or levofloxacin, and vancomycin or linezolid[36].

Bacteremia

Bacteremia without particular organ-specific source is increasingly common in cirrhosis and can be arbitrarily divided into 2 entities: (1) primary or spontaneous bacteremia; and (2) secondary bacteremia. True primary bacteremia shares the same initial step of pathogenesis as SBP, whereby bacteria flora in the gut lumen translocate into

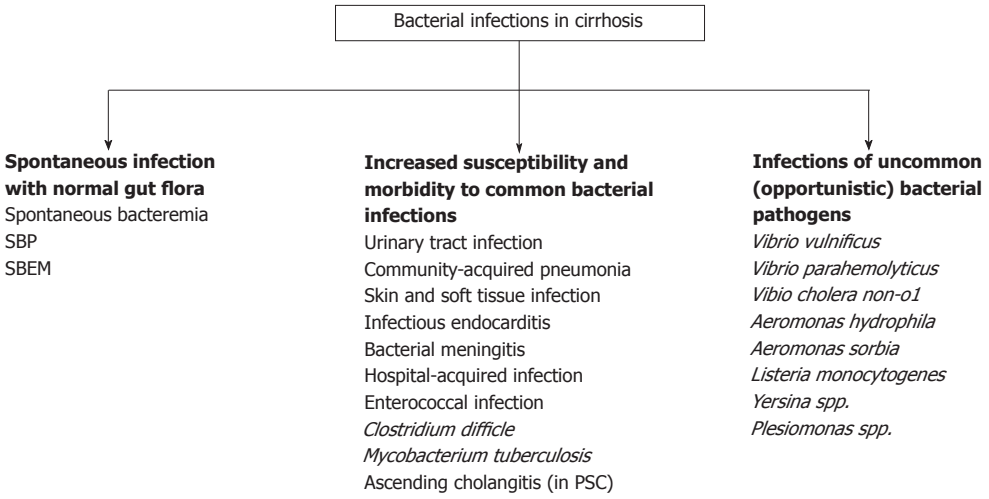

Figure 1 The outline of bacterial infection in cirrhotic patients. SBP: Spontaneous bacterial peritonitis; SBEM: Spontaneous bacterial empyema; PSC: Primary sclerosing cholangitis.

the bloodstream. It is generally encountered in the setting of advanced cirrhosis and is often caused by gram-negative enteric bacilli, enterococci and *Streptococcus spp.*[7,11].

Secondary bacteremia, in which pathogens come from an exogenous source, can be observed either in community-acquired settings, such as gastrointestinal bleeding, wound exposure and food-borne, or in healthcare-associated settings, such as transarterial chemoembolization[37], transjugular intrahepatic portosystemic shunt[38], therapeutic endoscopy[39] and intravenous catheters[7]. The causative organisms are largely dependent on the origin of bacteremia. Bacteremia and/or SBP occur in 17%-45% of patients following an episode of gastrointestinal bleeding[8] and, like those patients with primary bacteremia, the causative organisms are typically gram-negative enteric bacteria. Bacteremia associated with invasive procedures is commonly caused by *S. aureus* and *S. epidermidis*[7,11]. Notably, methicillin-resistant *S. aureus* (MRSA) was reported in up to 35% of nosocomial bacteremia in cirrhotic patients[11]. Although relatively uncommon, several case reports and case series have reported cases of severe community-acquired bacteremia in cirrhotic patients caused by *Vibrio spp.* and *Aeromonas spp.* without obviously localized infection[40-45]. Previous exposure to flood or seawater, or prior consumption of uncooked seafood may be a clue for diagnosis[40-45]. Intravenous third-generation cephalosporins and/or fluoroquinolones are commonly used as an empirical therapy for community-acquired bacteremia without other risk for specific or resistant pathogens. The use of antipseudomonal and glycopeptide antibiotics should be considered for hospital-acquired infection depending on the local pattern of resistant bacteria.

Spontaneous bacterial empyema

Spontaneous bacterial empyema (SBEM) is the infection of a pre-existing hydrothorax in which pneumonia has been excluded. It has been reported to be present in 10%-20% of hospitalized patients with hepatic hydrothorax[46-48]. The pathogenesis of SBEM, SBP and spontaneous bacteremia are closely interconnected; thus, they share the same types of common pathogens. SBEM can occur either with SBP, through transdiaphragmatic spread, or without SBP, through hematogenous spread (53% associated with SBP, 30% had no clinical ascites and 17% had non-infected ascites)[49]. Therefore, thoracocentesis should be performed when an infection is suspected in cirrhotic patients with ascites and hydrothorax, particularly in those with non-infected ascites. Risk factors for developing SBEM are the presence of SBP, low pleural fluid protein and complement levels, and advanced liver disease[47,50].

The criteria for diagnosis are: (1) pleural fluid polymorphonuclear neutrophil (PMN) $\geqslant$ 250 cell/mm^3 with a positive culture or $\geqslant$ 500 cells/mm^3 with a negative culture; and (2) exclusion of parapneumonic infection[46,49]. Notably, culture of pleural fluid should be performed by inoculating 10 mL of pleural fluid into a blood culture bottle at bedside, which is the same as the standard recommendation for SBP[49,51]. Analysis of pleural fluid by reagent strip for leukocyte esterase might be a rapid and easy-to-use tool for the detection of SBEM[52]. Hospital mortality has been reported as 20%-40% in cirrhotic patients with SBEM[46-48]. Treatment with intravenous third-generation cephalosporin should be initiated immediately when pleural fluid PMN $\geqslant$ 250 cell/mm^3 while awaiting culture result. In cases with slow clinical recovery, a repeat thoracocentesis is suggested to document the treatment response. Chest tube drainage can be harmful in cirrhotic patients with hepatic hydrothorax and should not be used in the treatment of SBEM[49].

Skin and soft tissue infection

Several reasons can contribute to an increased risk of skin and soft tissue infection (SSTI) in cirrhotic patients, such as fragile, thin and edematous skin, poor hygiene

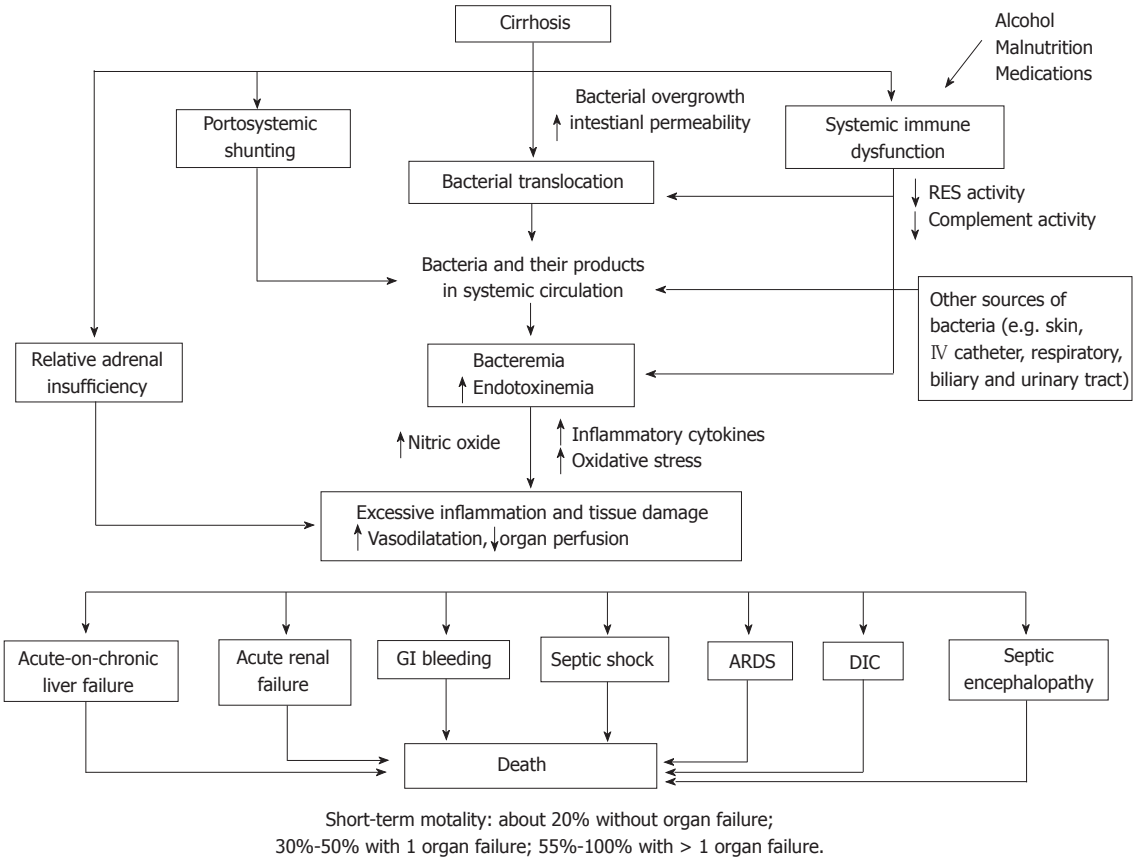

Figure 2 Proposed pathogenesis and consequences of bacterial infections in cirrhotic patients. GI: Gastroenterology; RES: Reticuloendothelial system; ARDS: Acute respiratory distress syndrome; DIC: Disseminated intravascular coagulation.

standards, malnutrition, frequent hospitalization and invasive procedures. Antibiotic therapy is generally effective for mild cellulitis; however, it is associated with a considerable recurrence rate of 21%[53]. Attention must be paid to severe cellulitis and necrotizing fasciitis that are increasingly reported and often carry a high mortality rate in cirrhotic patients, ranging from 6%-76% depending on the pathogens, extension of disease, presence of hemorrhagic bullae, severity of cirrhosis and management[54-56].

The common causative organisms are gram-positive cocci (*S. aureus,* beta-hemolytic streptococci) and gram-negative enteric bacteria (occasionally polymicrobial)[54]. Remarkably, the incidence of gram-negative pathogens, such as *E. coli, Klebsiella spp., P. aeruginosa, Aeromonas spp., Vibrio spp*., has evidently increased in cirrhotic patients[42-45,55-57]. Unlike the general population, necrotizing fasciitis in cirrhotic patients sometimes develops without an obvious portal of entry in the extremities, thereby suggesting a potential alternative pathway of bacterial translocation and bacteremia leading to SSTI[55,56]. In addition, approximately two thirds of cirrhotic patients with necrotizing fasciitis caused by gram-negative pathogens had concurrent bacteremia and/or initially presented with septic shock[55]. The presence of severe pain and/or SIRS out of proportion to the local wound appearance raises the possibility of necrotizing fasciitis. Careful evaluation with a high index of suspicion is mandatory since an early surgical intervention has been shown to reduce morbidity and mortality in necrotizing fasciitis[54-56,58].

There is no specific guideline for the empirical antibiotic therapy for severe SSTI in cirrhosis. Given a high morbidity/mortality and wide-range of possible pathogens in cirrhotic individuals, gram-stained smears from pus and/or infected tissue should be immediately obtained and broad-spectrum antibiotics should be prompt utilized, such as third or fourth generation cephalosporins, amoxicillin-clavulanate, piperacillin-tazobactam and carbapenams. Combination therapy with fluoroquinolones or cloxacillin may be considered if a gram-negative or gram-positive pathogen is highly suspicious, respectively. Empirical treatment is effective in approximately 80% of community-acquired SSTI. Importantly, it is effective in only half of cirrhotic patients with nosocomial SSTI, which is largely due to a higher incidence of MRSA and *P. aeruginosa*[32].

Endocarditis

Infectious endocarditis (IE) classically occurs in patients with underlying valvular heart disease and prosthetic valves. Interestingly, a recent review of 316 IE cases found that approximately 10% of patients had underlying liver cirrhosis[59]. IE in cirrhotic patients was often

observed in those patients who were hospitalized and/or had invasive procedures[7,59]. The common causative organisms are gram-positive such as Streptococci (*S. pyogenes, S. agalactiae, S. viridans), S. aureus, S epidemidis* and enterococci[59,60]. A minimum of 4-6 wk of antibiotic therapy is recommended. Caution should be taken since the majority of IE cases in cirrhosis are health-care associated and therefore the incidence of drug-resistant pathogens is considerably increased[59,60]. The mortality rate of cirrhotic patients with IE is high (27%-51%) despite treatment, especially in those patients with advanced cirrhosis and staphylococcal infection[59,60].

Meningitis

The incidence of community-acquired bacterial meningitis in the general population is estimated around 5/100 000 adults per year; the majority of these caused by *S. pneumoniae* and *N. meningitides*[61]. Several reports suggested that the incidence and the virulence of bacterial meningitis are substantially increased (up to 10-fold) in cirrhotic patients; thus, mortality rate in these patients is approximately 50%-63% and even higher in older patients and those with alcohol-related cirrhosis[62-65]. Cirrhotic patients, compared to non-cirrhotic patients, had a longer duration of symptoms before the time of diagnosis (> 4 d: 32% *vs* 16%, respectively), less obvious physical signs (nuchal rigidity: 75% *vs* 92%, respectively); greater incidence of relapse (18% *vs* 1%, respectively), and increasing incidence of *E. coli* and *L. monocytogenes*[62,64].

In the clinical setting of fever with headache and/or alteration of conscious in cirrhotic patients, the possibility of a central nervous system infection should not be overlooked. Neurological examinations are sometimes limited and ambiguous, particularly in the presence of concurrent hepatic encephalopathy. Prompt empirical central nervous system-dosed antibiotics and an appropriate diagnostic approach are key to decrease morbidity and mortality in patients with bacterial meningitis[66]. A combination of vancomycin, third generation cephalosporins and ampicillin is recommended for empirical therapy in cirrhotic patients with bacterial meningitis[66].

PATHOGENS AND THEIR CLINICAL FEATURES

Vibrio spp.

V. vulnificus is a gram-negative, motile, marine bacterium that is endemic in warm coastal water[42]. Exposure to this organism usually occurs through the ingestion of seafood (e.g. shellfish, raw oyster) or inoculation *via* traumatic injury in marine environments. *V. vulnificus* infection generally occurs in patients who are elderly or those who are compromised with comorbidities, especially cirrhosis[42,57]. It typically manifests as three clinical features: (1) SSTI: direct inoculation of organism causing wound infection or cellulitis, which generally occurs within 24-48 h after exposure. The lesions are typically painful and associated with rapid evolution to the hemorrhagic bullae and then to necrotic ulcers, necrotizing fasciitis and secondary bacteremia; (2) primary sepsis; and (3) gastrointestinal illnesses characterized by abdominal pain, diarrhea, and vomiting[42,67]. The virulence of *V. vulnificus* is linked to the availability of iron and its secreting toxin.

Infections from other marine *Vibrios* also increasingly occur and are associated with poor outcomes in cirrhotic patients[10,58,67-69]. *V. cholera* non-o1 infection occurs in endemic areas, such as the United States, Mexico, East and Southeast Asia[10,58,67-69]. The route of acquisition and clinical features can mimic *V. vulnificus* infection. *V. parahemolyticus* generally causes watery diarrhea, abdominal pain and vomiting.

An early recognition, aggressive treatment of shock and surgical management of SSTI is crucial. Most isolated marine *Vibrios* are susceptible to third generation cephalosporins, tetracyclines and fluoroquinolones. The combination of cefotaxime and minocycline or fluoroquinolones has been shown to have a synergistic effect against marine Vibrios[10,67,69].

Aeromonas spp.

Aeromonas spp. is a gram-negative, facultative anaerobic bacteria that is ubiquitous in fresh and brackish water. Infections are more frequently encountered in immunocompromised patients, particularly cirrhosis and malignancy[44,45,67,70-72]. *A. hydrophila* and *A. veronii* biovar *sobria* are the most often isolated species from symptomatic patients. Aeromonas bacteremia in cirrhotic patients tends to be monomicrobial, whereas polymicrobial bacteremia (frequently combined with *E. coli* or *Klebsiella spp.*) is commonly seen in patients with malignancy[44,72]. Drug of choice for empirical treatment is either intravenous carbapenams or a combination of intravenous third generation cephalosporins and aminoglycosides or fluoroquinolones.

Mycobacterial tuberculosis

The incidence and virulence of tuberculosis (TB) are increased in cirrhotic patients. Extrapulmonary involvement is more frequently observed (11%-31%)[73,74]. TB peritonitis possibly mimics SBP. TB peritonitis occurs in less advanced cirrhosis and its ascites has a lower white blood cell count, higher proportion of mononuclear cells, higher levels of protein and adenosine deaminase (ADA)[75]. More than 50% of TB peritonitis cases in the United States had underlying cirrhosis, especially alcohol-related[76]. Though ADA level is generally helpful in the detection of TB peritonitis, the presence of cirrhosis may reduce its sensitivity to 30%[76-78]. Laparoscopic biopsy sometimes is required for definitive diagnosis by revealing multiple whitish nodules scattered over the peritoneum, lymphocytic inflammation with granulomas and/or acid-fast organisms on the histopathological examination[77,79]. Patients with TB and cirrhosis often respond well to anti-TB treatment but are associated with more treatment-related hepatotoxicity incidence[73,77].

Table 2 Individual pathogens and their clinical manifestations in cirrhotic patients

Pathogens	Common clinical features	Key points
E.coli, Klebsiella spp. Enterobacter spp. and other gram-negative enteric bacteria[6,7,9,12,13]	SBP, bacteremia, UTI, biliary tract infection, meningitis	↑ Incidence of resistant organisms in hospital-acquired infection and in patients taking prophylactic quinolones
Plesiomonas shigelloides [10,81,99]	Septicemia, diarrhea, SBP, meningitis, SSTI	↑ Incidence in hemochromatosis Risk factors: contaminated food and water
Vibrio spp. (*V. vulnificus, non-o1 V. cholera, V. parahemolyticus*)[10,42,43,57,81]	SSTI, bacteremia, gastroenteritis	↑ Incidence in cirrhosis, particularly hemochromatosis ↑ Virulence; mortality 50%-60% in bacteremic form and about 24% for SSTI Risk factors: contaminated food and seawater
Aeromonas spp. (*A. hydrophilla, A. sobria*)[44,45,67,70-72]	Bacteremia, biliary tract infection, gastroenteritis, SBP, SSTI	↑ Incidence ↑ Virulence; mortality 20%-60% Risk factors: contaminated food and water
Yersinia spp. (*Y. enterocolitica, Y. pseodotuberculosis*)[10,81]	Bacteremia, SBP, hepatosplenic abscesses	↑ Incidence in hemochromatosis ↑ Virulence; mortality about 50% in bacteremic form
Campylobacter spp. (*C. jejuni, C. fetus*)[10,100]	Bacteremia, SBP	↑ Incidence Mortality about 10% in bacteremic form
Pateurella multocida[13, 101,102]	SSTI, bacteremia, arthritis, meningitis	↑ Incidence Mortality about 10% in bacteremic form Risk factors: cat and dog bites or scratches
Staphylocccus aureus[7,11,13]	Bacteremia, SSTI, endocarditis	↑ Incidence, particularly in those who are hospitalized and/or had invasive procedure ↑ Incidence of MRSA carriage and infection
Streptococcus pneumonia[94,95]	Bacteremia, pneumonia, SBP, SSTI, meningitis	↑ Incidence of invasive pneumococcal disease ↑ Virulence Vaccination is recommended
Streptococcus group B[103,104]	Bacteremia, SBP, SSTI, pneumonia	↑ Incidence Mortality 10%-25%
Clostridium difficile[80]	Antibiotic-associated diarrhea and colitis	↑ Incidence ↑ Virulence; mortality 14% Risk factors: hospitalization, antibiotics, proton pump inhibitors
Clostridium spp. (*C. perfringens, C. bifermentans, C. septicum*)[13,105]	Bacteremia, SSTI, peritonitis	↑ Incidence ↑ Virulence; mortality 54%-65%
Enterococcus spp (*E. faecium, E. faecalis, E. galinarum*)[7,11,59,106-109]	SBP, bacteremia, UTI, endocarditis, biliary tract infection	↑ Incidence, particularly in hospital-acquired infection and in patients taking prophylactic quinolones ↑ Virulence; mortality rate up to one third in bacteremic form and up to 60% in enterococcal peritonitis Pre-transplant VRE colonization (13%-15% from surveillance) is associated with increased morbidity and mortality following liver transplant
Listeria monocytogenes[10,64,81]	Bacteremia, meningitis, SBP	↑ Incidence in cirrhosis, particularly hemochromatosis
Mycobacterium tuberculosis (TB)[73,74,77]	Pulmonary TB, extra-pulmonary TB (e.g. peritonitis, disseminated TB)	↑ Incidence ↑ Virulence; mortality 22%-48% ↑ Extra-pulmonary forms ↑ Risk for multi-drug resistance TB ↑ Risk for anti-TB hepatotoxicity

SBP: Spontaneous bacterial peritonitis; UTI: Urinary tract infection; SSTI: Skin and soft tissue infection; TB: Tuberculosis.

Clostridium difficile

C. difficile infection has recently been recognized as a significant problem in hospitalized cirrhotic patients. US database of over 80 000 patients analysis suggested that *C. difficile*-associated diarrhea (CDAD) is an independent risk of death in hospitalized cirrhotic patients (OR 1.55, 95% CI: 1.29-1.85)[80]. It is also associated with an increase in the length of hospital stay and hospital cost in these patients. There was no correlation between severity of cirrhosis and the development of CDAD[80] (Table 2).

LIVER DISEASE-SPECIFIC ISSUES

Hemochromatosis

The association of hemochromatosis and certain pathogens has been well described. Several mechanisms have been proposed to explain this association. Iron excess induces oxidative stress which results in organ damage and impairment of immune function[81]. Hepcidin, a central iron-regulatory hormone, has recently been recognized for an immunomodulatory and broad antimicrobial property[82,83]. Inadequate expression and functional impairment of hepcidin in patients with hemochromatosis may connect to the increased susceptibility for infections[82,83]. Hemochromatosis is associated with a decrease in proliferation, migration, phagocytic activity and cytokines secreting ability of the immune cells, thereby, principally impairing cell-mediated immune response[81].

Aside from impaired host defense, the growth and virulence of various organisms are enhanced by a high

iron environment[81]. Interestingly, chelation therapy with desferoxamine in patients with hemochromatosis secondary to long-term transfusion may further stimulate the growth of particular bacteria, such as *V. vulnificus*, *Y. enterocolitica*, *K. pneumonia* and *S. aureus*, which can use it for efficient iron uptake *via* specific receptors[84,85]. On the other hand, newer iron chelators (deferasirox and deferiprone) do not act as siderophores and therefore may depress the growth of iron-dependent organisms[84,85].

A number of pathogens have been reported to be of increased susceptibility in patients with hemochromatosis, such as *E. coli*, *Vibrio spp.* (*V. vulnificus*, *V. cholerae*), *L. monocytogenes*, *Yersenia spp.* (*Y. pseudotuberculosis, Y. enterocolitica*), *Plesiomonas shigelloides*, *Mycobacterium tuberculosis*, cytomegalovirus and fungi (*A. fumigatus*, *Mucor spp.*)[81,86].

Primary sclerosing cholangitis

Patients with primary sclerosing cholangitis (PSC) are susceptible to repeated episodes of bacterial cholangitis, especially after biliary tract manipulation[87,88]. The incidence of cholangitis following endoscopic retrograde cholangiopancreatography (ERCP) is higher in PSC patients (4%-16%) compared to non-PSC patients, particularly in those who had therapeutic ERCP procedures[89]. If cholangitis occurs without biliary intervention, the presence of stones, dominant strictures or cholangiocarcinoma should be considered. Most common causative organisms are gram-negative enteric bacteria and enterococci[90]. Recurrent bacterial cholangitis may benefit from long term antibiotic prophylaxis[87].

PREVENTIVE MEASUREMENTS

All cirrhotic patients should be aware of the risk of infections and contact their physicians instantly when they are febrile or ill. Raw/uncooked foods, close contact to at-risk animals or sick people and wound exposure to flood or seawater should be avoided, particularly in those with advanced liver disease.

Prophylactic antibiotics should be utilized in cirrhotic patients at high risk of developing infection, including gastrointestinal bleeding and those undergoing invasive endoscopic or surgical procedures[28,39]. Long-term prophylaxis for patients with a history of SBP and those who have low ascitic fluid protein (< 1.5 gm/dL) is recommended[51]. On the other hand, overuse of antibiotic prophylaxis can lead to the development of resistant organisms and CDAD[7,80]. The rate of culture-positive infection caused by quinolone-resistant gram-negative bacilli was very high (65%) in patients on long-term norfloxacin prophylaxis[7]. Notably, prophylactic antibiotics are not recommended for routine endoscopy, elective variceal band ligation and abdominal paracentesis[39,51,91].

Immunization against hepatitis A and B viruses, influenza and pneumococcus are recommended in patients with cirrhosis[92,93]. Both cellular and humoral immune responses are suppressed in cirrhotic patients which may be associated with suboptimal early post-vaccination response and loss of long-term immunogenicity[92]. Therefore, a booster dose early during the follow-up is suggested in order to improve the immune response[92]. Cirrhotic patients are able to receive both inactivated and live vaccines according to the current guidelines[92,93]. *S. pneumoniae* infections are common, more severe and frequently associated with poor outcome in cirrhotic patients[94,95]. Anti-pneumococcal vaccination is recommended with booster injections every 5 years[92]. Incidence of seasonal flu is not evidently increased in cirrhotic patients; however, influenza infection may precipitate hepatic decompensation[92,96]. Influenza vaccine is well-tolerated and clinically effective in cirrhotic patients despite a slightly reduced immunogenicity[97,98].

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BRIEF ARTICLE

Global hypomethylation in hepatocellular carcinoma and its relationship to aflatoxin B_1 exposure

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Abstract

AIM: To determine global DNA methylation in paired hepatocellular carcinoma (HCC) samples using several different assays and explore the correlations between hypomethylation and clinical parameters and biomarkers, including that of aflatoxin B_1 exposure.

METHODS: Using the radio labeled methyl acceptance assay as a measure of global hypomethylation, as well as two repetitive elements, including satellite 2 (Sat2) by MethyLight and long interspersed nucleotide elements (LINE1), by pyrosequencing.

RESULTS: By all three assays, mean methylation levels in tumor tissues were significantly lower than that in adjacent tissues. Methyl acceptance assay log (mean ± SD) disintegrations/min/ng DNA are 70.0 ± 54.8 and 32.4 ± 15.6, respectively, $P = 0.040$; percent methylation of Sat2 42.2 ± 55.1 and 117.9 ± 88.8, respectively, $P < 0.0001$ and percent methylation LINE1 48.6 ± 14.8 and 71.7 ± 1.4, respectively, $P < 0.0001$. Aflatoxin B_1-albumin (AFB_1-Alb) adducts, a measure of exposure to this dietary carcinogen, were inversely correlated with LINE1 methylation ($r = -0.36$, $P = 0.034$).

CONCLUSION: Consistent hypomethylation in tumor compared to adjacent tissue was found by the three different methods. AFB_1 exposure is associated with DNA global hypomethylation, suggesting that chemical carcinogens may influence epigenetic changes in humans.

Key words: Hepatocellular carcinoma; Epigenetics; Hypomethylation; [^{3}H]-methyl acceptance assay; Satellite 2; Long interspersed nucleotide element-1; Aflatoxin B_1

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and a leading cause of death worldwide, especially in Saharan Africa and

southern Asia, including Taiwan, Thailand, Hong Kong and southern China[1,2]; it is also increasing in Western, developed countries such as the United States[3]. HCC incidence is associated with various risk factors, including chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcohol consumption and several environmental factors, especially aflatoxin B1 (AFB1), a dietary mold contaminant, and polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental contaminants[4-6].

As with other cancers, the development of HCC is a complex, multistep process, involving multiple genetic aberrations in the molecular control of hepatocyte proliferation, differentiation and death and the maintenance of genomic integrity. This process is influenced by the cumulative activation and inactivation of oncogenes, tumor suppressor genes and other genes[7,8]. Epigenetic alterations are also involved in cancer development and progression[9,10]. Human tumors often display changes in DNA methylation, including both gene-specific promoter hypermethylation and genome-wide hypomethylation[11,12]. Frequent promoter hypermethylation and subsequent loss of protein expression of tumor suppressor genes has been demonstrated in HCC[13]. Global hypomethylation, in both noncoding repetitive sequences and in genes, contributes to carcinogenesis by causing chromosome instability, reactivation of transposable elements, loss of imprinting and increased gene expression, and has been detected in different human cancer tissues, including HCC[14].

Hypomethylation of the genome mainly affects the intergenic and intronic regions of DNA, particularly repeat sequences and transposable elements[15]. Repetitive elements, which consist of interspersed and tandem repeats, comprise about 45% of the human genome[16,17]. More than 90% of all 5-methylcytosine (5^mC) lies within the transposons, including short interspersed nucleotide elements and long interspersed nucleotide elements (LINEs), which are comparatively rich in CpG dinucleotides[14]. Satellite 2 (Sat2) DNA sequences are located as tandem repeats in the pericentromeric and juxtacentromeric heterochromatin of several chromosomes[18]. Loss of DNA methylation in these sequences is believed to mainly account for global hypomethylation[19]. Analysis of methylation levels of Sat2 and LINE1 are frequently used as a measure of global methylation since levels measured using the MethyLight assay were significantly associated with methylation, as measured by high-performance liquid chromatography quantitation of 5^mC[20].

The methyl group acceptance assay also can be used to determine global DNA methylation levels. It is based on the ability of isolated DNA to "accept" radio labeled methyl groups from S-[^{3}H-methyl] adenosylmethionine, using the bacterial CpG methyltransferase SssI. As this enzyme methylates all unmethylated CpG dinucleotides in the genome, radio labeled methyl group acceptance is inversely proportional to the level of preexisting methylation[21].

In the current study, global DNA methylation status in paired HCC and their adjacent non-tumor tissues was measured using the methyl acceptance assay, analysis of Sat2 by MethyLight and LINE1 by pyrosequencing. Data were correlated to both clinical data and other available biomarker data on exposure to AFB1 and gene-specific promoter methylation.

MATERIALS AND METHODS

Patient population and data on clinical parameters

The study samples consisted of frozen dissected tumor and adjacent tissues from HCC patients, collected in the Department of Surgery, National Taiwan University Hospital. Informed consent was obtained from patients and the study was approved by the appropriate institutional review committees. Data on demographics and clinicopathological characteristics obtained from hospital charts, and HBV and HCV status, determined by immunoassay, were published previously[22]. Plasma collected at the time of surgery had been previously analyzed for the albumin adducts of AFB1. In addition, methylation of $p16^{Ink4A}$ and *HINT1* were previously determined in the tumor tissues by methylation specific PCR[22-23].

DNA extraction

DNA was isolated from frozen tissue samples, as previously described[24]. Briefly, tissue was placed in liquid nitrogen and pulverized with a blender. The tissue powder was lysed with a DNA lysing buffer (10 mmol/L Tris, 10 mmol/L NaCl, 0.1% sodium dodecyl sulfate at pH 7.9 and 200 μg/mL proteinase K). DNA was isolated by RNase treatment, phenol/chloroform extraction and ethanol precipitation. The laboratory investigator who performed the assays was blinded to epidemiological data.

Sat2 MethyLight assay

After sodium bisulfite conversion (EZ DNA methylation kit, Zymo Research, Orange, CA), genomic DNA was amplified using the previously reported Sat2 M1 and Alu C4 (control for DNA input) primers and probes[20]. Bisulfite-converted, CpGenome universal methylated DNA (Chemical International, Temecula, CA) served as the methylated reference. A pooled sample of DNA from 5 controls was used as a quality control and analyzed with each batch of test samples. All samples were analyzed in duplicate on an ABI Prism 7900 Sequence Detection System (Perkin-Elmer, Foster City, CA). Intra- and inter-assay coefficients of variation (CVs) were 1.2 and 1.9, respectively. The data are expressed as a percentage of methylated reference (PMR) values.

PMR = 100% * 2 exp - {Delta Ct (target gene in sample - control gene in sample) - Delta Ct (target gene in fully methylated reference sample - control gene in reference sample)}.

LINE1 amplification and pyrosequencing

The assay for LINE1 was carried out essentially as described previously, using reported primer and sequencing

Table 1 Methylation levels of hepatocellular carcinoma tumor and adjacent non-tumor liver tissue

	Tumor mean ± SD	Adjacent mean ± SD	*P* value
LINE1 (%)	48.6 (14.8)	71.7 (1.4)	< 0.0001
Sat2 (%)	42.2 (55.1)	117.9 (88.8)	< 0.0001
Methyl acceptance (DPM/ng)	70.0 (54.8)	32.4 (15.6)	0.040

LINE1: Long interspersed nucleotide element-1; Sat2: Satellite 2; DPM: Disintegrations/min.

Table 2 Correlations between methylation levels and aflatoxin B$_1$- albumin adducts in hepatocellular carcinoma tumor tissues

	AFB$_1$-Alb *r*	*P* value
LINE1 (%)	-0.36	0.034
Sat2 (%)	-0.30	0.082
Methylacceptance DPM/ng)	0.18	0.286

AFB1: Aflatoxin B$_1$; LINE1: Long interspersed nucleotide element-1; Sat2: Satellite 2; DPM: Disintegrations/min.

probe sequences as well as PCR conditions[25]. We used non-CpG cytosine residues as internal controls to verify efficient sodium bisulfate DNA conversion and controls were as in the MethyLight assay. Pyrosequencing was conducted using a PyroMark Q24 instrument (Qiagen), with subsequent quantitation of methylation levels determined with the PyroMark Q24 1.010 software. Relative peak height differences were used to calculate the percentage of methylated cytosines at each given site. Percent methylation within a sample was subsequently determined by averaging across all three interrogated CpG sites in the analysis. The inter-assay CV was 1.1.

[^{3}H]-Methyl acceptance assay

The [^{3}H]-methyl acceptance assay was carried out as described by Balaghi and Wagner[26] and Pilsner *et al*[27]. The DNA was incubated with [^{3}H]S-adenosylmethionine in the presence of the SssI prokaryotic methylase enzyme. Briefly, 200 ng of DNA was incubated with 3 U of *Sss*I methylase (New England Biolabs); 3.8 μmol/L (1.1 μCi) [^{3}H]-labeled S-adenosylmethionine (Perklin-Elmer); and EDTA, DTT, and Tris-HCL (pH 8.2) in a 30 μL mixture and incubated for 1 h at 37 ℃. The reaction was terminated on ice and 15 μL of the reaction mixture applied onto Whatman DE81 filter paper. The filter was washed on a vacuum filtration apparatus three times with 5 mL of 0.5 mol/L sodium phosphate buffer (pH 8.0), followed by 2 mL each of 70% and 100% ethanol. Dried filters were each placed in a vial with 5 mL of scintillation fluid (Scintisafe, Thermo Fisher, Waltham, MA) and analyzed by a Packard scintillation counter to determine counts/min then converted to disintegrations/min (DPM) based on counting efficiency. Each DNA sample was processed in duplicate and each processing run included samples for background (reaction mixture with all components except SssI enzyme) and controls as for the other assays. Intra- and inter-assay CVs were 2.0 and 3.9, respectively. DPM values were expressed per ng DNA as quantified by PicoGreen using double-strand DNA quantification reagent (Molecular Probes, Life Technologies, Grand Island, NY).

Statistical analysis

Paired *t*-test was used to examine differences in methylation levels between tumor and adjacent tissues after natural log-transformation to normalize the distribution. We present the values as arithmetic data for ease of interpretation. Spearman correlation coefficients were used to determine the correlation between methylation and AFB$_1$-Alb adducts. Wilcoxon signed-rank test was used to compare methylation levels and clinical characteristics. All analyses were performed with SAS software 9.0 (SAS Institute, Cary, NC). All statistical tests were based on two-tailed probability.

RESULTS

Methylation levels of DNA from HCC and adjacent non-tumor tissues were determined by the methyl acceptance assay, a measure of global methylation. Two repetitive elements were also analyzed as an additional measure of methylation, including Sat2 by MethyLight and LINE1 by pyrosequencing. For all three assays, mean methylation level was significantly lower in tumor compared to adjacent non-tumor tissues. For the methyl acceptance assay, mean levels of DPM/ng DNA were 70.0 ± 54.8 and 32.4 ± 15.6, respectively ($P = 0.040$); for Sat2 by the MethyLight assay, values were 42.2% ± 55.1% and 117.9% ± 88.8% ($P < 0.0001$); and for LINE1, 48.6 ± 14.8 and 71.7 ± 1.4% ($P < 0.0001$), respectively (Table 1).

For the methyl acceptance assay, in 28 of 37 paired samples (75.7%), methylation in tumor tissues was lower than that in adjacent non-tumor tissues. For Sat2 and LINE1 analysis, in 31 (83.8%) and 32 (86.5%) subjects, levels were lower in tumor than in adjacent non-tumor tissues, respectively.

Plasma levels of AFB$_1$-Alb adducts had been measured previously in bloods collected at the time of surgery[22]. As hypothesized, plasma levels of AFB$_1$-Alb adducts were statistically significantly inversely correlated with methylation levels of LINE1 ($r = -0.36$, $P = 0.034$) (Table 2 and Figure 1). Plasma levels of AFB$_1$-Alb adducts were also inversely correlated with tumor methylation levels measured by Sat2, but not statistically significantly ($r = -0.30$, $P = 0.082$, Table 2). Since higher values in the methyl acceptance assay indicate hypomethylation, the correlation between adducts and methylation in this assay is also in the correct direction but not significant ($r = 0.18$, $P = 0.286$, Table 2).

The associations of HBV and HCV infection sta-

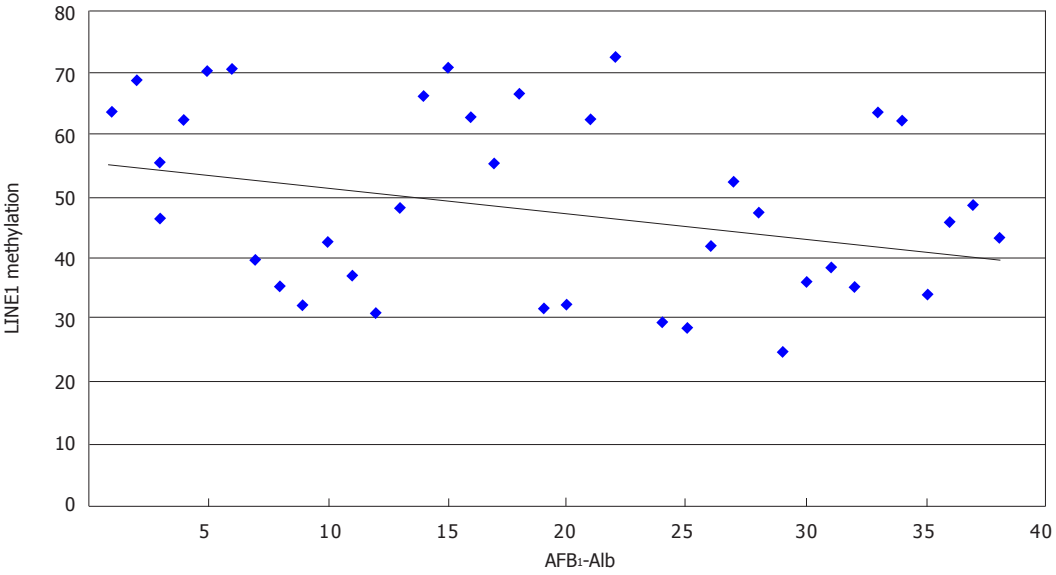

Figure 1 The correlation between aflatoxin B_1-Alb levels in plasma (aflatoxin B_1/μg) and long interspersed nucleotide element-1methylation (%) in tumor tissue. AFB_1: Aflatoxin B_1; LINE1: Long interspersed nucleotide element-1.

tus, cirrhosis status and promoter hypermethylation of $p16^{Ink4A}$ and *Hint1* with global hypomethylation in tumor tissue are given in Table 3. No statistically significantly correlations were found, except for LINE1 and being positive for both HBV and HCV infection. However, only one subject was negative for both HBV and HCV so this result is likely to be spurious.

DISCUSSION

Hypomethylation was observed in tumor compared to adjacent non-tumor tissues using three different assays that measure global methylation or methylation in two repetitive elements. The level of [^{3}H]-methyl acceptance of HCC tumor DNAs was statistically significantly higher compared to that of adjacent non-tumor DNAs ($P < 0.040$), indicating significantly lower methylation. This is the first study to report that global hypomethylation contributes to hepatocarcinogenesis using the [^{3}H]-methyl acceptance assay. Global loss of methylation in cancer may lead to alterations in the expression of proto oncogenes critical to carcinogenesis and facilitate chromosomal instability[28].

Repetitive DNA elements are normally heavily methylated and a previous study showed a correlation between Alu, Sat2 and LINE1 methylation by MethyLight and 5^mC content in normal and tumor samples[20], indicating the usefulness of these assays as surrogate measures of genomic methylation levels. In this study, tumor methylation was statistically significantly lower than in paired adjacent non-tumor tissue for Sat2 ($P < 0.0001$) by the MethyLight assay and for LINE1 ($P < 0.0001$) by pyrosequencing, consistent with the data from the methyl acceptance assay and as reported previously[14,27,28].

A previous study found that three repetitive DNA elements, Sat2, Alu and LINE1, showed discordance in timing of hypomethylation along the multistep pathway in hepatocarcinogenesis from normal liver to HCC; Sat2 hypomethylation occurred at the chronic hepatitis stage[29]. Hypomethylation also differed according to geographic location of the subjects and their hepatitis infection status; mean LINE1 methylation in tumor samples was lower in hepatitis-positive cases than in hepatitis-negative cases[30]. These findings suggest that HBV or HCV infection can influence global DNA hypomethylation status. This may be partially explained by the fact that the HBV X protein can induce altered DNA methyltransferase activity, hypermethylation of specific CpG islands and global hypomethylation[30,31]. In the present study, no associations between DNA global hypomethylation and HBV and HCV infection were observed (Table 3). However, only one case was negative for markers of infection for either HBV or HCV, limiting our ability to investigate the role of infection on methylation levels.

Exposure to AFB_1 is one of the major risk factors for the development of HCC. In our previous studies, we found a strong relationship between AFB_1 exposure and promoter hypermethylation in tumor suppressor and other cancer-related genes, including *RASSF1A*[32], $p16^{Ink4A}$[22,32] and *MGMT*[33] in tumor tissues and plasma DNA of HCC patients. AFB_1 may bind preferentially to methylated CpG sites and/or specific structures in chromatin, inducing damage to DNA and histones[33] that may impact on methylation. Several other environmental exposures have been associated with epigenetic changes. Increasing air levels of benzene, a chemical carcinogen, was associated with a significant reduction in LINE1 and Alu1 methylation in white blood cells[34]. LINE1 DNA methylation is also inversely associated with lead exposure in humans[35]. Even although the mechanisms are still not clear, these data suggest that exposure to some chemical carcinogens may cause changes in global methylation status. In the present study, plasma levels of

Table 3 Methylation levels in tumor tissues and clinical characteristic and gene-specific methylation in tumor tissues

Variable	*n*	LINE1 (%) mean ± SD	*P* value	Sat2 (%) mean ± SD	*P* value	Methyl acceptance (DPM/ng) mean ± SD	*P* value
HBsAg							
Negative	5	41.0 (16.6)	0.245	22.1 (19.2)	0.905	67.2 (37.6)	0.607
Positive	26	47.7 (13.6)		31.3 (34.5)		81.3 (58.3)	
AntiHCV							
Negative	18	49.6 (14.2)	0.449	27.6 (22.6)	0.737	72.1 (49.7)	0.759
Positive	10	45.2 (14.3)		38.0 (47.7)		86.5 (71.3)	
HBsAg and AntiHCV							
Both negative	1	24.9	0.047	5.7	0.323	64.5	0.877
Either one positive	31	47.2 (13.5)		31.1 (32.2)		78.4 (55.5)	
Cirrhosis							
No	17	44.8 (15.2)	0.388	28.1 (23.8)	0.791	78.6 (52.4)	0.927
Yes	13	48.9 (13.2)		32.5 (43.1)		82.3 (61.9)	
$p16^{Ink4A}$							
Unmethylated	15	50.9 (16.7)	0.569	63.4 (80.8)	0.437	62.0 (61.2)	0.155
Methylated	22	47.1 (13.5)		27.8 (21.1)		75.4 (50.8)	
Hint1							
Unmethylated	17	50.9 (14.8)	0.405	51.8 (75.8)	0.951	60.2 (48.9)	0.142
Methylated	20	46.7 (14.8)		34.1 (30.3)		78.3 (59.4)	

HCV: hepatitis V virus; LINE1: Long interspersed nucleotide element-1; Sat2: Satellite 2; DPM: Disintegrations/min.

AFB_1-Alb adducts were statistically significantly inversely correlated with methylation levels of LINE1, providing additional evidence that carcinogens may alter global methylation. Reactive oxygen species and the resulting DNA damage produced by AFB_1 may reduce binding affinity of methyl-CpG binding protein 2, therefore resulting in epigenetic alterations[36,37].

It is still uncertain whether or not gene-specific promoter hypermethylation and global hypomethylation are independent processes; in HCC, their correlation is still controversial. One recent study demonstrated that global hypomethylation in HCC was associated with gene-specific hypermethylation[30], but another showed variability between individual CpG islands' hypermethylation and repetitive DNA hypomethylation status and concluded that there is no mechanistic link in liver cancer cells[29]. We also found no association between promoter hypermethylation in the two specific genes investigated and global hypomethylation in HCC tissue DNAs. In addition, gene-specific hypermethylation and global hypomethylation appear to be independent processes in colon and urothelial cancers[38,39]. Further investigations are still needed to validate the relationship between global hypomethylation and gene-specific promoter hypermethylation.

In summary, this is the first study to investigate global hypomethylation, one of the most consistent epigenetic changes in cancer development in HCC, and paired adjacent non-tumor tissues using three different methods: the methyl acceptance assay and analysis of Sat2 and LINE1, two repetitive elements. Consistent hypomethylation in tumor compared to adjacent tissue was found by all three methods. AFB_1 exposure was also associated with DNA hypomethylation, suggesting that chemical carcinogens may influence epigenetic changes in human tissues.

COMMENTS

Background

Epigenetic alterations are involved in cancer development and progression. Promoter CpG island hypermethylation contributes to carcinogenesis by shutting off expression of tumor suppressor and DNA repair genes. Genomic DNA hypomethylation is implicated in carcinogenesis by inducing chromosome instability and loss of imprinting. Genome-wide hypomethylation has been reported in a variety of cancers, including hepatocellular carcinoma (HCC). Hypomethylation of the genome mainly affects the intergenic and intronic regions of DNA, particularly repeat sequences and transposable elements. Analysis of methylation levels of Satellite 2 (Sat2) and long interspersed nucleotide element-1 (LINE1) is frequently used as a measure of global methylation. The correlations between global hypomethylation and hepatitis infection status have been investigated, but the association between hypomethylation and aflatoxin B_1 (AFB_1) exposure in HCC is still unclear.

Research frontiers

Genomic DNA hypomethylation is a common finding in human cancers. Global DNA hypomethylation reflected in reduced levels of methylation in repeat regions, occurs in target tissues undergoing carcinogenic differentiation, and could be used as a biomarker of malignant tumors. Environmental factors such as geographic location and hepatitis status have been shown to contribute to hepatocarcinogenesis through global hypomethylation.

Innovations and breakthroughs

In the present study, the authors first investigated DNA methylation in HCC and paired adjacent non-tumor tissues using the methyl acceptance assay as a measure of global methylation. They also analyzed two repetitive elements, including Sat2 by MethyLight and LINE1 by pyrosequencing. With all three assays, mean methylation levels in tumor tissues were significantly lower than that in adjacent non-tumor tissues. They also first found that AFB_1-albumin adducts levels were inversely correlated with LINE1 methylation, providing an additional mechanism by which exposure to this dietary carcinogen may influence

hepatocarcinogenesis.

Applications

This work demonstrated that methyl acceptance assay could be used to accurately detect global hypomethylation in HCC samples. Finding that AFB_1 exposure is correlated with global hypomethylation, as well as hypermethylation in some genes, demonstrates the important role it plays in the development of HCC. This may help to develop new strategies to prevent HCC.

Terminology

Global hypomethylation is a decrease in the overall genomic 5-methylcytosine content (compared to total cytosines) from approximately 4% in normal tissues to 2%-3% in cancer tissues. This change was first observed in a number of studies in 1983, in lung and colon carcinomas compared to adjacent normal tissue, and in various malignancies compared to various postnatal tissues, demonstrating that overall genomic 5-methylcytosine levels were lower in cancer tissues. This observation has been reproducibly repeated in a wide range of cancers and matched normal tissues using a variety of different techniques.

Peer review

This manuscript addresses an interesting issue for the initiation and progression of HCC.

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BRIEF ARTICLE

Improved cryopreservation of human hepatocytes using a new xeno free cryoprotectant solution

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Abstract

AIM: To optimize a xeno-free cryopreservation protocol for primary human hepatocytes.

METHODS: The demand for cryopreserved hepatocytes is increasing for both clinical and research purposes. Despite several hepatocyte cryopreservation protocols being available, improvements are urgently needed. We first compared controlled rate freezing to polystyrene box freezing and did not find any significant change between the groups. Using the polystyrene box freezing, we compared two xeno-free freezing solutions for freezing of primary human hepatocytes: a new medium (STEM-CELLBANKER, CB), which contains dimethylsulphoxide (DMSO) and anhydrous dextrose, both permeating and non-permeating cryoprotectants, and the frequently used DMSO - University of Wisconsin (DMSO-UW) medium. The viability of the hepatocytes was assessed by the trypan blue exclusion method as well as a calcein-esterase based live-dead assay before and after cryopreservation. The function of the hepatocytes was evaluated before and after cryopreservation by assessing enzymatic activity of 6 major cytochrome P450 isoforms (CYPs): CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A7.

RESULTS: The new cryoprotectant combination preserved hepatocyte viability significantly better than the standard DMSO-UW protocol ($P < 0.01$). There was no significant difference in viability estimation between both the trypan blue (TB) and the Live-Dead Assay methods. There was a correlation between viability of fresh hepatocytes and the difference in cell viability between CB and DMSO protocols ($r^2 = 0.69$) using the TB method. However, due to high within-group variability in the activities of the major CYPs, any statistical between-group differences were precluded. Cryopreservation of human hepatocytes using the cryoprotectant combination was a simple and xeno-free procedure yielding better hepatocyte viability. Thus, it may be a better alternative to the standard DMSO-UW protocol. Estimating CYP activities did not seem to be a relevant way to compare hepatocyte function between different groups due to high normal variability between different liver samples.

CONCLUSION: The cryoprotectant combination may be a better alternative to the standard DMSO-UW protocol in primary human hepatocyte cryopreservation.

Key words: Human hepatocytes; Viability; Cytochrome P540; Dimethylsulphoxide; Cryoprotectant; Cryopreservation

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INTRODUCTION

Liver disease is a major health problem worldwide. Liver transplantation is still the golden standard treatment for acute liver failure and end-stage liver disease. Lack of donor organs, among others, is still a major obstacle[1]. Hepatocyte transplantation is gaining more attention as an alternative today[2,3]. Hepatocyte transplantation may function as a bridge to liver transplantation when donors are not available, especially in hepatic emergencies such as acute liver failure. Hepatocytes are also needed for drug metabolizing enzyme induction studies *in vitro*. Hepatocytes can be successfully isolated from resected livers and from livers not suitable for transplantation. In many situations, cryopreservation is desired to ship hepatocytes between laboratories and hospitals. Furthermore, hepatocytes isolated from liver samples are produced acutely when a tissue is available and often in larger amounts than immediately needed. In addition, hepatocyte cryopreservation might also be an advantage in research related to stem cell differentiation to hepatocytes[4-6]. Hence, an efficient cryopreservation method for human hepatocytes is essential.

The first fully investigated hepatocyte cryopreservation protocol was published in the 1980s[7,8]. Since then, many groups have put efforts into optimizing the cryopreservation method[9-14]. In spite of such efforts, significant loss of viability and function of hepatocytes after thawing is still a major problem. Quality of the starting liver tissue, warm and cold ischemia times, and hepatocyte isolation protocols may also influence the outcome of the cryopreservation. The cryopreservation process itself also has several components that still need to be fine-tuned in order to get a fully optimized protocol. Preincubation of hepatocytes with anti-oxidants, cryoprotectants included in freezing medium, addition and dilution of freezing medium, cell density in freezing medium, and medium cooling and warming rates are considered to be the most important steps to be adjusted.

Dimethylsulfoxide in the University-of-Wisconsin solution (DMSO-UW) is one of the most widely used cryoprotectant combinations for hepatocyte cryopreservation in many laboratories[15]. Although the theoretical arguments behind using a controlled rate freezer are convincing, many laboratories still use common laboratory polystyrene boxes placed into a low temperature freezer. As a first step, we compared the two methods, controlled-rate freezer (CRF) *vs* a polystyrene box (PSB) in an ordinary -70 ℃ freezer, using only DMSO-UW in hepatocyte preparations from 4 patients.

In a recently published study from our group, we evaluated the use of a new xeno-free cryopreservation solution (STEM-CELLBANKER™, CB) containing DMSO and anhydrous dextrose in cryopreservation of human embryonic and induced pluripotent stem cells[16].

In this study, we compared STEM-CELLBANKER™, CB and standard DMSO-UW medium using the PSB method. The viability of hepatocytes was assessed by two different methods, trypan blue exclusion and live-dead assay.

MATERIALS AND METHODS

Isolation of human hepatocytes

Isolated hepatocytes from thirteen adult liver samples were used in this study. Liver tissue was obtained after partial hepatectomy because of primary or secondary tumors (Table 1). Ethical approval for the study was granted by the Regional Ethical Review Board in Stockholm, Sweden. Hepatocytes were isolated using a three-step collagenase perfusion procedure as described before[17]. In brief, the liver sample was perfused using the following warm (37 ℃) solutions: Hank's Buffered Salt Solution (Cambrex, *in vitro*, Stockholm, Sweden) containing Ethylene Glycol Tetraacetic Acid (Sigma, Stockholm, Sweden); Hank's Buffered Salt Solution only; and finally Eagle's Minimum Essential Medium with Earle's salts (Cambrex, *in vitro*) containing Collagenase XI (Sigma). Digested tissue was then transferred to cold (4 ℃) Eagle's Minimum Essential Medium in a sterile beaker, chopped with scissors, and filtered through sterile gauze. The hepatocytes were collected by centrifugation and the Collagenase removed. Pellets were resuspended and washed twice in cold Eagle's Minimum Essential Medium by centrifugation at 50 g for 5 min at 4 ℃ to obtain hepatocytes.

Assessment of hepatocyte viability

Viability of freshly isolated hepatocytes was compared to thawed hepatocytes cryopreserved using either the CB protocol or the standard DMSO-UW protocol. The viability of the hepatocytes was first estimated using the trypan blue exclusion method[18]. Hepatocytes were diluted in trypan blue (TB) (Sigma) and TB negative and positive cells were immediately counted under light microscopy in triplicates using a hemocytometer. Viability was also estimated using calcein-esterase based Live-Dead Assay (LDA) using LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, United States) according to the manufacturer's instructions. Hepatocytes were washed in phosphate buffered saline and incubated on a

Table 1 Demographic data and viability for patient samples used

Liver	Gender	Age (yr)	Viability (%)	Diagnosis
L1	F	43	75	Deceased donor, head trauma
L2	F	16	74	Deceased donor, head trauma
L3	M	60	83	Deceased donor, anoxia
L4	M	55	73	Deceased donor, head trauma
L5	M	46	84	PSC, CCC
L6	M	72	76	Colorectal metastasis
L7	M	36	80	PSC, CCC
L8	M	49	78	CCC
L9	M	69	68	Gallbladder cancer
L10	F	60	70	Gallbladder cancer
L11	F	65	75	CCC
L12	F	73	83	Colorectal metastasis
L13	F	62	76	Colorectal metastasis

L1-L9: The 9 liver samples used in this study; M: Male; F: Female; PSC: Primary biliary cirrhosis; CCC: Cholangiocellular carcinoma.

cover slip at 37 ℃ for 15 min. The live-dead reagent was prepared freshly each time with a volume of 200 μL added to hepatocytes on cover slips. Hepatocytes were then incubated for 45 min in the dark at room temperature. Thereafter, hepatocytes were carefully mounted on glass slides and examined under the fluorescence microscope (Olympus, IX71, Japan). Three different high power fields were pictured for each sample.

Assessment of hepatocyte function

The activity of the major cytochrome P450 enzymes (CYPs) CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A7 were assessed for freshly isolated hepatocytes and for thawed hepatocytes cryopreserved using either the CB protocol or the standard DMSO-UW protocol. This was done using luminescence-based assays utilizing specific P450-Glo substrates and their specific luciferin detection reagents (Promega, Madison, WI, United States) according to the manufacturer's instructions. In brief, 4×10^4 hepatocytes suspended in William's E Medium (Lonza, Denmark) were incubated with their specific luminogenic substrates on a white opaque 96-well plate (Corning, Costar, United States). Substrate-specific Luciferin Detection Reagents were then added to detect the amount of free luciferin as an indication for different CYPs activity in a luminescence plate reader (BMG LABTECH, FLUstar OPTIMA, Germany). CYP activities were normalized to the amount of double-stranded DNA per well. Samples were transferred to a black opaque 96-well plate (Corning) and freshly prepared PicoGreen Reagent (Quant-iT PicoGreen dsDNA Reagent and Kit) (Molecular Probes) was then added directly to the wells according to the manufacturer's instructions. The plate was incubated in the dark at room temperature and PicoGreen fluorescence was measured at 480 nm^{Ex}/520 nm^{Em} using fluorescence plate reader (TECAN, infinite F500, Austria).

Table 2 Comparison of controlled rate freezer *vs* a polystyrene box in ordinary -70 ℃ freezer

	Fresh	PSB	CRF[a]
L1	75	22	6
L2	74	38	22
L3	83	42	18
L4	73	40	37

Data represent percent ± SD of viable cells using trypan blue exclusion method. [a]$P < 0.01$ *vs* fresh. CRF: Controlled rate freezer; PSB: Polystyrene box.

Controlled rate freezer and polystyrene box

Initially, isolated hepatocytes from four different patients were used to test two different methods of freezing down the hepatocytes. The controlled rate freezer Planer Kryo 10 series Ⅲ model K10/16 using the program described by Diener *et al*[19] was compared to placing the tubes in a closed polystyrene box in -70 ℃. Hepatocytes in UW + 12% DMSO were transferred to 3 mL cryopreservation tubes on ice. Half of the tubes were frozen in a controlled rate freezer and transferred to the vapor phase of a liquid nitrogen tank when the cycle was completed. The other half of the tubes were wrapped in tissue paper and put into a common laboratory PSB. The box was sealed shut with tape and quickly placed into a -70 ℃ freezer. After 2 d, the frozen tubes were transferred to the vapor phase of a liquid nitrogen tank for storage.

PSB freezing and thawing of hepatocytes

Primary human hepatocytes from nine different preparations were cryopreserved using either the CB protocol or the standard DMSO-UW protocol. CB is a new xeno-free, chemically defined cryopreservation solution, containing a mixture of both permeating as well as non-permeating cryoprotectants (ZENOAQ, 1-1 Tairanoue, Sasagawa, Asaka-machi, Koriyama, Fukushima 963-0196, Japan). It contains 10% DMSO, glucose and the high polymer anhydrous dextrose described in the Japanese Pharmacopeia as cryoprotectants. For cryopreservation of hepatocytes using the standard method, a cryoprotection solution composed of 12% DMSO in UW was prepared. Ice-cold freezing solution was then added to the cell pellet in a concentration of 7×10^6 cells/mL. Hepatocytes were brought into suspension by gently inverting the tubes. Cell suspension was distributed to 3.5 mL cryotubes. Cryotubes were transferred to a polystyrene box and kept in a -70 ℃ freezer overnight. Cryotubes were then transferred to liquid nitrogen and kept in the vapor phase. For freezing hepatocytes using the CB protocol, the same procedure, cold CB, was added directly to the cell pellet in a concentration of 2×10^6 cells/mL.

For thawing of frozen hepatocytes, the cryotubes were incubated in a 37 ℃ water bath for 1-2 min until ice crystals started to melt. Hepatocytes were reconstituted in two different ways according to the protocol used. For hepatocytes cryopreserved in DMSO-UW, the

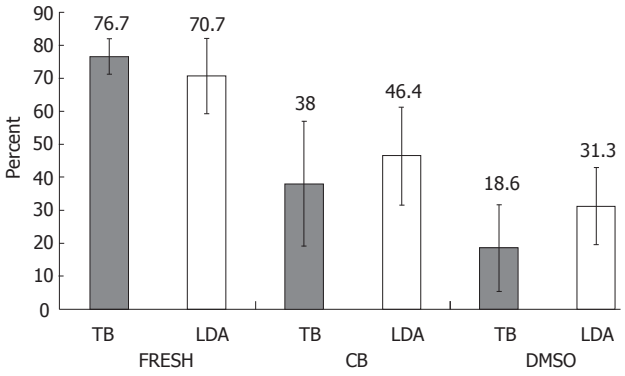

Figure 1 Hepatocyte viability before and after freezing. Viability in the three groups: fresh hepatocytes (FRESH) and hepatocytes frozen using either the STEM-CELLBANKER protocol (CB) or the standard dimethylsulfoxide in the University-of-Wisconsin solution (DMSO-UW) protocol using both the trypan blue exclusion and the Live/Dead Assay (LDA) methods. There was a significant difference in viability between CB and DMSO using the two-way analysis of variance test ($P < 0.05$). TB: Trypan blue.

contents of the cryotubes were transferred to a 50 mL tube. An equal volume of cold William's E Medium was then added gradually to the hepatocytes on ice. This was repeated 3 times, 5 min apart. For hepatocytes cryopreserved in CB, the contents of the cryotubes were similarly transferred to a 50 mL tube. An equal volume of a cold washing solution, a thawing buffer containing NaCl (CELLOTION; ZENOAQ), was directly added to the hepatocytes once on ice. Hepatocytes were then washed twice in cold William's E Medium by centrifugation at 50 g for 5 min at 4 ℃.

Viability and function of thawed hepatocytes from both methods were then assessed as described above for the freshly isolated hepatocytes.

Statistical analysis

Analysis of variance and the non-parametric Kruskal-Wallis tests were carried out using the PASW statistics 18 software. Test results were considered statistically significant when P values were < 0.05.

RESULTS

Controlled rate freezing

Hepatocyte viability for the four samples used to compare CRF to PSB in an ordinary -70 ℃ freezer is presented in Table 2. Preserving hepatocytes in a PSB gives viability (35.5 ± 9.2) not significantly different from the freshly isolated. However the use of CRF gives a significantly lower viability (2.8 ± 12.8) ($P < 0.01$) compared to fresh (76.3 ± 4.6). Therefore, we only used the PSB method when comparing the two cryoprotectants.

Viability of hepatocytes

Viability of hepatocytes from nine liver samples cryopreserved by CB or DMSO-UW protocols was estimated using the two different methods TB and LDA (Figure 1). In the LDA method, live hepatocytes showed "green" fluorescence in their cytoplasm upon active uptake and conversion of calcein AM to the more fluorescent Calcein. Ethidium-1 entered dead hepatocytes through their damaged cell membranes and bound to nucleic acids showing "red" fluorescence in the nuclei. Upon thawing, hepatocytes cryopreserved in DMSO-UW were sticking together in clumps, which were difficult to dissolve in contrast to hepatocytes cryopreserved in CB (Figure 2).

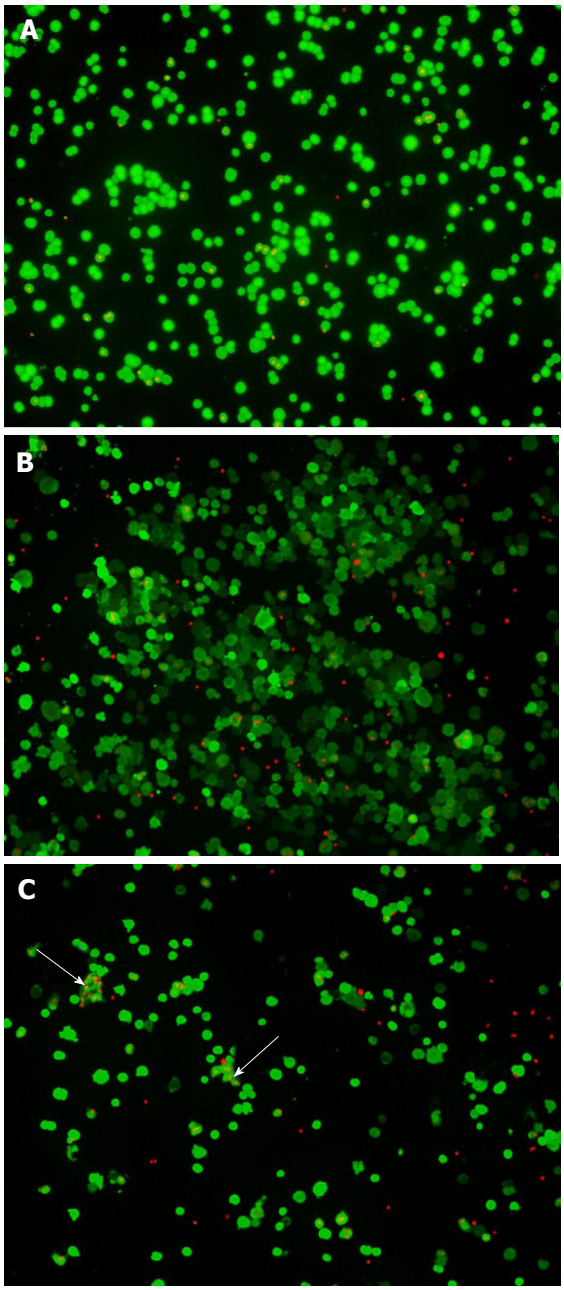

Figure 2 LIVE/DEAD Assay for hepatocytes. Fluorescence staining of the live and dead hepatocytes in fresh hepatocytes (A) and freshly thawed hepatocytes cryopreserved using either the STEM-CELLBANKER protocol (B) or the dimethylsulfoxide in the University-of-Wisconsin solution (DMSO-UW) protocol (C). Live hepatocytes showed "green" fluorescence in their cytoplasm upon their active uptake and conversion of calcein AM to calcein. Ethidium-1 entered dead hepatocytes through their damaged cell membranes and bound nucleic acids showing "red" fluorescence in their nuclei. It was not uncommon to see hepatocytes cryopreserved in DMSO sticking together in clumps (arrows).

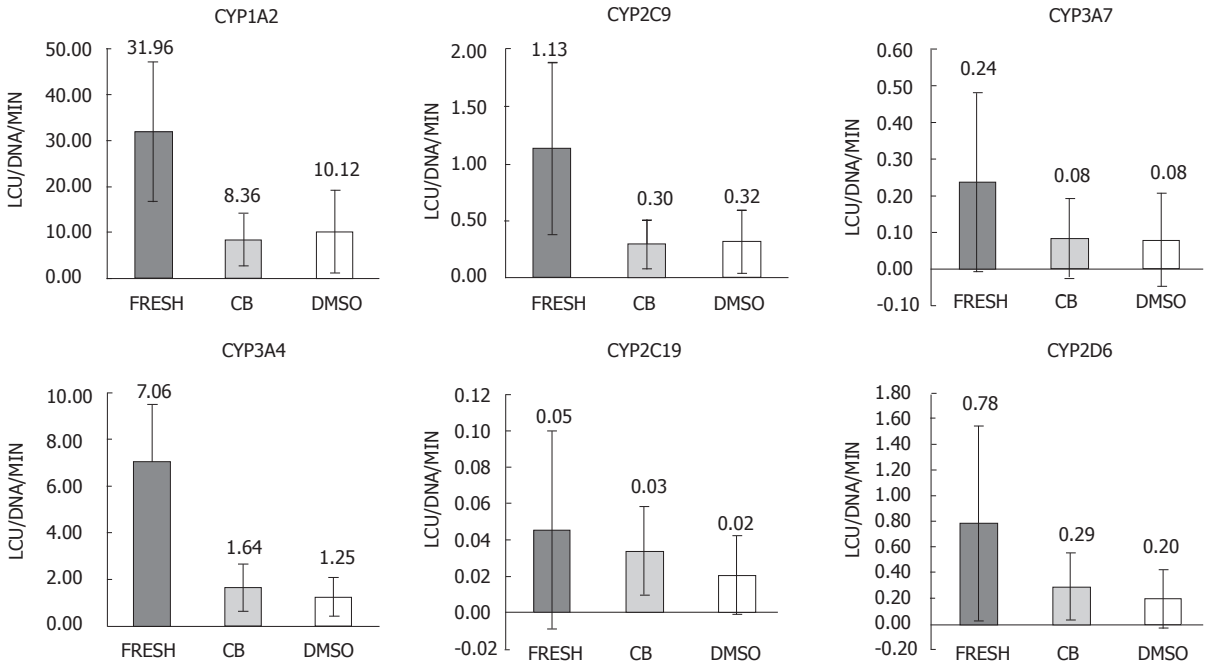

Figure 3 Hepatocyte functionality before and after freezing. Activity of the major cytochrome P450 enzymes; CYP1A2, CYP2C9, CYP3A7, CYP3A4, CYP2C19, CYP2D6 for the fresh hepatocytes (FRESH) compared to hepatocytes cryopreserved using either the STEM-CELLBANKER protocol (CB) or the standard dimethylsulfoxide in the University-of-Wisconsin solution protocol. The standard deviation exceeded the mean in many cases, illustrating the high within-groups variability. Data is presented as luminescence (LCU) per minute per DNA in nanograms. **CYPs: Cytochrome P450 enzymes.**

A two-way analysis of variance was performed to investigate the influence of the two different cryopreservation protocols on hepatocyte viability and if this was influenced by the method used for viability estimation. The change in hepatocyte condition (fresh, cryopreserved in CB, or cryopreserved in DMSO-UW) did have a significant effect on viability; $F(2, 48) = 62.9$, $P < 0.001$, $\eta^2 = 0.724$. In a pairwise comparison, hepatocytes cryopreserved in CB did have better viability after thawing than hepatocytes cryopreserved in DMSO-UW ($P < 0.05$). There was no significant difference in viability estimation between both the TB and the LDA methods; $F(1, 48) = 1.99$, $P > 0.05$, $\eta^2 = 0.040$. There was no significant interaction between hepatocyte condition and the viability estimation method; $F(2, 48) = 2.50$, $P > 0.05$, $\eta^2 = 0.094$, indicating that the difference in hepatocyte viability was mainly due to changes in their condition and not due to the method used.

The higher the viability of fresh hepatocytes, the better the survival was after cryopreservation using the CB protocol. This was indicated by a positive correlation between viability of fresh hepatocytes and the difference in cell viability between CB and DMSO protocols with $R^2 = 0.69$ using the TB method. This might indicate that hepatocytes with good viability preserve better in CB than in DMSO-UW.

Function of hepatocytes

Function of thawed hepatocytes cryopreserved using either CB protocol or DMSO-UW protocol was compared to freshly isolated primary human hepatocytes. The activities of the following CYPs were estimated in three groups; CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A7. CYP activities were normalized to the amount of double-stranded DNA. The within-groups variability was high as manifested by the high standard deviation values that exceeded the mean values in many cases (Figure 3).

One-way analysis of variance was carried out to compare activity of each CYP in the three groups. Changes in hepatocyte condition between the three groups had significant effect on CYP1A2 activity; $F(2, 24) = 12.21$, $P < 0.001$, $\eta^2 = 0.504$. This effect was mainly due to differences between the fresh group and both the cryopreserved groups as there was no statistically significant difference between CB and DMSO-UW groups in pairwise comparisons. For CYP3A4 activity, there was also significant difference between the three groups; $F(2, 24) = 11.90$, $P < 0.001$, $\eta^2 = 0.498$, which was mainly due to differences between the fresh group and both the cryopreserved groups as there was no significant difference between the CB and DMSO-UW groups. Similarly, for CYP2C9 activity; $F(2, 24) = 13.97$, $P < 0.001$, $\eta^2 = 0.538$, and there was no significant difference between the CB and DMSO-UW groups.

There was no significant difference between the three groups regarding activities of the following CYPs; CYP3A7 [$F(2, 24) = 1.38$, $P > 0.05$, $\eta^2 = 0.103$], CYP2C19 [$F(2, 24) = 1.08$, $P > 0.05$, $\eta^2 = 0.082$], and CYP2D6 [$F(2, 24) = 3.35$, $P > 0.05$, $\eta^2 = 0.218$]. There were no statisti-

cally significant differences between the CB and DMSO-UW groups regarding the activities of these CYPs.

DISCUSSION

The potentially high demand of primary human hepatocytes necessitates the need for a fully optimized cryopreservation protocol. Presently, there is no fully optimized cryopreservation protocol for hepatocytes. This is despite many efforts with varying degrees of success[9-14]. Much effort is still needed to be put in testing, for instance hepatocyte pre-incubation with anti-oxidants prior to cryopreservation or including non-permeating cryoprotectants in the freezing solution[20]. In this study, we introduced a new experimentally optimized xeno-free cryoprotectant medium (CB), for the first time for cryopreservation of hepatocytes. In comparison to DMSO-UW, CB is also a xeno-free freezing solution but it further contains both permeating and non-permeating cryoprotectants at carefully tested concentrations[16].

The corner stone in evaluating the success of any hepatocyte cryopreservation protocol is to compare viability of hepatocytes, their function and their plating efficiency before and after freezing[20]. Here, we evaluated viability using two methods, TB and LDA. We also evaluated function of hepatocytes by testing the enzymatic activity of major CYPs isoforms, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A7, before and after freezing.

During hepatocyte cryopreservation, the density at which hepatocytes are frozen may affect their viability on thawing. A cell density between 3 and 10 × 10^6 cells/mL is usually recommended[13]. Lower cooling and higher warming rates usually have a lower incidence of intracellular ice crystal formation that dramatically affects hepatocyte viability after cryopreservation. The rate at which the cryopreservation solution is diluted may affect viability as well[14]. Controlled rate freezing is gaining more interest as a better alternative to using a polystyrene box in -70 ℃ freezer[13,21]. However, in our small pilot study where we compared both freezers, we did not find using the CRF better than the ordinary -70 ℃ freezer. These findings were supported in reports by others[20,22,23] where no difference was shown between CRF, the Nalgene propan-2-ol device or simply using -20 ℃ and -70 ℃ freezers. The aim of our study was to compare the efficacy of two complete cryopreservation protocols, CB protocol and DMSO-UW protocol. There were few differences between the two protocols. The CB protocol had lower freezing cell density compared to the DMSO-UW protocol, while the latter had gradual dilution of the freezing medium upon thawing compared to the CB protocol.

Upon evaluating the viability of hepatocytes before and after cryopreservation using the two protocols, we could conclude that the CB protocol, in addition to being simpler and faster, yielded a better cell survival of the cells in comparison to the DMSO-UW protocol. Using the TB or LDA method in assessing viability of hepatocytes, the results were similar. Hence, it is possible to use only the LDA method in the future because the LDA method had some advantages over the TB method. For example, the active uptake of calcein AM by the live hepatocytes indirectly tests their transport function at the same time. However, one drawback with the LDA is the long time it takes to perform, in contrast to the rapid TB method.

In general, assessing hepatocyte function is not an easy task. Hepatocytes perform a vast number of different functions ranging from energy metabolism, synthesis of proteins and hormones to metabolism of xenobiotics and bile production. Choosing one or a few functions to represent the overall vitality of the cell is therefore difficult. Moreover, the high variability between one liver to another usually makes it difficult to define "the normal liver". There are many reasons for variability: genetic polymorphism, gene expression modulation, the tissue quality, and tissue handling before and during hepatocyte isolation are potential reasons[24]. Gene expression modulation can occur due to various environmental factors e.g. food and xenobiotics. In this study, we could see a high within-group variability depicted by the high standard deviation values that exceeded the mean values in many of the cases.

There was an obvious tendency for hepatocyte function to be higher in fresh in comparison to cryopreserved hepatocytes. The same tendency was seen in the CB group, as well as the DMSO-UW group. This hierarchy was seen in 33 out of 54 comparisons. However, there was no significant difference between fresh and cryopreserved groups in the case of CYP2C19, CYP2D6 and CYP3A7 activities in contrast to the activities of CYP1A2, CYP2C19 and CYP3A4. This might be due to the high within-group variability. In some cases, CYPs activity was higher in cryopreserved hepatocytes compared to fresh hepatocytes or in the DMSO-UW group compared to the CB group. This is in line with what was found by Li *et al*[11] where there was no significant difference between the fresh and the cryopreserved hepatocytes regarding their drug-metabolizing enzyme activities or their bile acid conjugation and secretion[25]. Results from those two previous studies suggested that the functions of the hepatocytes were equally good before and after cryopreservation. In other words, cryopreservation had no impact on hepatocyte function when it comes to their drug-metabolizing enzyme activities[11].

In this study, we conclude that CYP activity might not be the best choice in choosing between different hepatocyte cryopreservation protocols and more stringent measurements of function might be needed when evaluating advanced functions of liver cells. Both protocols tested yielded hepatocytes with good P450 function; however, the CB protocol gave a higher viability than the widely-used hepatocyte cryoprotectant DMSO-UW. CB is also xeno-free and might be useful in cryopreservation of clinical-grade primary human hepatocytes. In conclusion, in this study we show that CB is a good freezing solution for hepatocytes.

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COMMENTS

Background

Efficient preservation of primary human hepatocytes by freezing is very important both for research and clinical purposes. An efficient preservation method is also needed for hepatocytes obtained from stem cells in the future. There are many hepatocyte freezing protocols available with varying degrees of success. Protocols which do not include any animal substances are mandatory for clinical use of human hepatocytes. The different protocols tested different freezing methods and different freezing solutions. Up to now, there is no ideal protocol available for preserving hepatocytes. The new freezing solution the authors are using contains chemicals which protect inside and outside cells during freezing.

Research frontiers

Research in the field of human hepatocyte freezing focuses, among others, on testing several freezing solutions. The new freezing solution they are using was tested before with other cell types with great preservation capacity.

Innovations and breakthroughs

The new freezing solution we tested here has not been tested before with human hepatocytes. It was tested with embryonic stem cells previously in the group and proved to be much more effective than the standard protocol. The results suggest that this new solution can be a better alternative to the standard freezing solution. Being xeno-free makes it suitable for freezing of hepatocytes in clinical settings. The results also argue against using liver cytochrome P450 enzymes in evaluating a given hepatocyte freezing protocol because of the huge between-samples variability and since even dying hepatocytes can still express them.

Applications

The study results suggest that the new freezing solution can be a better alternative to the standard solution in freezing primary human hepatocytes and hepatocytes derived from stem cells.

Terminology

Dimethylsulphoxide (DMSO) is an organosulfur compound used as a solvent and constitutes a very important part in many freezing solutions. University of Wisconsin solution is an intracellular-like preservation medium typically used during organ transplantation. (STEM-CELLBANKER, CB) is the new freezing medium and contains mainly DMSO and anhydrous dextrose. Freezing solutions can protect cells, either by penetrating through their cell membrane (permeating cryoprotectant) or by stabilizing the outside of the cells (non-permeating cryoprotectant). Cytochrome P450 isoforms (CYPs) are drug-metabolizing enzymes present mainly in the liver.

Peer review

Overall, the paper demonstrates very little difference between preservation methods. Viability is low with both storage and freezing methods. Similarly, there is much variability in CYP activities in fresh hepatocytes and frozen hepatocytes. Based on this, it is not clear that this study rises to the level of a full research paper. The authors have miscalculated the viability of controlled-rate freezer hepatocytes in Table 2-the viability is 20.8%, not 2.8%. Additional tests of viability and functionality could be included - MTT, mitochondrial function.

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Events Calendar 2012

January 18, 2012
AHPBA Sponsored Consensus Conference on the Multidisciplinary Treatment of Colorectal Cancer Liver Metastases
San Francisco, CA, United States

January 20-21, 2012
AGA Clinical Congress of Gastroenterology and Hepatology: Practice, Evidence and Quality in 2012
Miami, FL, United States

January 27-28, 2012
28th Annual Meeting of the German Association for the Study of the Liver
Hamburg, Germany

January 30-31, 2012
5th International Conference on the Management of Patients with Viral Hepatitis
Paris, France

February 8-10, 2012
Stockholm Liver Week 2012
Stockholm, Sweden

February 16-19, 2012
22nd Conference of the Asian Pacific Association for the Study of the Liver
Taipei, Taiwan, China

March 16 -17, 2012
Hepatitis Single Topic Conference
Atlanta, GA, United States

March 16-17, 2012
ESGE - Workshop on Advanced Endoscopy with Live Demonstrations
Vienna, Austria

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA, United States

April 18-22, 2012
The International Liver Congress by EASL
Barcelona, Spain

April 27-28, 2012
The European Society for Paediatric Gastroenterology, Hepatology and Nutrition
Stockholm, Sweden

May 16-19, 2012
International Liver Transplant Society 18th Annual International Congress 2012
San Francisco, CA, United States

May 19-22, 2012
Digestive Disease Week 2012
San Diego, CA, United States

June 22-23, 2012
EASL Monothematic Conference: Vascular Liver Diseases
Tallin, Estonia

July 1-5, 2012
10th World Congress of the International Hepato-Pancreato-Biliary Association 2012
Paris, France

September 5-8, 2012
International Congress of Pediatric Hepatology, Gastroenterology and Nutrition
Sharm El-Sheikh, Egypt

September 7-9, 2012
Viral Hepatitis Congress 2012
Macclesfield, United Kingdom

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 14-16, 2012
The International Liver Cancer Association's 6th Annual Conference
Berlin, Germany

September 20-22, 2012
Prague Hepatology Meeting 2012
Prague, Czech Republic

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech Republic

October 18-20, 2012
2nd World Congress on Controversies in the Management of Viral Hepatitis
Berlin, Germany

November 9-13, 2012
AASLD - The Liver Meeting 2012
Boston, MA, United States

November 9-13, 2012
The Liver Meeting - 63rd Annual Meeting and Postgraduate Course of the American Association for the Study of Liver Diseases
Boston, MA, United States

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December 26-28, 2012
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INSTRUCTIONS TO AUTHORS

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Acknowledgments

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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