15.

THE "HEMOLYSIS MODEL" FOR THE STUDY OF CYTO-TOXICITY AND CYTO-PROTECTION BY BILE SALTS AND PHOSPHOLIPIDS

Piero Portincasa, Antonio Moschetta, Michele Petruzzelli, Michele Vacca, Marcin Krawczyk, Francesco Minerva, Vincenzo O. Palmieri and Giuseppe Palasciano*

1. INTRODUCTION

Hemolysis of human erythrocytes is a simple *in vitro* model to assess both toxic and protective properties of different molecules. The method was extensively used to characterize the detergent properties of bile salts. Erythrocyte lysis strongly depends on bile salt concentration.[1] We systematically characterized the protection offered by different phospholipids and elucidated molecular mechanisms underlining this phenomenon. Here we describe the hemolysis model, review its use in lipid research, and underscore the similarities with other commonly used *in vitro* models for cyto-toxicity and cyto-protection.

2. THE "HEMOLYSIS MODEL"

Aliquots of fresh blood obtained from healthy subjects with a syringe containing 100 units of heparin[2] are sedimented 3 times by centrifugation at 3000 rpm for 15 min; plasma and buffy coat are discarded and pellet reconstituted to the original blood volume in TRIS-buffer of the following composition (mM): TRIS 10, NaCl 130, glucose 10, pH 7.4 (Figure 1). Although the buffer solution provides an isotonic environment and glucose sustains erythrocytes activity, experiments must be performed within a few hours to avoid erythrocyte distress. For the incubation, washed erythrocytes are added to testing substances dissolved in the same buffer solution in a 1:4 ratio and final vol. of 1 mL, at 37°C for 30 to 45 min.[2-5] Thereafter, 7 mL of buffer are added to reduce hemolysis to negligible levels. Samples are centrifuged to sediment pellet and the absorbance of

* Piero Portincasa, Antonio Moschetta, Michele Petruzzelli, Michele Vacca, Marcin Krawczyk, Francesco Minerva, Vincenzo O. Palmieri and Giuseppe Palasciano, Clinica Medica "*A. Murri*", Department of Internal & Public Medicine, University Medical School, Piazza G. Cesare 11, 70124 Bari, Italy.

hemoglobin is assayed in the supernatant at 540 nm. Hemoglobin release (a sensitive index of hemolysis) is then normalised to maximal hemolysis of erythrocytes incubated with distilled water.[2-5]

3. BILE SALTS AND HEMOLYSIS

Bile salts are essential lipid components in bile, with phospholipids and cholesterol. Bile salts have a potent intrinsic detergent activity which allows the solubilization of cholesterol and phospholipids in the biliary tract and intestine. This step interaction is essential for intestinal lipid absorption, bile formation and cholesterol disposal.[6] The "bad" side of bile salt,[7] however, is their cellular toxicity which depends on the degree of hydrophobicity[5, 8] and on cell membrane composition.[9] Although bile salt toxicity has long been known, the mechanisms by which bile salts injure cells have been troublesome to elucidate. Since both toxicity and detergency of bile salts increase with increasing hydrophobicity, it was suggested that the toxicity of this molecule was due to plasma membrane disruption. The hemolysis model was particularly suitable to test this hypothesis: in fact, since erythrocytes lack the nucleus and are poor in intracellular organelles, they can not respond to bile salts injury by adapting some tissue-specific pathways such as bile salt uptake, transport or metabolism,[10] and most effects result from interaction between bile salts and the plasma membrane.[11] In pioneering studies, Coleman et al. [1-12] showed that the hemolytic activity of bile salts occurs exclusively at concentrations above their critical micellar concentration and strongly depends on the degree of hydrophobicity. Later, we systematically studied the effects of bile salts hydrophilic-hydrophobic balance on membrane damage.[5] The detergent effect induced by increasing concentrations of various bile salts was analysed: the most hydrophobic taurodeoxycholate caused 100% hemolysis at low concentrations (5 mM), while slightly higher concentrations were needed for the less hydrophobic taurocholate (40 mM). These bile salt concentrations are in line with those measured in the gallbladder bile under normal conditions. In contrast, hydrophilic tauroursodeoxycholate caused only moderate hemolysis, even at very high -not physiological- concentrations (see Figure 2A).[5]

The hemolysis model is also valid to study the protective role of molecules. Heuman et al.[8] demonstrated the protective role of conjugates of ursodeoxycholate on total bile salt toxicity. The protection offered by lecithins against bile salt-induced damage on plasma membrane was the object of investigation by our group.[2, 4] Velardi et al.[4] studied the effect of inclusion of phosphatydilcholine within bile salt micelles on bile salt induced hemolysis: phosphatydilcholine decreased hemolysis induced by 50 mM sodium-taurocholate in a concentration-dependent manner. These studies have a clear pathophysiological relevance. At the concentrations occurring in hepatic and gallbladder biles, bile salts could theoretically damage the hepatocyte, but the biliary phosphatidylcholine plays a complete protective role against this phenomenon. This scenario is further supported by the fact that mice with homozygous disruption of the *mdr*2 gene exhibit severe bile salt-induced hepatocyte damage in vivo. Since *mdr*2 encoded a P-glycoprotein that facilitates the phosphatidylcholine secretion in bile, there is no phosphatidylcholine protecting against bile salt-induced toxicity in the bile of these mice.[13] Interestingly, the amounts of phosphatidylcholine needed to protect against bile salt-induced cyto-toxicity positively correlated with the bile salt degree of hydrophobicity (see Figure 2B);[5] at increasing bile salt hydrophobicity index, both toxicity and amounts of protection needed increase.