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The antibacterial effects of bilirubin on gram-negative bacterial agents of sepsis.

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Abstract

In this study, we investigated the antibacterial effects of bilirubin on certain Gram-negative bacteria using both agar dilution and liquid microdilution methods. Twenty-five strains each of *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* strains, isolated from various clinical samples in the medical microbiology laboratory of our hospital, were evaluated. Stock solutions of bilirubin (5 mg, 10 mg, and 15 mg/dl) (AppliChem, GmbH, Darmstadt, Germany) were prepared for agar dilution method. Aliquots of bilirubin stock solutions were added to liquid brain heart infusion medium in sterile microplates for liquid dilution methods. The average bilirubin MICs were 61.44, 62.72, ≥ 64.00, and ≥ 64.00 μg/mL for *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*. In agar and liquid dilution methods, all tested bacteria grew at all bilirubin concentrations used. Bilirubin had no *in vitro* antibacterial effect on *E. coli*, *K. pneumoniae*, *A. baumannii*, or *P. aeruginosa*.

Keywords: Bilirubin, Antibacterial effect, Newborn, Jaundice, Sepsis.

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Introduction

Bilirubin is an end product of heme metabolism which is released in association with the breakdown of hemoglobin. Previously, the products of heme metabolism considered negligible waste products. But unlike the other livings, only in mammals biliverdin undergoes additional metabolism that consumes the energy to produce bilirubin [1]. Elevated serum bilirubin levels are associated with the manifestation named as hyperbilirubinemi-jaundice in many newborn [2].

The natural antioxidant characteristics of bilirubin were first discovered by Stocker and colleagues [3]. Recent studies have suggested that bilirubin causes oxidative damage at pathological levels but exerts antioxidant effects at physiological levels [4]. Bilirubin can trigger apoptosis of cell cultures and stimulates the inflammatory response to lipopolysaccharide [5]. Bilirubin protected mice subjected to experimental sepsis [6]. Santangelo and colleagues found that bilirubin exhibited antiviral activity when added to infected cell cultures [7]. Bilirubin can be elevated in cases of sepsis, intra-abdominal abscesses from urological, gynecological or gastroenterological origins, and antiviral therapy [8].

Bilirubin has been suggested to be an effective antibacterial, but few data are available. Thus, we explored this topic by measuring the antibacterial effects of bilirubin on certain Gram-negative bacteria using both agar dilution and liquid microdilution methods.

Materials and Methods

Strains and bacterial identification

Gram-negative bacterial agents of sepsis were divided into four test groups according to their species. Twenty-five strains each of *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* strains, isolated from various clinical samples in the medical microbiology laboratory of our hospital, were evaluated. Cultures were inoculated on blood agar, eosin methylene blue (EMB) agar, or chocolate agar, depending on the sample type and incubated by standard conventional microbiological methods [9]. Species identification was performed using the Vitek 2 automated system (bioMérieux, Marcy-l'Étoile, France), and evaluated based upon the recommendations of the Clinical Laboratory Standards Institute (CLSI) [10]. Patient laboratory records

were examined retrospectively. If several isolates were obtained from the same patient, only one was included in the evaluation.

Medium preparation and dilution studies

Agar dilution: Stock solutions of bilirubin (5 mg, 10 mg, and 15 mg/dl) (AppliChem, GmbH, Darmstadt, Germany) containing EDTA and $NaHCO_3$ were prepared and mixed in the appropriate amounts with Mueller-Hinton agar (Oxoid, Hampshire, England) at 50°C, which was used to prepare solid culture plates with 4-mm-thick agar. Bacterial suspensions of McFarland turbidity 0.5 were prepared, and 5-µl aliquots were spotted onto the agar plates within circles 5 mm in diameter (final bilirubin concentrations: 5, 10, and 15 mg/dl). Bacterial growth was evaluated after 18 h of incubation at 35°C. Control plates contained no bilirubin.

Microdilution Test: Bacterial suspensions of McFarland turbidity 0.5 were prepared from 24-h cultures using the Densi-Check 2 system (bioMerieux, Durham, NC, USA) and diluted 1/100 to 5×10^5 colony-forming units (cfu)/ml, as suggested by CLSI [11]. Growth, negative, and positive controls were included. Aliquots of bilirubin stock solutions were added to 120 µl liquid brain heart infusion medium in sterile microplates using an automatic pipette. Serial dilution yielded final bilirubin concentrations of 64, 32, 16, 8, 4, 2, 1, and 0.5 µg/ml. Bacterial suspensions were added to 5×10^5 cfu/ml, and initial optical densities (ODs) at 450 nm were recorded. After an 18-h incubation at 35°C, OD changes were recorded using an EPOC (Biotek, USA) device. The concentration of bilirubin that reduced the final OD value to 50% that of the growth control was defined as the MIC.

Statistical Analysis: *E. coli* ATCC 25922, K. pneumoniae ATCC 13883, A. baumannii ATCC 19606, and P. aeruginosa ATCC 27853 were used as control isolates. Agar and microdilution tests was carried out in triplicate using three samples for all isolates under the same conditions. Descriptive statistics are presented as mean ± standard deviation (SD). One Way ANOVA test was used for comparing the data. Statistical significance was assumed if $p<0.05$. All statistical evaluation was performed using SPSS (Version 17.0 for Windows; SPSS, Inc., Chicago, IL, USA).

Results

Liquid microdilution

The average bilirubin MICs were 61.44, 62.72, ≥ 64.00, and ≥ 64.00 µg/mL for *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* respectively. Between *E. coli* and *K. pneumoniae* MIC values was found differences but there was no statistical significant ($p>0.05$). There was no difference between *A. baumannii*, and *P. aeruginosa* (Table 1). The detected MIC levels were found higher than the amount required that can be used in antibiotherapy.

Table 1: *Statistical analysis of MIC values.*

	Mean	Minimum	Maximum	Standard	Variance
	value	value	value	deviation	
E. coli	61.44	32.00	64.00	8.86.	78.51
K. pneumoniae	62.72	32.00	64.00	6.41.	40.96
A. baumannii	64.00	64.00	64.00	-	-
P. aeruginosa	64.00	64.00	64.00	-	-

Agar dilution

The *E. coli* isolates, which had different resistance profiles, and the control *E. coli* ATCC 25922 were resistant to bilirubin at all levels tested. This was also true of the *K. pneumoniae* isolates and ATCC 13883 control, the *A. baumannii* isolates and ATCC 19606 control, and the *P. aeruginosa* isolates and ATCC 27853 control. Moreover, there was no sensitive clinical isolate in the study at all levels tested.

Discussion

Despite extensive work, it remains unclear whether bilirubin, a heme catabolite, is a non-functional end-product or a biologically significant compound [4]. However, studies on the antioxidant properties of bilirubin have defined treatment threshold values [12]. Certain questions have arisen: “Are phototherapy and blood changes over-prescribed?”, “To what level should the plasma bilirubin level of a newborn be decreased?”, and “Are we interfering with a useful defense mechanism?”

Although advances in neonatal care have reduced complications in and increased the survival of preterm infants, neonatal sepsis is still associated with significant mortality and morbidity, particularly, of low birth-weight infants (<1,500 g) [13]. Such infants have high bilirubin levels. Maisels et al. found that jaundice in 306 newborns was neither the cause of nor a risk factor for bacteremia or sepsis [14]. However, a Nigerian study found that septicemia in preterm infants was associated with high bilirubin levels and high-level mortality [15]. In Turkey, Ergür et al. reported that 11% of infants with prolonged jaundice experienced sepsis and other infections [16].

Increased interest in the antioxidant effects of bilirubin has encouraged work on the antibacterial properties of biliverdin/bilirubin, which are heme catabolites. Overhaus et al. found that intraperitoneal injection of biliverdin protected mice from sepsis-induced inflammation and intestinal dysmotility [12]. Biliverdin decreased intestinal morbidity by selectively decreasing inflammation. Wang et al. found that a single intravenous injection of bilirubin protected mice from mortality and liver dysfunction induced by injection of *E. coli* endotoxin [6]. Recently, Lanone et al. showed that mouse mortality caused by endotoxins decreased as the bilirubin level increased [17]. Supporting such data [18], the presence of high levels of oxidative metabolites of bilirubin in the urine of adult patients with sepsis suggests that bilirubin degradation parallels the oxidative stress of sepsis [19]. Goddard et al.

inoculated group B streptococcus (GBS), coagulase-negative staphylococcus (CNS), and *E. coli* (all isolated from blood cultures) into media containing bilirubin at concentrations of 0-100 µmol/L [20]. *E. coli* was bilirubin-resistant, while GBS and CNS were not. It was suggested that physiological jaundice prevented the growth of Gram-positive bacteria such as GBS and CNS, thus protecting against sepsis in premature infants. In our study we evaluated the effects of bilirubin on Gram-negative agents of sepsis using both agar and liquid dilution methods. All tested bacteria grew at all bilirubin concentrations used; thus, bilirubin had no antibacterial effect.

Santangelo et al. investigated the antiviral effect of bilirubin *in vivo* conditions. Bilirubin at 1-10 µM concentrations significantly decreased the growth of human herpes virus type 1 (HSV-1) and enterovirus 71 (EV 71) in Hep-2 and Vero cells [7]. In our study the absence of any *in vitro* antibacterial effect of bilirubin may indicate that bilirubin is effective only *in vivo*. Furthermore, the antioxidant characteristic of bilirubin may have an *in vivo* antibacterial effect. Also *in vivo* detection of the any antibacterial effect of bilirubin may be useful for the regulation of treatment protocols in neonatal jaundice. In addition, we evaluated only low numbers of bacterial isolates. Device constraints rendered us unable to test bilirubin concentrations greater than 64 µg/ml (liquid culture) or 15 mg/dl (agar culture). Moreover, bilirubin at concentrations exceeding 15 mg/dl is toxic to newborns.

Conclusion

Bilirubin had no *in vitro* antibacterial effect on *E. coli*, *K. pneumoniae*, *A. baumannii*, or *P. aeruginosa*. As explained above, *in vivo* work using animal models is required. Also, different microorganisms should be tested.

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