Review Article

The biology of bilirubin production: overview of detection and inhibition

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Contributions: (I) Conception and design: Both authors; (II) Administrative support: Both authors; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: None; (V) Data analysis and interpretation: None; (VI) Manuscript writing: Both authors; (VII) Final approval of manuscript: Both authors.

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Abstract: Newborn jaundice is a benign condition commonly seen in the first postnatal week of life (or the transitional period). It is primarily due to an imbalance between the rate of production of the yellow-orange pigment bilirubin and its elimination by the liver. Infants with high bilirubin production rates (such as those who are undergoing hemolysis) or with insufficient hepatic bilirubin conjugating ability [such as those with uridine 5'-diphosho-glucuronosyltransferase (UGT1A1) deficiencies] can subsequently develop excessive circulating total serum/plasma bilirubin (TB) levels or hyperbilirubinemia. Bilirubin is formed during the degradation of heme, derived from the turnover of red blood cells (RBCs). In this reaction, which is catalyzed by the rate-limiting enzyme heme oxygenase (HO), carbon monoxide (CO), iron (Fe^{2+}), and bilirubin are produced in equimolar quantities. As a result, measurements of total body CO production rates can be used as indices of bilirubin production. Standard treatment strategies for hyperbilirubinemia involves the use of phototherapy (specifically narrow-band blue wavelength light) and/or exchange transfusion. However, if infants with excessive hyperbilirubinemia are not identified or treated in a timely manner, they are at risk for developing bilirubin neurotoxicity, which can manifest as bilirubin-induced neurologic dysfunction (BIND) and result in neurologic sequelae (such as acute or chronic bilirubin encephalopathy. Here, we review the biology of bilirubin production and current technologies and approaches to identify and treat these high-risk infants.

Keywords: End-tidal carbon monoxide (end-tidal CO); hemolysis; hyperbilirubinemia; jaundice; metalloporphyrin

Received: 24 January 2021; Accepted: 01 March 2021; Published: 28 May 2021.
doi: 10.21037/pm-21-8
View this article at: http://dx.doi.org/10.21037/pm-21-8

Introduction

Neonatal jaundice is a syndrome arising from many different causes, but it can be easily understood by an analogy to a sink (*Figure 1*) (1). The turned-on spigot represents the process of bilirubin production and the drain represents the process of bilirubin elimination. The volume of the sink represents the capacity of the circulation to store bilirubin, which is determined primarily by the amount of albumin, the main binding protein for bilirubin, in the blood. Thus, if the rate of bilirubin production exceeds the rate of bilirubin elimination, then the level of total serum/plasma bilirubin (TB) in the circulation begins to rise (1,2). Moreover, when the ability of albumin to bind bilirubin is exceeded, the sink begins to "overflow"—representing bilirubin moving from the circulation into tissues. This latter phenomenon becomes manifest in the skin and conjunctiva as "jaundice," but also occurs in other tissues less easily seen with the naked eye, for example, the brain. The accumulation of bilirubin in discrete regions of the brain (i.e., globus pallidus) can lead to the syndrome of bilirubin-induced neurologic dysfunction (BIND) (3-5)

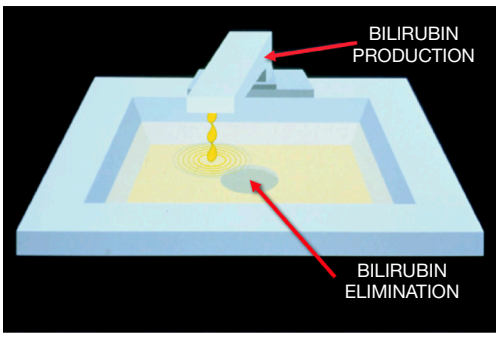

Figure 1 Syndrome of neonatal jaundice. Reproduced from Stevenson DK, Dennery PA, Hintz SR. Understanding newborn jaundice, *J Perinatol* 2001;21 Suppl 1:S21-4; discussion S35-9 with permission from Springer Nature.

and to acute or irreversible—known as "acute bilirubin encephalopathy (ABE)"—or chronic or permanent [traditionally called "kernicterus" or now "chronic bilirubin encephalopathy (CBE)"] clinical manifestations (6).

Simply put, jaundice cannot occur without first, the production of the pigment and second, the excessive production of the pigment relative to its elimination, as is the case in most neonates in the transitional period after birth (1,7). This circumstance is fundamental to the occurrence of all kinds of jaundice, physiologic or pathophysiologic. Most pathophysiologic jaundice requiring treatment is caused by increased bilirubin production rates (reflected as hyperbilirubinemia) above what is considered normal in all term newborns (approximately 2 to 3 times higher compared to an adult) (8), which is caused by a relatively large red cell mass and a shorter red blood cell (RBC) lifespan as a function of age-in-hours (9). In preterm infants, the RBC mass is slightly less than term infants, but the RBC lifespan is shorter resulting in corresponding higher bilirubin production rates on a body weight basis (1,10). Thus, the traditional epidemiologic risk factors for neonatal jaundice are easily understood, such as hemolysis, bruising, closed-space bleeding, and polycythemia, as they all contribute to an increase in heme catabolism and thus increased production of bilirubin (1,2,7). Of course, in all newborns, the conjugation of bilirubin is transiently impaired (and again even more immature in preterm infants), and together with any genetic polymorphisms that contribute to impairment of uridine 5'-diphosho-glucuronosyltransferase (UGT1A1) activity (11,12), result in further impairment of bilirubin elimination and can further exacerbate the risk for neonatal jaundice in the context of increased bilirubin production rates.

The spigot in the analogy is the heme oxygenase (HO)-catalyzed step in the two-step process of heme catabolism (13). This reaction, which occurs in the endoplasmic reticulum, requires NADPH donated from the cytochrome P450 system and molecular oxygen and results in the equimolar production of carbon monoxide (CO), iron (Fe^{2+}), and biliverdin, which is rapidly reduced in the cytosol to bilirubin. Because the reaction is ubiquitous and occurs in all nucleated cells, it would be impossible to know the total body production rate of bilirubin, except that CO is bound to hemoglobin, forming carboxyhemoglobin (COHb), is transported to the lungs and is continually excreted in breath (14,15). With certain assumptions about steady state, the CO excretion rate in breath, the end-tidal CO (ETCO) concentration, and the COHb concentration, when corrected for inhaled CO (ETCOc and COHbc, respectively) can each be used as indices of total body bilirubin production rates (2,16), making it possible to identify infants who are high producers of the pigment (2), and are therefore also at higher risk of neurologic injury (see below). To this end, various technologies and devices have been developed (2,16).

The association between increased bilirubin production and the risk for bilirubin neurotoxicity exists because babies with increased production of the pigment, for example due to hemolysis, are more likely to have greater amounts of bilirubin outside the circulation and in tissues as they exceed their bilirubin binding capacity (BBC) more rapidly (17-19). This phenomenon is often reflected in a rapid rate of rise in the circulating TB levels, and has been empirically taken by clinicians as a sign of increased bilirubin production (20,21). Moreover, two apparently identical TB levels can represent two very different situations of risk for neurologic injury, independent of the actual TB level (1). Consider the well, breastfeeding infant who reaches a TB level of 20 mg/dL at the end of the first week of life, and the hemolyzing infant who reaches the same level at 24 h of life (1). Because of individual variations in conjugating capacity among infants, not all high producers become hyperbilirubinemic to the point of requiring intervention, but most will at least become visibly jaundiced. However, high producers of the pigment, if they require treatment with phototherapy or exchange transfusion, are more likely to have rebound hyperbilirubinemia after treatment (22).

The most common treatment for neonatal hyperbilirubinemia is phototherapy, which was first

suggested as a therapy in 1958 by Cremer (23). Of course, there are alternative therapeutic approaches, such as exchange transfusion, and other less common treatments. However, phototherapy is very effective with currently available devices, making other options less necessary today in most cases. It works because the bilirubin molecule interacts with certain wavelengths of light (peak absorbance at 478 nm) and undergoes photo-oxidation (a minor effect) and structural and configurational isomerization, the latter very rapidly (24,25). These products can be eliminated without conjugation, thus bypassing the temporary impairment in elimination because of immaturity or a genetic polymorphism affecting UGT1A1 activity (11,12). So what is the rationale for why inhibition of bilirubin production might be an alternative to phototherapy, at least in some cases?

In 2008, a paper described a randomized clinical trial of aggressive versus conservative phototherapy for infants with extremely low birthweight (ELBW) (26). There had not been a large trial of this sort since the large National Institutes of Health collaborative trial (27) reported on the efficacy of phototherapy to prevent exchange transfusion in predominately larger, more mature infants. However, in that trial, there was the suggestion that phototherapy might not be safe in the smallest babies, although the trial was not designed for making this determination. In the 2008 trial (26), the primary outcome was that aggressive phototherapy did not significantly reduce the rate of death or neurodevelopment impairment. Nonetheless, there were two planned secondary analyses: one showed that the rate of neurodevelopmental impairment alone was significantly reduced with aggressive phototherapy, confirming the importance of limiting the rise of TB levels in these infants; and the other showed that this reduction was offset by an increase in mortality among infants weighing 501 to 750 grams at birth, suggesting that there might be an adverse effect of using light. The paper raised the possibility that what Bill Silverman had described as "ambitious overgeneralization" had occurred in the case phototherapy (28). We, as a profession, had "overgeneralized" the use of phototherapy extending its application to ever smaller and more translucent patients for longer and longer timeframes without studying its safety. We should have asked the question sooner, "*Is it possible that visible light has adverse effects in small premature infants?*" The paper reinforced the need for reconsidering how best to apply phototherapy safely in these infants, resurrecting the notion of cycled phototherapy to reduce the dose of light as well as restrict the wavelengths of light to those known to be most effective with respect to interacting with the bilirubin molecule, namely narrow-wavelength blue light (29). But the paper also suggested a rationale for wanting to avoid phototherapy altogether in these very immature, antioxidant-deficient, small translucent patients (30,31), and consider a pharmacologic approach to control rising TB levels, such as inhibition of bilirubin production (32). Although controlling the spigot (*Figure 1*) would seem to be a conceptually simple and rational approach, it presents some challenges which are the subject of the subsequent discussion below.

Although the HO-mediated catabolic pathway for heme has been generally considered to be a source of potential toxins, including bilirubin, CO, and Fe^{2+}, causing neurologic disturbances (33), mitochondrial dysfunction (34), and reactive oxygen species (ROS) production (35), respectively, it has many other roles in biology (33). For example, the biliverdin-bilirubin shunt has antioxidant, anti-inflammatory, and anti-apoptotic effects, and is important is maintaining the redox state of the cell (36). CO is an important signaling molecule in its own right, causing vessel relaxation through calcium and potassium dependent channels, as well through soluble guanylyl cyclase (sGC) and cyclic GMP, and mediating additional anti-platelet, anti-apoptotic (endothelial cells), anti-proliferative (vascular smooth muscle cells), and neurotransmission effects (37). CO also acts through p38MAPK to cause inhibition of pro-inflammatory cytokines, such as tissue necrosis factor-alpha (TNF-α) (38), and through vascular endothelial growth factor (VEGF) to stimulate angiogenesis (39). Finally, even Fe^{2+} with its binding to ferritin and the iron ATPase pump can have antioxidant, anti-inflammatory and anti-apoptotic effects (40). So wholesale inhibition of HO could have a myriad of potential adverse side effects while trying to modulate bilirubin production for the purpose of controlling TB levels during the transitional period after birth (41).

There are also a number of endogenous sources of CO (15). Heme degradation accounts for about 86% coming from senescing RBCs (~70%), ineffective erythropoiesis (~9%), and other hemoproteins (~21%) (42). The remaining 14% comes from lipid peroxidation (variable) and photo-oxidation (variable) (42). The latter sources can be quite large under pathologic conditions, such as lung injury or infection (43). Estimates of total bilirubin production by measuring CO excretion in

breath, ETCOc, or COHbc levels have been performed in rodents and primates, including human infants (2,16). The validity of the method has been proven in a rat model of hemolysis in which a precise amount of heme can be recovered as CO in the breath of the animals. However, in most circumstances, certainly in clinical settings, the estimates are only approximations of the actual production rate of bilirubin because the exact contribution from non-heme sources cannot be known for certain without labeling techniques, which are not feasible in the clinical setting or without labeling of the carbon atom in the animal models (44,45). Nonetheless, estimates of total bilirubin production have been made for most clinical conditions in the human newborn. A variety of ETCOc devices have been used for estimating bilirubin production in babies (16).

With tools to estimate total bilirubin production, the ability to test drugs that could inhibit the process could be easily screened. The most important category of drugs for this purpose has been the heme analogs or metalloporphyrins (Mps), which are competitive inhibitors of HO (46-50). The criteria for selecting one of these compounds, most of which are synthetic, include the following desirable characteristics: contains a biocompatible central metal, potent HO inhibition, negligible degradation, negligible inhibition of other enzymes, negligible photoreactivity, optimal duration of action, and negligible HO upregulation (49). The HO-1-*luciferase* (-*luc*) transgenic mouse was created in order to monitor the effect of such inhibitors on HO-1 gene expression in living mice treated with the compounds by monitoring the emission of photons (bioluminescence) as HO-1 was expressed (51). This technique is useful for studying *in vivo* expression patterns and served originally as a model system for this technology because the HO reaction is tightly regulated due to the toxicity of CO, Fe^{2+}, and bilirubin; tissue-specific expression; developmental regulation; its potential as a target for therapy; and the fact the *ex vivo* assays are slow and provide only a "snapshot" of the biological process (52). Thus, rapid screening for homozygosity is possible with bioluminescence imaging (BLI), and selected Mps could be easily screened for the effects on *HO-1* gene expression (53). Although tin mesoporphyrin (SnMP) had many desirable characteristics, especially potency, and was introduced into clinical trials (54-57), it is photoreactive, had a protracted duration of action, and was not rapidly excreted or metabolized (58). Its approval for human use has been delayed for this reason (57,58). Zinc protoporphyrin (ZnPP), although less potent, is still effective, short-acting, and can be metabolized with release of an essential trace metal (49). Moreover, it is naturally occurring in humans (59). Studies in Rhesus monkeys (60) and newborn rats (61) with increased bilirubin production caused by hemolysis demonstrated its efficacy. Because it is difficult to keep in solution, a special formulation had to be prepared for its administration which could be delivered orally, giving it one more desirable characteristic (62,63). Compared to various possible formulations, a lipid preparation of ZnPP had adequate *in vitro* inhibitory potency and no chemical toxicity or phototoxicity (63).

Collectively, these studies have set the stage for further animal and ultimately human studies in order to ensure safety and efficacy. Notably, the ZnPP lipid formulation looks promising as an inhibitor of *in vivo* bilirubin production in a heme-loaded newborn mouse model (62). Combined with the identification of high bilirubin producers, such an approach would revolutionize the management of neonatal jaundice and provide a viable alternative to phototherapy where its risks might outweigh its benefits in the ELBW babies. Moreover, a new therapeutic paradigm might be possible. It would begin with identification and isolation of fetal cell-free (cf) RNA or cfDNA in the circulation of the mother (64,65), the creation of a genetic profile of jaundice risk looking for polymorphisms associated with decrease UGT1A1 activity (12), or HO-1 polymorphisms (66) followed by confirmation of any existing pathophysiology with noninvasive monitoring after birth, and targeting individual babies for chemoprevention, thus avoiding phototherapy altogether or at least minimizing its use. In this way, all the toxic effects of the HO/CO pathway might be mitigated while all the beneficial effects are retained.

Knowing whether a baby is a high producer of bilirubin is important for the clinician and should be available as a clinical measure, as it can be used to identify babies who would be likely to benefit from inhibition of bilirubin production. This approach would be disruptive to existing markets for phototherapy and change dramatically the management strategies for ELBW infants with hyperbilirubinemia.

Acknowledgments

Funding: This work was supported by the Charles B. and Ann L. Johnson Research Fund; the Christopher Hess Research Fund; the Providence Foundation Research Fund; the Roberts Foundation Research Fund; and

the Stanford Maternal and Child Health Research Institute.

Footnote

Provenance and Peer Review: This article was commissioned by the editorial office, *Pediatric Medicine* for the series "Neonatal Jaundice". The article has undergone external peer review.

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at https://pm.amegroups.com/article/view/10.21037/pm-21-8/coif). The series "Neonatal Jaundice" was commissioned by the editorial office without any funding or sponsorship. DKS serves as the unpaid Guest Editor of the series and serves as an unpaid editorial board member of *Pediatric Medicine* from Oct 2020 to Sep 2022. RJW serves as the unpaid Guest Editor of the series. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/pm-21-8
Cite this article as: Stevenson DK, Wong RJ. The biology of bilirubin production: overview of detection and inhibition. Pediatr Med 2021;4:16.