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REVIEW ARTICLE

Review of bilirubin neurotoxicity I: molecular biology and neuropathology of disease

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Despite the availability of successful prevention strategies to prevent excessive hyperbilirubinemia, the neurological sequelae of bilirubin neurotoxicity (BNTx) still occur throughout the world. Kernicterus, encephalopathy due to BNTx, is now understood to be a spectrum of severity and phenotypes known as kernicterus spectrum disorder (KSD). A better understanding of the selective neuropathology and molecular biology of BNTx and using consistent clinical definitions of KSDs as outcome measure can lead to more accurately predicting the risk and causes of BNTx and KSDs. In Part I of our two-part review, we will summarize current and recent advances in the understanding of the selective neuropathology and molecular biology of the disease. Herein we emphasize the role of unbound, free unconjugated bilirubin as well as genetic contributions to the susceptibility BNTx and the development of KSDs. In Part II, we focus on current and possible novel methods to prevent BNTx and ABE and treat ABE and KSDs.

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BACKGROUND

There is for some the misconception that kernicterus is a disease of the past. Families of children with kernicterus spectrum disorders (KSDs) are frequently told this by physicians leading to delays in treatment, underdiagnosing, and underreporting. Sadly, preventable cases of KSDs continue to occur because of a lack of concern and failure to follow established local guidelines for the management of neonatal hyperbilirubinemia in term and near-term infants.[1,2]

In the past, kernicterus was common, estimated to cause about 10% of "cerebral palsy" (CP a.k.a., static encephalopathy), notably the dystonic or athetoid form of CP. With the description of the selective neuropathological syndrome of kernicterus, the subsequent understanding of the role of bilirubin in producing kernicterus,[3] the importance of bilirubin binding and pH,[4–10] the adaptation of effective treatments to prevent excessive neonatal hyperbilirubinemia,[3,11,12] and the understanding and treatment and prevention of Rh disease, kernicterus became a rarity. However, kernicterus did not completely disappear. This misconception is likely because major symptoms of KSDs, especially dystonia, may not appear for months after experiencing severe hyperbilirubinemia, and there was a time when objective methods of determining bilirubin neurotoxicity (BNTx) in neonates were unavailable. Current methods of determining clinical signs of acute bilirubin encephalopathy (ABE) include the subjective BIND-A and BIND-M scoring systems[13] and the objective automated brainstem response (ABR) as well as magnetic resonance imaging (MRI) of the brain. All these tests can be performed in the first few weeks of life and have greatly improved the ability of clinicians to successfully diagnose kernicterus in the neonatal period. Finally, without a clear definition of or diagnostic criteria for kernicterus, evidence-based outcome studies suffered from a lack of meaningful outcome measures. Thus the outcome measure for trials of different treatments of neonatal hyperbilirubinemia was either prevention of hyperbilirubinemia or ABE but rarely severity of KSD. Note that KSDs may occur in children who never showed signs of ABE neonatally, especially in those born prematurely.[14]

Data on the incidence of kernicterus after hyperbilirubinemia has been scarce until recently. Mollison and Cutbush's landmark 1954 paper was the sole outcome study that linked the maximum total bilirubin concentration to the number of cases with kernicterus in newborns with Rh hemolytic disease (Fig. 1). Note that kernicterus developed in 1 of 13 (8%) with total bilirubin (TB) 19–24 mg/dL increasing to 8 of 11 (73%) with TB 30–40 mg/dL. Note that the etiology was Rh isoimmune hemolytic anemia in the 1950s (or earlier) and Rh disease is known to carry a greater risk of BNTx for a given TB, possibly due to more rapid hemolysis and formation of bilirubin. Also notable was that no definition of kernicterus was given and no method for how the diagnosis was obtained.

Kernicterus remains a serious problem and cause of disability worldwide.[15–17] Even in developed countries with neonatal surveillance and prevention programs, the incidence can be as high as 1 per 40,000 live births,[18,19] which predicts an estimated 96 new cases of classical kernicterus per year in the USA. Even though rare, the result with most severe classic kernicterus is a cognitively normal individual afflicted with a lifetime of painful muscle cramps and loss of all voluntary movements and communication. Because the incidence of KSDs is much higher in low-and-middle-income countries, and in environments with poverty, famine, war, and other conflicts, any strategy to eliminate ABE and KSDs must account for environmental risk factors, available resources, as well as genetic risk factors, e.g., high rates of G6PD deficiency specific to a population.

One of the concerns for a prevention program is that its success in preventing KSDs will lead to a return to complacency. It is well

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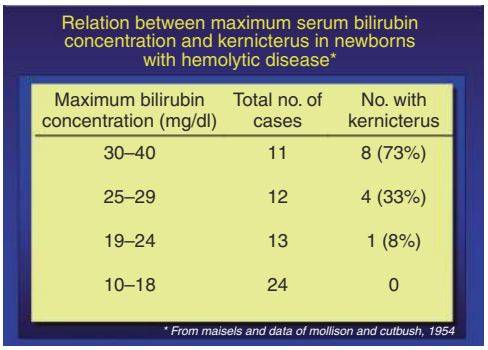

Fig. 1 Relation between maximum serum bilirubin concentration and kernicterus in newborns with hemolytic disease. As cited by Maisels MJ in Avery's Neonatology: Pathophysiology and Management of the Newborn, 2015 (ISBN-13: 978-1451192681 ISBN-10: 9781451192681), Ref# 122. Mollison PL, Cutbush M. Haemolytic disease of the newborn. In: Gairdner D, ed. Recent advances in pediatrics. New York: P. Blakiston & Son, 1954:110

to be reminded that neonatal hyperbilirubinemia is part of our biology, it has some benefits, e.g., as an antioxidant, and it will therefore never be eliminated. Therefore, even if kernicterus were to disappear completely, if we are not vigilant, it will inevitably return. Understanding of BNTx is important not only for prevention but also for treatment of ABE and KSDs. We still treat symptomatic hyperbilirubinemia and ABE with basically the same methods we have used for over 50 years, and treatments for KSDs are still lacking.

CLINICAL AND PATHOLOGICAL CONSIDERATIONS OF THE KSDS RELEVANT TO BNTX

Clinical and pathological considerations are important for understanding KSD, the sequelae of BNTx, and assessing the information that can and cannot be obtained by different experimental models. Specifically, the selectivity for certain brain regions or the developmental susceptibility of BNTx may be assessed in some models and not others, and the reader should be aware of what issues a particular experimental model can and cannot address.

Clinically classic kernicterus presents as (1) motor symptoms with abnormal movements and tone, (2) auditory symptoms of auditory neuropathy spectrum disorder (ANSD) with or without hearing loss, (3) oculomotor impairments, and (4) dental enamel dysplasia of the deciduous teeth.[20] The motor tone abnormality is dystonia with slow writhing movements, athetosis, sometimes accompanied by the faster movements of chorea due primarily to lesions of the basal ganglia localized to the globus pallidus (GP) and subthalamic nucleus (STN). The motor and auditory symptoms may range from subtle to severe. The dental enamel dysplasia is variably present. Cognitive function is invariably intact unless there has been an additional neuronal injury.

Bilirubin toxicity is brain region-selective in humans and our jj-sulfa Gunn rat model. Pathological studies from human autopsies and the Gunn rat show specific abnormalities of brainstem nuclei, basal ganglia, hippocampus, and cerebellum.[21–30] The brainstem nuclei affected are the auditory (cochlear, superior olivary, trapezoid body, lateral lemniscus, inferior colliculi), oculomotor, and vestibular. Primarily affected related to motor control are the GP and STN in the basal ganglia and the cerebellar Purkinje cells.[26,27] In addition, the hippocampal CA2 region has been seen to exhibit more intense bilirubin deposition than the CA1 or CA3 regions.

Understanding BNTx through nomenclature

Clinical efforts to understand BNTx and prevent KSDs suffer from a lack of definitions of outcomes. Consistent definitions are important for outcomes-based research. Over the past decade and a half, we have proposed standard definitions.[20,31,32] In previous publications, we proposed and defined clinical criteria for the clinical spectrum of kernicterus and used the terms kernicterus, chronic bilirubin encephalopathy, and bilirubin-induced neurological disorders (BIND) somewhat interchangeably.[20,31,33] In discussing the clinical spectrum of kernicterus, we have now abandoned the use of chronic bilirubin encephalopathy in favor of KSD because chronic bilirubin encephalopathy incorrectly suggests an ongoing neurological injury, which does not occur after the acute event, ABE. The term BIND has also been used inconsistently to mean either all KSDs or just mildly affected individuals.[32] We have also suggested criteria to indicate the probability of a neurological disorder as being due to BNTx, i.e., being a KSD, and further classified the subtypes and severity of the KSD. We are beginning to validate these definitions in patients referred to our clinic using historical data, physical examination, objective auditory criteria for ANSD, and specific, arguably pathognomonic MRI findings.

Auditory system

In the auditory system, the brainstem auditory nuclei, as well as the primary afferent auditory neurons in the spiral ganglion, are affected in BNTx.[28,33] The abnormalities of KSDs are different from the more common causes of hearing loss that are localized to the middle ear (conductive) or the hair cells of the inner ear (sensorineural hearing loss). Clinical neurophysiological testing with ABRs (also known as BAEPs) assesses the fast-conducting auditory nerve and brainstem fibers, while cochlear microphonic (CM) and otoacoustic emissions assess the hair cells and the basilar membrane of the cochlea. The auditory sequelae of BNTx, abnormal or absent ABRs with normal CMs,[33] define the clinical syndrome of ANSD. Note that lesions of the brainstem nuclei and nerve with kernicterus are beyond the resolution of conventional MRIs. Neurophysiology, i.e., ABRs, in humans and the animal models of kernicterus are highly sensitive to BNTx and indicate the auditory brainstem as more sensitive than auditory nerve to BNTx.[33]

In the cochlea, primary auditory bipolar neurons synapse on the receptor inner ear hair cells. These neurons have cell bodies in the spiral ganglion of the cochlea, traverse the auditory nerve to the brainstem, and synapse in the cochlear nuclei in the pons. A report using rat organotypic brain culture (OBC) slices showed that auditory nerve fibers and vestibular nerve endings were destroyed with exposure to bilirubin in culture.[34] Based on a dose–response curve that peaked at 250 µM bilirubin (14.6 mg/dL), inner ear neurons and fibers were shown to be more sensitive to bilirubin-induced neurotoxicity than sensory hair cells.[34]

Similarly, electron microscopy and immunohistochemistry of the spiral ganglia, auditory nerve in sulfonamide-treated Gunn rats from our laboratory, have shown that cell bodies of the primary afferent neurons in the spiral ganglia and large diameter axons in the auditory nerve are preferentially affected by BNTx.[30] Furthermore, abnormal immunohistochemistry of brainstem auditory[29] and vestibular[28] nuclei, as well as electrophysiology evidence,[24,25] argue that the auditory brainstem is involved. Evidence from human brainstem auditory-evoked potential recordings supports this hypothesis. This combined evidence helps explain the phenotype of ANSD seen in KSD patients, although a mechanism for this selectivity still needs to be fully elucidated.

Motor system

Damage to the GP is believed to underlie dystonia in kernicterus. This is evidenced by the fact that dystonic CP due to hyperbilirubinemia is highly correlated to bilateral hyperintense lesions in the GP on T2-weighted MRI.[20,31,35,36] Deep brain stimulation used to treat some dystonia is usually placed

downstream in the GP interna. Moreover, autopsy studies indicate a decrease in GP neurons marked with the calcium-binding protein parvalbumin (PV),[37] and we have previously reported decreased PV immunoreactivity in brainstem nuclei of dystonic jj-sulfa Gunn rats.[28,29] The STN is also frequently reported damaged.[37] It has been hypothesized that dystonia in kernicterus is caused by destroying the output of the GP, which reduces inhibitory input to the motor thalamus leading to the excessive movement of dystonia.[31,38] Since 95% of the neurons in the GP are gamma-aminobutyric acid (GABA)-ergic and among these, 50–60% co-express PV[39–42] and project to the STN and substantia nigra,[40,43] we have proposed these PV-GABA neurons in the GP as the specific target of BNTx causing the disabling dystonia. Based on these findings, we hypothesize that the degeneration of GP neurons, especially PV-GABAergic neurons, is the most important cause of dystonic kernicterus.

We believe that replacing the damaged cells by transplanting stem cells or neural progenitor cell differentiated into phenotype (s) that resemble these damaged neurons and inducing them to connect to normal targets over a relatively short neuroanatomic distance will be a promising strategy to restore motor function. The tools currently exist to use specific transcription regulators to identify PV-GABA and other types of GP neurons in animal models and then to create and transplant with specific neural progenitor cells. We see these as preclinical studies that may lead to so-called stem cell treatment for this devastating condition of dystonic kernicterus cerebral palsy. The fact that kernicterus is a static encephalopathy and not a chronic progressive encephalopathy and that individuals with severe classical and/or motor-predominant KSDs have remarkably similar clinical symptoms and neuropathology gives individuals with this type of KSD and their families hope that a treatment found effective in one or a few individuals will be effective for most or all.

PATHOPHYSIOLOGY OF BNTX IN THE NEONATE

Region-specific damage of the brain has long been known as a hallmark of BNTx and distinct sequelae of severe KSD. Studies in both humans and rodents have shown that bilirubin deposition occurs in the basal ganglia, cerebellum, hippocampus, and brainstem, including the pons and midbrain and more as described above in section "Clinical and pathological considerations of the KSDs relevant to BNTx". For a detailed review of currently available in vitro and in vivo models of hyperbilirubinemia and kernicterus, see the recently published review in *Pediatric Research*.[44]

Recent work from Dal Ben et al.[45] using OBC slices from four regions of rat pups showed that the hippocampus was the most sensitive of these regions to BNTx followed by the inferior colliculus, cortex, and cerebellum as measured by lactate dehydrogenase (LDH) release (LDH release is a marker for damage to cells and tissue as it leaks from damaged membranes), Hoechst staining, and methylthiazolyldiphenyl-tetrazolium bromide (MTT) (MTT dye is used to assessing cell metabolic activity) assay. The response to BNTx was significantly increased in 8-day-old (P8) compared to P2 OBCs and corresponded with previously identified regions of bilirubin deposition.

Dose of bilirubin

It is well established that unconjugated bilirubin (UCB) is the toxin in BNTx and that unbound or free UCB (Bf) is the best measure of BNTx in vitro and in vivo.[46,47] A landmark review by Ostrow et al. in 2003 in *Pediatric Research*[48] revealed that most in vitro studies of neurons and astrocytes were done at vastly higher UCB levels than neonates are exposed to in vivo. A meta-analysis of in vitro studies of neurons and astrocytes done at clinically relevant Bf values revealed that at Bf > 70 nmol/L UCB might be toxic.[46,47] After this, most in vitro studies with Bf values in the range of 70–140 nmol/L led to more consistent results. Ostrow also made the point that high UCB concentration in vitro with short exposure does not equal low UCB concentration with a long exposure. He found that in a Bf concentration of 140 nmol/L in aqueous solution for 1 h about 50% is dissolved and 50% is aggregated, whereas at 55 nmol/L, below the estimated aqueous saturation of 70 nmol/L, for 3 h bilirubin is 100% dissolved (J. Donald Ostrow, personal communication). While these results have led to changes in the in vitro methodology, they are specific to the presence of albumin in the culture media and as such may not be an appropriate representation of the albumin-free environment of the brain. Investigations into brain region-specific concentrations of bilirubin such as Gazzin et al. in 2012[49] showed brain bilirubin concentrations to be between 2 and 60 nmol/g of wet tissue (2–60 µmol/kg, or roughly equivalent to 2–60 µmol/L) in jaundiced rats depending on the region of the brain and whether the animals were treated with sulfadimethoxine.[49] One historical report of bilirubin measurements in the brains of severely hyperbilirubinemic patients reported concentrations of (34 nmol/g of wet tissue) in the human basal ganglia,[50] the same range as Gazzin et al. reported in kernicteric Gunn rats.[49] It should be noted that this emiprically determined toxic concentration of Bf in cells is about 1000-fold lower than the concentration of bilirubin measured in kernicteric human and rat brain tissue. Owing to a lack of understanding of how bilirubin behaves in the brain, it is difficult to estimate what the in-brain minimum toxic concentration is. Further complicating this matter is that we do not understand why bilirubin deposition is markedly different in various regions of the brain.[49] Therefore, we contend that the concentration of bilirubin to be used in the cell culture model of BNTx should be that which has been empirically determined to cause markers of neurotoxicity in the cell line being used. While in vitro model are of course imperfect, they still have some functional advantages over in vivo models and, as such, we support their continued use.

Selectivity of BNTx

Numerous studies have attempted to explain the exquisitely selective sensitivity of certain areas of the CNS to BNTx clinically and pathologically. Theories abound including the role of calcium and calcium-binding proteins in sensitive areas.[27,28,30] Previously excluded theories include the role of blood–brain barrier and *N*-methyl-D-aspartate receptors.[51] Furthermore, neurodevelopmental sensitivity to BNTx has been observed in the jaundiced (jj) Gunn rat. Cerebellar hypoplasia, which is common in jj Gunn rats, can be prevented with a single 24-h dose of phototherapy delivered at postnatal days 4–11 but not before or after, with maximum effect at postnatal day 7.[52] We have shown that lobules in the cerebellum undergoing differentiation are more sensitive to BNTx that those before or after.[26] These findings relate to new reports of differences of ABE in extremely premature neonates[53,54] and suggest that the clinical syndrome of KSD may need to be expanded to include sequelae of BNTx in extremely low birth weight premature babies.

Key new information about the regional CNS susceptibility to BNTx was reported in a landmark study by Gazzin et al. in the jj-sulfa Gunn rat model of ABE and kernicterus.[49] In this study, the authors reported the time dependency of the massive accumulation and gradual clearance of UCB in different brain regions. Greater and longer-lasting accumulations of UCB were reported in BNTx-susceptible areas of the cerebellum and inferior colliculus (involved in motor coordination and auditory function, respectively) and more gradual onset in jaundiced neonates and jj Gunn rats. By contrast, UCB accumulation in the same jj-sulfa Gunn rats was limited and brief in resistant areas, cortex and superior colliculus, whose functions (cognition and vision, respectively) are unimpaired in KSDs.

We have hypothesized that PV, a calcium-binding protein present in the brain, may serve a role in BNTx, but to our

knowledge this relationship has not been thoroughly investigated in vivo. Previously, we have shown reduced PV immunohistochemistry in brainstem auditory, oculomotor, and vestibular nuclei in jaundiced Gunn rats with severe kernicterus following sulfonamide administration to displace bilirubin from albumin.[28,29] Additional studies of different brain regions are needed to better understand whether PV has a role in BNTx or merely serves as a marker for susceptible neurons. We note that the majority of neurons in the BNTx-sensitive GP are PV-expressing GABA-ergic neurons and hypothesize that these are especially sensitive and vulnerable to BNTx (see section "Auditory system" and Part II, section "Novel KSD treatment: brain–computer interfaces").

Genetic contributions to severe hyperbilirubinemia and BNTx

The production and elimination of bilirubin in the body is a well-understood process. As such, multiple genetic mutations have been identified that cause either increased production through hemolysis or decreased elimination through impaired enzymatic function of the liver. Most notably among those related to bilirubin production is *G6PD* deficiency.[55] The most notable genes related to improper bilirubin processing are *UGT1A1* and *SLCO1B1*.[56,57] Until recently, hyperbilirubinemia-related genes were the only genetic component considered relevant to KSD. However, recent in vitro and in vivo studies have identified additional genes and pathways that are part of the BNTx response. Studies in various brain sections from sulfa-treated Gunn rats have confirmed differential expression of *Cyp1a1*, *Cyp1a2*, and *Cyp2a3* in the BNTx-resistant regions of the brain compared to the more sensitive regions, indicating a likely protective effect.[49] These genes all code for bilirubin-oxidizing proteins making it likely that they serve a role in response to BNTx. Additional studies have been performed in human and murine neuronal and glial cell lines that have indicated a significant genetic response to bilirubin treatments in vitro.[58–63] These studies point toward a major role for the glial cell inflammatory response in the neuroprotective process. We have hypothesized that variants present in key genes in this BNTx response process could help explain the significantly different outcomes of infants exposed to similar levels of bilirubin.[64] With better understanding of these key genetic contributors, it is possible that new screening methods could be devised to help identify those at higher risk of brain damage from severe hyperbilirubinemia and BNTx.

CONCLUSION

While there exist effective treatments for severe hyperbilirubinemia to prevent the development of ABE and KSD, the incidence of this disease remains surprisingly high throughout the world. Understanding the molecular biology and selective neuropathology of BNTx and kernicterus is essential to further development of enhanced prevention and novel treatments for both ABE and the auditory and motor dysfunction found in patients with KSD. In Part I of this review, we have presented what we believe to be key information in developing this understanding, and in Part II, we will present the current and developing treatments for these conditions moving forward.

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AUTHOR CONTRIBUTIONS

Both authors drafted and critically edited the manuscript together and gave final approval of the version to be published.

ADDITIONAL INFORMATION

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