Review

THE PHARMACOLOGICAL FEATURES OF BILIRUBIN: THE QUESTION OF THE CENTURY

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Abstract: This review looks at the toxicity and metabolism of bilirubin in terms of its pharmacological potential. Its role has gained importance as more research has revealed the functional significance and interrelationship between the gasotransmitters nitric oxide and carbon monoxide. The biological actions of bilirubin have mostly been characterized in the high micromolar range where toxic effects occur. However, it could also prove to be an important cytoprotector for brain tissue, which is inherently less equipped for antioxidant defense. Plasma bilirubin levels negatively correlate to a number of disease states. Higher levels of bilirubin that are still within the normal range provide a protective effect to the body. The effects on various disorders could be tested using controlled pharmacological upregulation of the molecule with animal

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models. At nanomolar concentrations, considerable benefits have been obtained when the molecule was delivered pharmacologically under in vitro or in vivo test conditions, particularly in neurodegenerative disorders and after tissue or organ transplantation. The induction of heme oxygenase-1 (HMOX-1) via the activation of nuclear factor erythroid 2-related factor or the use of bile pigments in the harvesting of diseased tissue are novel applications, and like every new therapy, should be used with caution. HMOX-1 is tissue specific, and in exceptional states, such as schizophrenia and specific types of renal disorder, the same therapy may have disastrous effects.

Keywords: Antioxidant, Bilirubin, Cardioprotectant, Heme oxigenase, Neuroprotectant, Neonatal hyperbilirubinimia

INTRODUCTION

For many years, bilirubin was considered only as a useless or toxic waste product of heme catabolism. It is only since the aspiration of bile for biochemical estimation (bilirubin, amylase, lipase, pH and electrolytes) [1–4] that researchers have linked bilirubin to normal hepatic function and seen any alteration in its normal level as a reflection of hepatic disorder (Fig. 1).
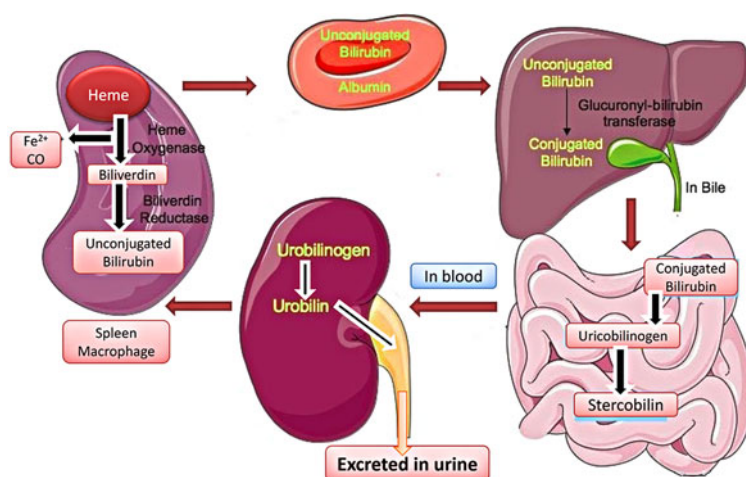

Fig. 1. Transport, conjugation and excretion of bilirubin. The production of bilirubin from heme mainly occurs in macrophages in the spleen and Kupfer cells in the liver, but also takes place in macrophages throughout the organism and in renal tubular cells. The cells that perform this function are collectively known as the reticuloendothelial system. After bilirubin is released from reticuloendothelial cells, it travels in albumin in the blood. This ensures that no bilirubin is excreted in the urine. At very high concentrations, bilirubin can slowly diffuse into the peripheral tissues, where it is toxic. It is removed from circulation by hepatocytes in the sinusoids. This is a passive process that occurs down a concentration gradient. To see this figure in colour, go online: http://www.degruyter.com/view/j/cmble.

In recent years, a number of intriguing biochemical properties of bilirubin have been discovered. Bilirubin belongs to a superfamily of tetrapyrrolic compounds that perform multiple functions in plants and animals [5]. Ranging from light harvesting (higher plants, algae, cyanobacteria), energy generation (bacteria and eukaryotes), transport and homeostasis of oxygen (hemoglobin/myoglobin in animals and leghemoglobin in plants), antioxidant effects (biliverdin, bilirubin and phycocyanobilin), generation of reactive oxygen species (ROS), cell signaling (through bilirubin, carbon monoxide and Fe^{2+}), pigmentation (chlorophyll in plants and heme/porphyrin/biliverdin in animal and birds), to assimilation of nitrogen and sulfur in plants by seroheme). It is noteworthy that bilirubin is only found in higher organisms, while lower animals and birds have its precursor, biliverdin. The enzyme involved in the generation of bilirubin from biliverdin is biliverdin reductase. It is a pleiotropic enzyme with role in cell signaling, metabolism and gene control.

This review focuses on circumstantial pharmaceutical advantages of bilirubin and looks at recent advancements in its study. Bilirubin is a biplanar molecule, chemically regarded as 2,2' dipyrrolylmethane [6]. It occurs in mammals and other species [7]. It was first considered to be lipophilic but extensive studies performed in the 1970s overturned this concept. It has been successfully demonstrated that the solubility of purified bilirubin in apolar solvents and triglycerides is low and that it increases with solvent polarity [8]. It has six internal hydrogen bonds that are responsible for its physicochemical properties [9]. In the liver–spleen system, it is the final product of heme catabolism at the end of the normal lifespan of erythrocytes. The heme dissociates from hemoglobin and is oxidized by the membrane-bound enzyme heme oxygenase (EC 1.14.99.3) to biliverdin, with carbon monoxide and iron as by-products. Subsequent metabolism of the water-soluble green-colored biliverdin by the cytosolic enzyme biliverdin reductase (EC 1.3.1.24) gives rise to bilirubin.

This unconjugated bilirubin (UCB) is tightly bound to albumin for transport in the blood [10]. It later gets conjugated with glucuronic acid, which renders it water-soluble, and is secreted in the bile to be mostly excreted in the feces as urobilinoids after biotransformation [11, 12]. The remaining 0.01% of the total UCB circulates in the blood in an unbound form known as free bilirubin (Bf). This in turn regulates the diffusion of UCB into tissues [9].

Under cholestatic conditions, a variable proportion up to 85% of accumulated bilirubin diglucoronides undergo transesterification with plasma albumin, yielding the so-called delta bilirubin [13]. This pigment cannot cross the glomerular membrane. Therefore, it is not excreted in the urine or bile, accounting for the slow decline in plasma bilirubin levels following relief of biliary obstruction [9, 14].

The toxic effects of bilirubin are due to inhibition of DNA synthesis. Bilirubin may also uncouple oxidative phosphorylation and inhibit the adenosine triphosphatase (ATPase) activity of brain mitochondria. Bilirubin-mediated inhibition of various enzyme systems, RNA synthesis and protein synthesis in

the brain and liver, and/or alteration of carbohydrate metabolism in the brain can also contribute to its toxicity. The accumulation of bilirubin in the plasma and tissues results in a characteristic yellow discoloration of tissues, known as icterus or jaundice.

HISTORICAL OVERVIEW

In 1937, Najib-Farah [15] postulated that bilirubin forms part of a protective mechanism designed to overcome infection. One mole of albumin-bound bilirubin can scavenge two moles of peroxyl radicals, and small amounts of plasma bilirubin are sufficient to prevent oxidation of albumin-bound fatty acids and oxidation of the protein itself [16]. Under 2% oxygen in liposomes, bilirubin suppresses oxidation more than alpha-tocopherol, which is regarded as the best antioxidant of lipid peroxidation [17]. Bilirubin has been identified as an extremely potent antioxidant, with studies also claiming that biliverdin has antioxidant activities. Conjugated bilirubin and biliverdin have been observed to directly scavenge lipid radicals to some extent and both can act synergistically with membrane-bound vitamin E to prevent lipid peroxidation initiated in the lipid phase [18], most likely through regeneration of the vitamin from its chromanoxyl radical [19].

Evidence supports bilirubin's status as a powerful chain-breaking antioxidant in biological systems. This may contribute to the cellular and tissue protection seen with increased heme oxygenase levels [20]. It is probably the most abundant endogenous antioxidant in mammalian tissues, accounting for the majority of the antioxidant activity of human serum [21]. In an extensive series of antioxidant studies by Farrera et al., bilirubin displayed the most potent superoxide and peroxyl radical scavenger activity [22]. Free bilirubin and biliverdin showed superoxide scavenging activities near to that of serum albumin, higher than that of the water-soluble vitamin E analog trolox, and lower than that of vitamin C. The peroxyl radical-trapping antioxidant abilities of these tested bile pigments were much higher than those of the serum albumin and of the same order as their serum albumin complexes [22]. Moreover, bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation [23]. Frei et al. found that bilirubin was more effective in protecting lipids from peroxidative damage than other endogenous antioxidants [24]. Bilirubin reportedly inhibits L-dopa-Cu^{++}-mediated DNA cleavage and directly quenches OH radicals generated by the L-dopa-Cu^{++} system [25]. Adding bilirubin to erythrocytes incubated with cumene-OOH induced an inhibition of lipid peroxidation processes, preserving superoxide dismutase activity, increasing catalase and glucose 6-phosphate dehydrogenase activity, and reducing the glutathione concentration [26]. Studies of bilirubin spectra at 316 nm with respect to S-nitrosocysteine (SNOC) and S-nitrosoglutathione (GSNO), which are nitric oxide donors, suggest that bilirubin should be considered not only as an endogenous antioxidant but also as a molecule with the potential to counteract intracellular nitrosative stress

reactions [27]. Moreover, ultraviolet and mass spectrometry analysis revealed that in a pro-oxidant atmosphere, bilirubin binds to nitric oxide forming an N-nitroso derivative (BR-NO), which can be used as a biomarker of nitrosative stress [28].

BILIRUBIN AND BILIVERDIN AMPLIFICATION CYCLE

The bilirubin and biliverdin amplification cycle is currently a topic of considerable discussion. In a key experiment published in Cell Biology, Barañano et al. reported that bilirubin is a major physiological antioxidant that acts at concentrations as low as 10 nM and can protect brain cells in culture from a 10,000-fold excess of H_2O_2 [29]. They postulated that the potent physiological antioxidant actions of bilirubin reflect an amplification cycle whereby bilirubin, acting as an antioxidant, is itself oxidized to biliverdin and then recycled back to bilirubin by biliverdin reductase. This redox cycle may constitute the principal physiological function of bilirubin. This explains how a low concentration of bilirubin in tissues, i.e., 20–50 nM, which is < 0.1% of the levels of established antioxidants such as glutathione (GSH), remains relevant to intracellular oxidative stress. Therefore, they argued it to be a more potent antioxidant than glutathione [29].
In an antecedent report, it was further stated that bilirubin and glutathione are complementary to each other [30]. It was established that reduced glutathione depletion activates the HO-1 gene through nuclear factor erythroid 2-related factor (Nrf2) [31]. Despite strong disagreement to the above amplification cycle, Stocker et al. [19] accepted a limited role for the bilirubin–biliverdin redox amplification cycle in cellular antioxidant protection by biliverdin reductase. A gene silencing study in primary endothelial culture revealed conversion of biliverdin to bilirubin by bilirubin reductase [32]. A recent experimental study on young and senescent human diploid fibroblasts in the presence of H_2O_2 demonstrated that biliverdin reductase activity is upregulated in young cells, suggesting age-dependent differential upregulation of the enzyme [33].

BILIRUBIN AS A NEUROPROTECTANT

Heme oxygenase catalyzes the conversion of heme to carbon monoxide, iron and biliverdin, which is immediately reduced to bilirubin [34]. Two heme oxygenase active isozymes exist: heme oxygenase-1 (HMOX-1), which is an inducible heat shock protein also known as HSP-32; and heme oxygenase-2 (HMOX-2), which is constitutive and highly concentrated in neurons. Bilirubin, formed by the activation of HMOX-2, protects neurons against oxidative stress injury (Figs 2 and 3).
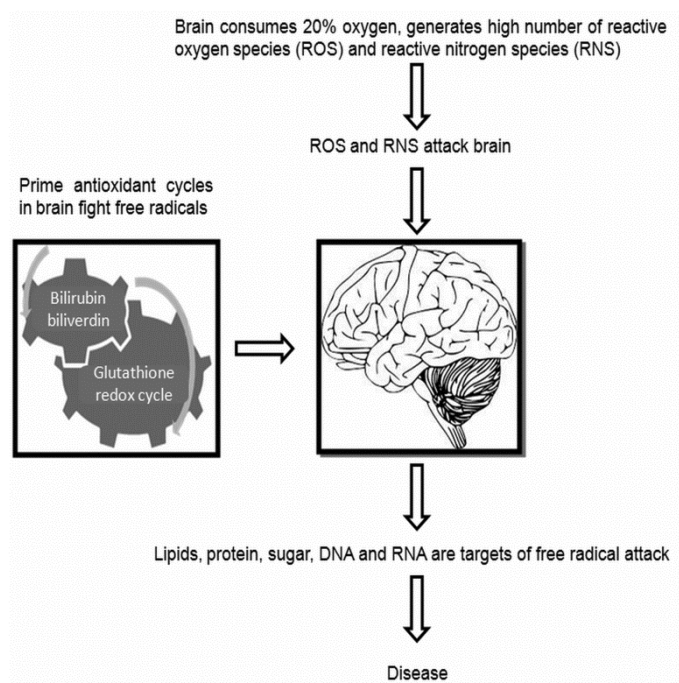
Fig. 2. The bilirubin–biliverdin cycle and glutathione redox cycle are inherent antioxidant defenses inside the brain. Disease occurs when the antioxidant defense fails to protect cells from free radicals in the brain tissues.
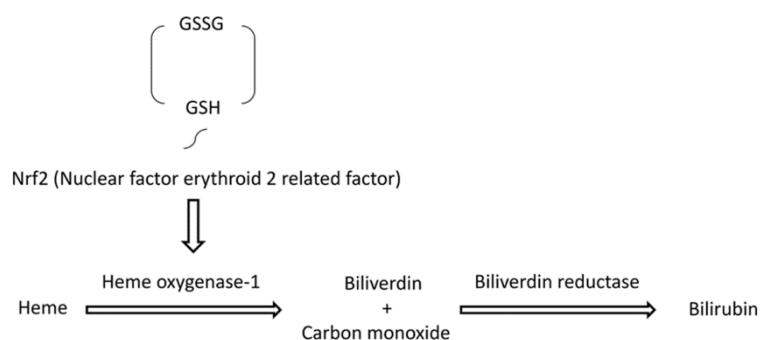
Fig. 3. Conversion of heme to bilirubin. Reduced glutathione depletion activates the HO-1 gene through Nrf2 [31].

MOLECULAR MECHANISMS LINKING BILIRUBIN TO NEURODEGENERATIVE DISEASES

It has been shown that bilirubin conjugated to human serum albumin has a neuroprotective effect. It reverses the neurotoxic effects of H_2O_2 on neuronal hippocampal cultures at a concentration of 10 nM, but as the bilirubin concentration reaches higher levels, the survival of neurons is diminished [35].

A recent study showed that HMOX-2 knockout neurons are less vulnerable to hemoglobin toxicity [36] and HMOX-2 protects against glutathione depletion-induced neuronal apoptosis [37]. It is well known that astrocytes confer protection of neurons against oxidative stress by scavenging ROS and producing factors that induce antioxidant enzymes [38, 39]. This is supported by a study on astrocyte cultures from mice lacking the HO-1 gene: the level of oxidative injury was found to be twice as high [40]. Bilirubin also protects astrocytes from its own toxicity by inducing upregulation and translocation of multidrug resistance-associated protein 1 (Mrp1) [41]. Bilirubin and biliverdin successfully protected HT22 cells (cell line originated from mouse hippocampal neuron) when HO-1 expression was suppressed by siRNA technology and the cells were exposed to H_2O_2 and 4-hydroxyneonal, which are both pro-oxidant compounds [42]. An additional report showed that co-treatment of ethanol-treated HT22 cells with 50 nM bilirubin reduced the cell viability. Surprisingly, the same report documents that supplementation of 5 μM zinc protoporphyrin IX (ZnPP IX), a competitive HO inhibitor to the identical HT22 cell culture, could not affect cell viability [43]. Another study of heme oxygenase-1 knock-out mice showed that heme oxygenase-1 protects brain cells, in particular neurons, against NMDA-induced excitotoxicity [44]. Exposure of PC12 and primary rat cerebellar granule neurons to bilirubin (0.5–10 μM) demonstrated that bilirubin could be an endogenous modulator of neurotrophin redox signaling [45].

THE HMOX-1 LEVEL INCREASES IN NEURODEGENERATIVE DISEASES

Clinical studies have indicated that in humans, there is a progressive increase in the expression of HMOX-1 between 3 and 84 years of age in the neurons and neuroglia [46]. Superfluous expression of HMOX-1 was observed in the temporal cortex and hippocampus from post-mortem tissue of Alzheimer's disease patients [47]. Upregulation of biliverdin reductase-A, an enzyme that converts biliverdin to bilirubin, was found in the hippocampus of subjects with Alzheimer's disease or mild cognitive impairment, whereas its levels remained unchanged in the cerebellum [48]. Likewise, Lewy bodies in substantia nigra neurons exhibited intense expression of HMOX-1 when immuno-histochemistry was conducted post-mortem on tissue from Parkinson's disease patients [49]. It has been established that HMOX-1 increases within 24 h in instances of brain trauma [50] and returns to its baseline after a period of months [51]. Oxidative and nitrosative stress-induced activation of the heme oxygenase–biliverdin reductase-A axis has been demonstrated in neurodegenerative disorders and is now under consideration as a biomarker for conditions like Alzheimer's disease [52]. In a recent pre-clinical study on dogs, biliverdin reductase-A was identified as a novel drug target for atorvastatin, which differentially upregulates the level of the enzyme in the parietal cortex [53].

MOLECULAR MECHANISMS LINKING BILIRUBIN TO CARDIAC DISEASES

In a cross-sectional study on a Utah population, low serum bilirubin was found to be associated with a gene that was linked to coronary heart disease [54]. Close associations were observed between low serum bilirubin concentrations and increased cardiovascular disease risk factors in the Hong Kong Chinese population [55]. Serum bilirubin levels have been identified as an inverse risk factor for coronary artery disease [56, 57] and carotid plaques [58]. Surprisingly, mildly elevated serum bilirubin, has a protective effect comparable to that of high-density lipoprotein cholesterol [59]. Low serum bilirubin levels are also independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness, two well-known indicators for atherosclerosis [60]. Quite expectedly in atherosclerosis, the concentration of the primary antioxidant glutathione is found to be reduced [61]. Hyperbilirubinemic individuals with Gilbert's syndrome demonstrated reduced coronary artery disease, which the authors reasoned could be due to antioxidant function rendered by bilirubin [62, 63]. Irinotecan is a key chemotherapeutic drug used to treat many tumors, including cervical and ovarian cancers, but it can cause toxicity, particularly in the presence of uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) gene polymorphisms, which are associated with reduced enzyme activity [64]. Bilirubin concentrations controlled by UGT1A1 and SLCO1B1 genes respectively coding for bilirubin glucuronosylation and transport protein have been found to be inversely related to cardiovascular disorders and related diseases, such as diabetes and metabolic syndrome [65, 66]. A linkage study indicates that individuals homozygous for the UGT1A1*28 gene have a lower risk of developing cardiovascular disease than carriers of wild-type alleles [5].

BILIRUBIN AS A CARDIOPROTECTANT

Cardioprotection of bilirubin in vascular proliferative disorders like atherosclerosis was successfully demonstrated in vivo with hyperbilirubinemic Gunn and wild-type rats. The animals treated with biliverdin, the precursor of bilirubin, exhibited less balloon injury-induced neointima than controls when serum-driven smooth muscle cell cycle progression was inhibited at the G1 phase via inhibition of the mitogen-activated protein kinase signal transduction pathways and inhibition of phosphorylation of the retinoblastoma tumor suppressor protein [67]. It is speculated that HMOX-1 degrades the pro-oxidant heme inducing oxidative stress stimuli and leading to a series of events that result in dysfunction of endothelial cells and ultimately in cardiovascular disease.

MOLECULAR MECHANISMS LINKING BILIRUBIN TO HEPATIC DISEASE

There is enough experimental evidence in favor of bilirubin co-treatment or pre-treatment as a preventive measure for liver disease [68]. Pretreatment of rats with biliverdin (40 μM/kg, ip) was found to block acetaminophen-induced injury in rat hepatocytes [69]. Administration of bilirubin (5 μM/kg body weight) 24 h before the end of the experiment in Wistar rats entirely prevented pre-hepatic portal hypertension-induced toxicity [70]. Current data corroborate that the controversial effect of bilirubin in hyperbilirubinemia in neonates may be a transitional backup procedure. Hydrophobic bile acids contribute to hepatocellular injury in cholestasis and rapidly induce apoptosis in vitro. However, when freshly isolated rat hepatocytes were incubated with 100 μM glycochenodeoxycholate (GCDC) alone or with increasing concentrations of unconjugated (UCB) or conjugated bilirubin (CB), apoptosis was prevented in a concentration-dependent manner [71]. This study has evidence that hyperbilirubinemia may have a protective role in liver disease, since bilirubin was found to prevent cell death. It has also been found that enhanced HO-1 expression in human liver could help to prevent chronic ethanol-induced cytotoxicity in normal human liver cells [72]. A recent study concluded that bilirubin counteracts the pro-oxidant effect of bile acids in chronic endotoxic shock and cholestasis-induced liver damage [73].

HYPERBILIRUBINEMIA IN NEONATES

Most neonates experience temporary, mild to moderate physiological jaundice, due to the immaturity of their hepatic conjugation and the clearance processes for unconjugated bilirubin (UCB). Although the majority of neonatal jaundice is benign, some neonates with severe hyperbilirubinemia develop bilirubin encephalopathy or kernicterus [74]. Increased levels of UCB may result in various disorders, ranging from mild mental retardation and subtle cognitive disturbances to deafness and severe cerebral palsy, seizure, or death from kernicterus [75, 76]. Controlled studies have also revealed cognitive disturbances in children with elevated levels of bilirubin in the infant period [62]. The cerebellum, cochlear and oculomotor nuclei of the brain stem, the hippocampus, and the basal ganglia are brain regions susceptible to UCB toxicity [77].
It is well known that bilirubin encephalopathy or kernicterus are preventable causes of brain injury. The first week of an infant's life should be critically monitored according to recent guidelines on the management of the disease. Infants that fall into the high risk category for the development of hyperbilirubinemia-related disorders that could largely be identified using gestational age and pre-discharge measurements of serum/transcutaneous bilirubin [78], tracking those with rapid rates of bilirubin rise 0.2 mg/100 ml/h [79] and glucose-6-phosphate deficiency [80]. The reports of kernicturus

resurgence after 20 years [81, 82] were contradicted by a recent study [83] that had evidence that the disease is a rare one. The antioxidant defense in neonates is poorly developed [84]. The possible protective role of bilirubin in neonates has long been debated. It could represent a transitional preventive mechanism in neonatal circulation. At physiologically relevant concentrations, bilirubin is useful in protecting the body against oxidative damage (Fig. 4).
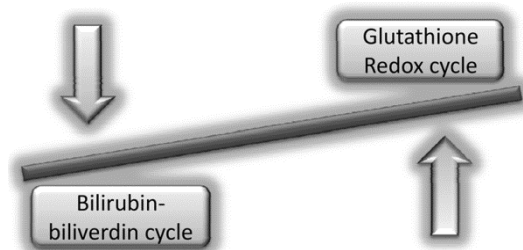
Fig. 4. Antioxidant defense inside the human brain has a delicate balance between the glutathione redox cycle and the bilirubin–biliverdin cycle.
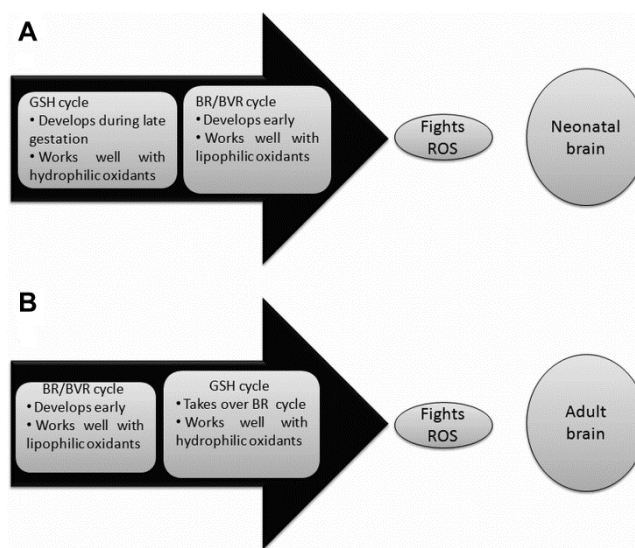
Fig. 5. The comparative roles of the prime antioxidant cycles in the neonatal (A) and adult (B) brain.

Preliminary reports said that the beneficial effects of breastfeeding were often accompanied by high bilirubin levels [85]. Pre-term infants endowed with an immature antioxidant defense system are prone to oxidative stress. Recently, it has also been reported that human milk enhances antioxidant defenses against hydroxyl radical aggression in pre-term infants [86]. Experimental evidence has been found for the protective effect of bilirubin to neonatal rats exposed to hyperoxia against serum oxidative damage in first few days of life [87]. The

earliest indicator of the antioxidant effect of bilirubin in pre-term infants was demonstrated in 1989, when it was observed that a higher serum bilirubin level is associated with diminished incidence of retinal damage [88]. There has been limited evidence found since then. In pre-term infants, higher bilirubin levels are associated with a lower incidence of oxygen radical-mediated injury (Fig. 5A) [89]. In vivo, the plasma antioxidant capacity of jaundiced newborn infants is related to the level of bilirubin [90]. In a study of 20 premature infants with jaundice, high levels of plasma nitric oxide were seen in the study group, indicating free radical stress. In addition, antioxidant defense was found to be weak since the activities of the principal antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase were found to be significantly lower than in the controls, even though the plasma bilirubin levels were found to be significantly high (Fig. 5B) [91, 92]. It is interesting to note that the decrease in the plasma total bilirubin level was found to increase the total antioxidant capacity and decrease oxidative stress in pre-term infants [93]. A positive correlation was found between malondialdehyde and bilirubin levels in full-term infants [94]. In a clinical study of full-term normal neonates, it was concluded that bilirubin acts as a physiological antioxidant until the concentration is 200 mg/l [95].

ISCHEMIA–REPERFUSION INJURY (IRI)

Experimental models have demonstrated that HO-1 activity is induced in response to IRI in the heart [96], brain [97], liver [98] and intestine [99], and that the cytoprotective effects of HO-1 in these organs are mediated by bilirubin. In a detailed review on the role of HO-1, it was suggested that an absence of HO-1 has detrimental consequences, such as atherosclerotic lesion formation and vein graft disease, while overexpression of HO-1 plays a protective role in IRI in vivo in a mouse model [100]. Overexpression in the cardiomyocytes of transgenic mice can protect against IRI [101]. In experimental models, exogenous administration of bilirubin in isolated, perfused rat heart and liver models had a protective effect against IRI and the efficacy of this protective effect was improved by administration of bilirubin before or during the ischemic event [102]. Pretreatment with bilirubin produced the most consistent improvements in vascular resistance, urine output, GFR, and tubular function after IRI in the rat kidney [103].

BILIRUBIN AND CANCER

The Third National Health and Nutrition Examination Survey, a comprehensive assessment of health and nutrition in the United States, demonstrated that individuals with a history of non-dermatological malignancy exhibit significantly lower serum bilirubin concentrations than those who do not have a history of non-dermatological cancer [104]. Based on the 10-year follow-up mortality data from the Belgium Inter-University Research on Nutrition and Health (BIRNH), it was concluded that populations with high serum bilirubin

that still remains within the normal range were associated with low cancer mortality, especially in males [105].

Photodynamic therapy (PDT) is an emerging treatment method that is increasingly used to treat thoracic malignancies. PDT employs a photosensitizing agent that is activated by light of a specific wavelength to produce reactive singlet oxygen ($^1O_2$) that mediates cellular cytotoxicity. PDT is approved for the management of both early and advanced tumors. HMOX-1 protected tumor cells against photodynamic therapy-mediated cytotoxicity [106]. It has been demonstrated that HMOX-1/CO cooperates with NF-kappa-B-dependent anti-apoptotic genes to protect endothelial cells from tumor necrosis factor (TNF) alpha-mediated apoptosis [107]. HMOX-1 expression was reported to be enhanced in response to proteasomal inhibition in melanoma and breast cancer cell lines [108]. Tissue microarray studies report upregulation of HMOX-1 in lung cancer patients [109]. Altered expression of HMOX-1 has been found to be associated with pancreatic cancer, hepatoma and colorectal cancer, and a review article on gastrointestinal tumors suggested that therapeutic modulation of HMOX-1 could contain the malignancy [110]. Discovery of HMOX-1 specific regulatory CD8^{+} T cells in cancer patients open new avenues for treatment [111]. A case control study of sporadic colorectal cancer patients found that male allele carriers of the UGT1A1*28 gene were less susceptible to the disease due to high bilirubin levels [112]. However, in a study of squamous cell carcinoma of mice it was found that while HMOX-1 protected normal tissue against carcinogen-induced injury, it seemed to promote the malignancy in already developing tumors [113].

BILIRUBIN AND INFLAMMATION

Neutrophilic and macrophage inflammation is a double-edged sword. In small and controlled amounts it helps in healing, but uncontrolled inflammation is a principal etiologic factor of many diseases, including asthma. Vascular cell adhesion molecule (VCAM-1) is an essential molecule with a role in tissue injury, particularly in response to low levels of ROS. Unconjugated bilirubin inhibits VCAM-1-mediated transendothelial leukocyte migration, as observed with intraperitoneal administration of bilirubin to C57BL6/J mice (murine asthma model). Bilirubin blocks VCAM-1-dependent lymphocyte migration in vitro and ameliorates VCAM-1-mediated airway inflammation in vivo through the suppression of cellular ROS production [114]. In rats pretreated with bilirubin before exposure to smoke, the development of emphysema was found to be low. Bilirubin exerted protective effects on alveolar macrophages by regulating the expression of inducible nitric oxide synthase (iNOS) and nitric oxide [115].

It is notable that HMOX, the rate-limiting enzyme in bilirubin synthesis, is upregulated at sites of inflammation, and the stimulated activity of this enzyme is associated with diminished tissue injury in a host of model systems [116].

Treatment of microglia with astrocyte culture-conditioned media (ACM) increased the expression level and activity of HMOX-1. This could be a feasible mechanism for preventing excessive brain inflammation [117].

Inflammation is an important trigger of bone damage. Increased HMOX-1 expression was observed to be important in protecting against TNF alpha-induced apoptosis in osteoblasts [118]. Serum levels of bilirubin were found to be elevated in rheumatoid arthritis patients without bone damage. In vitro and in vivo experiments on HMOX-1 reported to negatively regulate osteoclastogenesis and inflammatory bone loss in arthritis [119].

Bilirubin has been found to inhibit human pro-inflammatory secretory phospholipase A(2) (sPLA(2)) [37]. Enzyme activities from *Vipera russellii* and *Naja naja* venom and partially purified sPLA(2) enzymes from human ascitic fluid, pleural fluid and normal serum in a dose-dependent manner. Inflammatory human sPLA(2) was found to be more sensitive to inhibition by bilirubin than snake venom sPLA(2). Inhibition of sPLA(2)-induced mouse paw edema by bilirubin confirms its sPLA(2) inhibitory activity in vivo [120]. In a population-based study using measurement of hsCRP as an indicator of inflammation, a high level of bilirubin was found to be associated with favorable coronary endothelial function [121]. Low bilirubin levels were found to inversely correlate with high coronary artery calcification and reduced hsCRP level [122].

ROLE OF BILIRUBIN IN ORGAN TRANSPLANTATION

Recent organ transplantation research has focused on finding ways to enhance and predict the efficacy of graft survival. The success and endurance of the graft depends on a multitude of factors. Expression of the stress protein HO-1 has recently been associated with long-term survival of various allografts in various animal models, including liver [123], heart [124] and xenografts [125]. Numerous publications of various research groups advocate HMOX as a protective gene that confers protection from graft rejection and supports long-term survival [126]. Clinical studies have also demonstrated upregulation of HMOX-1 gene in renal allografts in response to immune injury inferred by acute rejection [127]. Moreover, reduced expression level of the HMOX-1 gene in chronic rejection as compared with acute rejection (AR) represents either an inadequate response to injury or a sequel of prior injury that jeopardizes further tissue response to immune attack [127].

Administering bilirubin to the recipient (B6AF1) or incubating islets in a bilirubin-containing solution ex vivo led to long-term survival of allogeneic islets in a significant percentage of cases. In addition, administering bilirubin to only the donor frequently led to long-term survival of DBA/2 islets in B6AF1 recipients and significantly long-term graft survival has been seen in BALB/c islets in C57BL/6 recipients. Such approaches are also appealing in donor patient treatment with bilirubin upregulated mRNA expression of protective genes such as HO-1 and *BCL*-2 and suppressed pro-inflammatory and pro-

apoptotic genes, including monocyte chemoattractant protein-1 and caspase-3 and -8 in the islet grafts before transplantation [5].

In another study a brief course of treatment with biliverdin lead to long-term survival of H-2 incompatible heart allografts. Furthermore, recipients harboring long-surviving (> 100 days) allografts were tolerant to donor antigens indicated by the acceptance of second donor strain hearts but not third-party grafts. Treatment with biliverdin decreased intragraft leukocyte infiltration and inhibited T cell proliferation [128]. Bilirubin protects grafts against nonspecific inflammation-induced injury in syngeneic intraportal islet transplantation in rat model [129]. Injection of bilirubin in mice islet allografts led to generation of T-regulatory cells (Treg cells), suggesting that bilirubin administration leads to graft tolerance by Treg cell generation [130]. Another study suggested that Treg cell populations regulate immunity by regulating the expression of HMOX-1 as one of the anti-inflammatory factors [131]. At the site, overexpression of the HMOX-1 gene in the pancreas and liver induced Treg cell proliferation and subsequently enhanced graft survival in rat models [132, 133].

BILIRUBIN IN IMMUNOLOGICAL DISORDERS

It was found that bilirubin effectively prevents experimental autoimmune encephalomyelitis (EAE) when administered both before and after the clinical onset of disease [134]. There are many studies indicating the dysfunction of the endogenous antioxidant system in chronic disorders, including alteration in bilirubin levels. For instance, the bilirubin level decreases in patients of amyotrophic lateral sclerosis [135].

Systemic lupus erythematosus (SLE) is a disease with a complex pathology. It is characterized by high oxidative stress. Low bilirubin levels were reported to be negatively associated with SLE in a clinical survey. The research group claimed that each 1 µmol/l decrease in bilirubin leads to a 37% increase in the chance for SLE [136]. Other groups have also confirmed these findings. In a recent study on lupus-prone mice, HO-1 induction and CO exposure improved the symptoms of the disease [137].

In a pilot study on 90 patients, each 1 µmol/l increase in serum bilirubin was associated with 15% odds for the occurrence of Crohn's disease [138] while in a genomic study of patients with Crohn's disease, a homozygous state of UGT1A1*28 associated with higher bilirubin rendered protection against the disease [131].

CONCLUSION

This review article advocates for bilirubin as having various potential pharmacological advantages. It has manifold biological properties: antioxidant, anti-apoptotic, anti-excitotoxic, anti-nitrosative, immunomodulatory, anti-inflammatory, anti-complementary and anti-mutagenic (Table 1). A number of in vitro and in vivo studies have demonstrated that bilirubin is a crucial intermediary of the cytoprotective chain of events regulated by heat shock

protein 32 (Hsp32) or heme oxygenase (HMOX) and that it ameliorates cellular damage in these situations [139]. It has been shown to be a more potent antioxidant than glutathione [29], one of the principal antioxidants in our body. The role of bilirubin gets more important as the functional significance and interrelationship between the gasotransmitters nitric oxide and carbon monoxide gets fully deciphered. Multiple reviews conclude that carbon monoxide is a regulator of vascular functions like nitric oxide and both stimulate guanyl cyclase [140, 141]. Additionally, nitric oxide has also been found to stimulate HMOX-1 transcription [142].

Table 1. Biological properties of bilirubin

Properties	References
Immunomodulatory agent	[91]
Anti-inflammatory	[116, 120]
Anti-oxidant	[133]
Anti-apoptotic	[116]
Anti-mutagenic	[119]
Anti-exitotxic	[156]
Anti-complement	[25]
Anti-nitrosative	[45, 123]
Cytoprotective	[35]

Numerous reviews have been written about the unquestionable toxicity of bilirubin at high concentrations [143]. However, mild physiological jaundice is no longer considered a threat [144]. Hyperbilirubinemia in neonates should long since have been considered as a transitional mechanism in neonatal circulation, where high bilirubin levels provide back up to the neonate with a weak immune system and antioxidant defense. The toxic effects of hyperbilirubinemia in neonates will largely be transient provided prompt treatment is initiated [145].

In humans, the biological actions of bilirubin have been mostly characterized in the high micromolar range or higher, where a wide range of toxic effects may occur. However, bilirubin could also prove to be an important cytoprotector for the same brain tissue, which is inherently naturally less equipped for antioxidant defense. The situation obviously deteriorates in instances of brain trauma or in neurodegenerative disease states such as Parkinson's disease or Alzheimer's disease (Fig. 2) [146].

A large number of studies document the role of bilirubin or its precursor heme oxygenase as neuroprotectant [35, 42]. A number of non-hepatic disease conditions are associated with altered bilirubin levels (Table 2). Plasma bilirubin levels are negatively related to a number of disease states particularly those of cardiovascular origin, non-dermatological cancers or inflammatory disorders like asthma. Bilirubin levels could actually serve as a biomarker for a number of non-hepatic disease states (Table 3).

Table 2. Non-hepatic disease states associated with altered bilirubin levels

Disease	References
Ageing	[46]
Alzheimer's disease	[49]
Parkinson's disease	[157]
Multiple sclerosis	[130]
Schizophrenia	[73, 130]
Cancer	[84, 119]
Cardiovascular disease	[58, 59]
Arthritis	[158]
Nitrosative stress	[28]

Table 3. Non-hepatic disease states where bilirubin levels could be used as biomarker

Disease	References
Ageing	[46]
Alzheimer's disease	[49, 55]
Parkinson's disease	[157]
Schizophrenia	[82, 130, 159]
Trauma	[50, 51]
Bacterial meningitis	[160]
Human cerebral malaria	[85]
Friedreich's ataxia	[149]
Amyotrophic lateral sclerosis	[135]
Ischemia-reperfusion Injury	[96, 97]
Multiple sclerosis	[117]
Cancer	[84, 119]
Carotid plaques	[58]
Cardiovascular disease	[43]
Artherosclerosis	[60, 136]
Atopic dermatitis	[62]
Rheumatoid arthritis	[158]
Diabetes	[44, 161]
Asthma	[162]
Transplantation	[116]

Bilirubin appears as a potential candidate for increasing both graft tolerance and long-term graft survival by maintaining cell viability and controlling inflammation thanks to its anti-apoptotic and anti-inflammatory properties. Quite expectedly, heme oxygenase-1, the rate-limiting enzyme in bilirubin synthesis, is upregulated at the sites of inflammation and that the stimulated activity of this enzyme is associated with diminished tissue injury in a host of model systems [50, 116]. Since higher levels of bilirubin within normal range render a protective effect to the body, localized delivery of controlled doses of bilirubin to the diseased tissue could turn out to be a safe strategy. Gene therapy and gene transfer, including site- and organ-specific targeted gene transfer could be tested for various disease states in animal models.

NEPP11 is the first molecular probe reported to have a neuroprotective action through induction of HO-1 in neuronal cells [147]. Acetylcarnitine treatment induces heme oxygenase-1 (HMOX-1) in rat astrocytes [148] and the same compound in senescent rats raises HMOX-1, Hsp 70 and SOD-2 [149]. Extensive reviews conclude that activation of Nrf-2 induces HMOX-1 [150, 151]. Polyphenolic HMOX-1 inducers of plant origin include curcumin and green tea [152], but there are contradicting reports from other quarters [153, 154].

It is unanimously agreed by researchers and medical professionals worldwide that high levels of bilirubin are dangerously toxic. Research has identified a novel class of natural substances that can be used in a particular disease in site- and tissue-specific transfer of HO-1 in the protection of tissues against the inflammatory response. Adeno-associated viral (AAV) vector-mediated muscle-directed gene therapy in the preclinical animal model of Crigler-Najjar syndrome, an autosomal recessive disorder with severe unconjugated hyperbilirubinemia due to deficiency of bilirubin uridine diphospho-glucuronosyl transferase (UGT1A1) encoded by the UGT1A1 gene was successful [155].

Very recently, Indian scientists successfully demonstrated bilirubin as a topical applicant for wound healing in diabetic rats [162] in a severe cutaneous wound model [163]. The approach towards induction of HMOX-1 or the use of bile pigments towards harvesting benefits in diseased tissue is novel, but like every new therapy it should be exercised with caution, since HO-1 is tissue specific and in exceptional states, such as schizophrenia and specific types of renal disorder, it could have disastrous effects.

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