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Bilirubin and Ischemic Stroke: Rendering the Current Paradigm to Better Understand the Protective Effects of Bilirubin

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Abstract

Novel and innovative methods are critical in fostering new treatments and improving clinical outcomes in patients who suffer from ischemic stroke. Bilirubin has long been considered metabolic waste that can be harmful to the body; however, it is now becoming recognized as one of the body's most potent antioxidant, anti-inflammatory, and neuroprotective molecules. These properties facilitate bilirubin's anti-atherogenic effects to impede and prevent the formation of thrombi in ischemic stroke. These functions allow for protection from neuronal injury during an ischemic state and suggest that elevated bilirubin levels may be linked to a lower rate of morbidity and mortality. Therefore, here we discuss the pathophysiology of stroke and the molecular properties of bilirubin to better understand their beneficial relationship. We outline clinical studies looking at the relationship between serum bilirubin levels and ischemic stroke prevalence. At this time, few studies have rigorously looked at the relationship between bilirubin and ischemic stroke, whether it is positive or negative. Thus, rigorous research is needed to provide evidence supporting the current studies, expand on these studies, and facilitate their translation to bedside therapy for patients who suffer from ischemic stroke.

Keywords Antioxidant · Ischemia · Outcomes · Protection · Treatment

Introduction

Stroke is the fifth leading cause of mortality in the USA behind heart disease, cancer, chronic lower respiratory disease, and unintentional injuries; however, it is the leading cause of disability. According to the World Health Organization, 15 million people suffer from stroke worldwide, of which 5 million die and another 5 million are left permanently disabled [1]. Stroke is categorized into two types: ischemic and hemorrhagic. In this paper, we will focus on ischemic stroke. In particular, we will focus on the relationship between bilirubin and ischemic stroke severity and clinical outcomes. Bilirubin is a vital, endogenous molecule with anti-inflammatory, antioxidant, and cytoprotective properties to combat atherosclerosis. This condition results in occlusion of a vessel and ultimately ischemia [2]. Previously, research has shown that increasing bilirubin levels have had deleterious effects on stroke severity and outcomes; however, recent studies have contradicted these conclusions. Here, we will discuss in detail the pathogenesis of ischemic stroke, the molecular properties of bilirubin, and how bilirubin confers protection in the setting of cerebrovascular ischemia.

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Ischemic Stroke

Ischemic stroke is a term used to describe a variety of pathological conditions that result in decreased blood flow to a certain area of the brain. It occurs as a result of decreased systemic perfusion, stenosis of a cerebral vessel, or occlusion of a vessel that causes diminished blow flow distally. Decreased systemic perfusion can result from significant blood loss leading to hypovolemic shock, heart failure leading to cardiogenic shock, or a significant drop in blood pressure

[3]. Stenosis of a cerebral vessel can occur due to thrombosis or an embolism. Thrombosis, or obstruction of a blood vessel, in most cases occurs over time due to atherogenesis that causes stenosis of the vessel. Embolism occurs from a clot obstructing the vessel from a distal site; the heart tends to be a common source in the setting of atrial fibrillation [4]. All of these lead to diminished blood flow to the brain, which causes decreased oxygen delivery to vital tissues.

In juxtaposition, the mechanisms of pathology differ between ischemic and hemorrhagic stroke; however, both facilitate the onset of neuronal cell death. At a molecular level, ATP supply, the body's main source of energy, is contingent on continuous perfusion and can reach dangerously low levels within 4 min of complete ischemia [5]. A cascade of molecular events that ultimately leads to cellular death occurs in response to this depletion of energy [6, 7]. One such event—glutamate release—causes excitotoxic neuronal damage and plays a key role in the pathogenesis of ischemic stroke [8, 9]. Decreased ATP levels in response to hypoxia lead to the impairment of glutamate receptors; this results in neuronal depolarization and an accumulation of glutamate [10]. This accumulation of glutamate causes an influx of calcium, increased creation of reactive nitrogen species, mitochondrial dysfunction, and the accumulation of reactive oxygen species [10].

As the duration of ischemia lengthens, the damage then becomes irreversible and tissue infarction occurs, leading to symptomatic ramifications. According to The National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator (NINDS r-tPA) Stroke Study, early detection and treatment of stroke decreases mortality and enhances clinical outcomes [11]. As the population continues to age in the USA and in the world, the prevalence of stroke continues to grow due to an array of factors [12, 13] including hypertension [13, 14], diabetes [15–17], obesity [18], smoking, and metabolic syndrome [19].

Current Treatment Options

The current gold standard for treatment of ischemic stroke is intravenous (IV) alteplase, given that it is initiated within 4.5 h of stroke onset. Alteplase, a tissue plasminogen activator (tPA), behaves as a co-factor in the activation of plasminogen to plasmin to facilitate the degradation of the obstructing clot [20]. At this time, IV-tPA is the only FDA-approved drug treatment for patients who have an acute ischemic stroke [21]. However, the utilization of IV-tPA does have its limitations in the form of side effects. Intracerebral hemorrhage (ICH) is one of the more serious side effects of IV-tPA utilization. According to the NINDS trials, symptomatic ICH (evidenced by computed tomography) occurs in approximately 6.4% of patients who receive IV-tPA [22]. Of the 6.4% of patients who convert to ICH, there is an associated 83% mortality rate according to the PROACT II trial [23].

Patients who have an acute ischemic stroke caused by occlusion of a large artery in the anterior circulation can undergo mechanical thrombectomy. Patients 18 years or older should undergo a mechanical thrombectomy with a stent retriever if they have minimal stroke disability, a NIHSS score ≥ 6, a causative occlusion of the internal carotid artery or proximal cerebral artery, an affirming non-contrast CT scan (ASPECT score of ≥ 6), and if they can be treated within 6 h of their last known normal [24–28]. According to five multicenter, open-label randomized controlled trials (MR CLEAN, ESCAPE, SWIFT PRIME, EXTEND-IA, and REVASCAT), mechanical thrombectomy initiated within 6 h of symptoms can effectively reduce disability in ischemic stroke patients [24–28]. The results from these studies are promising; however, mechanical thrombectomy does have adverse effects. The most common adverse effect is a new ischemic stroke in a different area of the brain within 90 days of treatment; according to the MR CLEAN study, the occurrence was 5.6% versus 0.4% in the non-intervention group [24].

In addition to intravascular therapy, stroke management includes medical therapy that is associated with increased functional capacity, reduced complications, and reduced risk of stroke recurrence. Medical therapy can be used in conjunction with either IV-tPA or mechanical thrombectomy, or it can be used as a stand-alone treatment. The medical regimen includes an anti-thrombotic agent at discharge (i.e., aspirin), a high-intensity statin (lipid lowering agent), blood pressure reduction after the acute phase has passed, and behavioral and lifestyle modifications [29].

Need for Additional Treatment Options

IV-tPA and mechanical thrombectomy are the only two invasive options for treatment of acute ischemic stroke. At this time, IV-tPA is the only FDA-approved treatment of acute ischemic stroke. However, fewer than 2% of patients are actually given the therapy because of concern of conversion to ICH and delays in reaching a stroke center within the 4.5-h window [30]. Patients who have been diagnosed with a transient ischemic attack (TIA) or minor stroke and patients who are in the hyperacute phase of recovery from a major ischemic stroke are at the greatest risk of early deterioration [31]. Under current guidelines, these patients do not qualify for acute treatment; however, they are equally at risk for progression to a major ischemic event. The peak risk of conversion to a major ischemic stroke is in the first hours to 7 days from the initial symptom onset. Patients have a 7% risk at 48 h and a 10% risk by 7 days [31]. Furthermore, there are currently no viable treatment options for these patients. Stroke is trending toward becoming the leading cause of death in the USA, and it is crucial that we continue to conduct research and trials to facilitate the implementation of additional treatment options for these patients.

Bilirubin

Bilirubin, generated as an end-product of heme catabolism, has long been seen as a potentially toxic agent at high levels in the human body. However, evidence strongly indicates that bilirubin contains antioxidant, anti-inflammatory, and cytoprotective properties [32]. These specific properties are vital for organs that do not have strong endogenous cytoprotective defenses such as myocardial and neural tissue [33]. Table 1 summarizes some of these unique properties of bilirubin.

Chemistry and Metabolism

Bilirubin exists in the body in three different forms: unconjugated bilirubin, conjugated bilirubin, and unbound (free) bilirubin. In various cell types, notably the microcytic cell lineage, heme is broken down to biliverdin via heme oxygenase and is subsequently broken down to bilirubin via biliverdin reductase [48]. Approximately 96% of the bilirubin in normal plasma flows in the unconjugated form and is tightly bound to albumin to be transferred to the liver for conjugation [49]. A liver–blood shuttling loop in humans with MRP3, OATP1B1, and OATP1B3 plays a key role in maintaining stable bilirubin blood levels between the liver and the surrounding vasculature [50]. Once in the hepatocytes of the liver, bilirubin is conjugated via the enzyme uridine diphosphate glucuronyl transferase (UDGPT) and is subsequently excreted into bile [51]. Hyperbilirubinemia in the unconjugated form suggests some form of hemolysis, whereas hyperbilirubinemia in the conjugated form suggests liver disease or an inherited disorder of conjugation [48]. The conjugated form of hyperbilirubinemia is rarely associated with bilirubin neurotoxicity [52]. In neonates, the visual identification of jaundice requires that their total serum bilirubin concentrations be greater than 5–6 mg/dL (85–100 μmol/L) [52]. Jaundice in neonates can even become a more severe hyperbilirubinemia case involving infant mortality and morbidity [52]. However, accumulating clinical studies have indicated that hyperbilirubinemia provides many benefits to the human body [53]. One such benefit is that hyperbilirubinemia increases the antioxidant capacity in the blood, leading to lower oxidative stress and superior outcomes in free radical-associated diseases, like grade III intraventricular hemorrhage, severe fungal infection, and sepsis [53].

Clinically, bilirubin levels are reported as total bilirubin (direct and indirect) and direct bilirubin (conjugated bilirubin); indirect bilirubin (unconjugated bilirubin) can be calculated separately. In addition, bilirubin also flows in the plasma in the unbound (free) form [54]. This unbound, unconjugated form of bilirubin can more readily cross the blood–brain barrier, as it is lipid soluble and can passively diffuse across cells [55]. This relationship can be seen in Fig. 1 below. This is logical, as bilirubin that is bound to albumin is a large compound with hydrophilic properties that prevent it from passively crossing the lipophilic blood–brain barrier. Factors that can increase the amount of unbound bilirubin include hypoalbuminemia, metabolic acidosis (low pH weakens the albumin–bilirubin bond), and pharmacologic agents (salicylates, sulfonamides) [55]. Other binding proteins, such as lipocalin-type prostaglandin D synthase, can also theoretically alter the levels of bound and unbound bilirubin in the serum [56]. In 1999, Beuckmann et al. showed that the binding of this protein to bilirubin, via the resonant mirror technique and surface plasmon resonance detection, could theoretically alter the amount of bound and unbound bilirubin in the serum [56]. At this time, limited clinical studies and data are available to determine the best method for calculation of unbound bilirubin and its clinical importance, demonstrating the need for further research.

Genetic diseases have also shed light on bilirubin and its membrane transporters. Specifically, Dubin–Johnson syndrome, a conjugated hyperbilirubinemia, is caused by a mutation in multidrug resistance-associated protein, which significantly reduces the liver's ability to excrete conjugated pigment into the bile [50]. Gilbert's syndrome, or unconjugated hyperbilirubinemia, is due to a UGT1A1 mutation resulting in defective conjugation of bilirubin [50].

Bilirubin and Antioxidation

In 1987, Stocker et al. showed that the antioxidant activity of bilirubin significantly increased when the body's oxygen concentration dropped from 20% (room air) to 2% [57]. Oxidative damage, as a result of ischemia, contributes significantly to the pathogenesis and progression of ischemic stroke [58]. Specifically, excessive free radical production causes oxidative lipoprotein modification, which accelerates the development of atherosclerosis, a key pathological mechanism leading to ischemia [59]. Ischemia in the brain causes the oxygen concentration in the infarct area to significantly drop; therefore, bilirubin's antioxidant activity would increase to counter the harmful effects from the oxidative stress. Bilirubin contains an extended system of conjugated double bonds and a reactive hydrogen atom that gives the compound its strong antioxidant properties [57]. This reaction allows for the inhibition of NADPH oxidase-dependent proliferation of vascular smooth muscle cells, which have a pro-oxidant effect in vessels [58]. All types of bilirubin share some of the same antioxidant properties, yet during an ischemic stroke, only unbound, bioactive bilirubin is effective in treatment, as it can cross the blood–brain barrier [55].

During an ischemic stroke, much of the damage is caused by reactive oxygen species (ROS) and superoxide production in the infarct area due to hypoxia. Furthermore, when there is successful reperfusion, this reperfusion injury is known to be associated with a surge in free radicals. Bilirubin has the

Table 1 Summary of reported clinical studies that show how bilirubin alters stroke prevalence and outcomes

Reference	Study design	*N*	Age	Sex	Study span	Type of bilirubin	Bilirubin level	Clinical endpoints	*p* value	Clinical outcomes
Oda et al. [34]	Retrospective cohort study	5444	55 ± 15	62.0% Male	2008–2010	Total bilirubin	Quintile 1 (Q1) in male 2.6–9.3 μmol/L	Odds ratio (OR) for stroke and congestive heart disease (CHD) for male: reference	Male: referenceFe-male: reference	Lower quartiles of total bilirubin are associated to a greater prevalence of stroke and CHD in male and stroke in female.
							Q1 in female 0.9–9.3 μmol/L	OR for stroke for female: reference		
							Q2 in male 9.4–12.7 μmol/L	OR for stroke and CHD for male 0.49 and 0.63	Male 0.050 Female 0.040	
							Q2 in female 9.4–12.0 μmol/L	OR for stroke for female 0.35		
							Q3 in male 12.8–14.4 μmol/L	OR for stroke and CHD for male 0.65 and 0.45	Male 0.030 Female 0.030	
							Q3 in female 12.1–54.7 μmol/L	OR for stroke for female 0.34		
							Q4 in male 14.5–17.8 μmol/L	OR for stroke and CHD for male 0.37 and 0.67	Male 0.020	
							Q5 in male 17.9–71.8 μmol/L	OR for stroke and CHD for male 0.40 and 0.61	Male 0.030	
Boon et al. [35]	Retrospective cohort study	87	65 ± 15 (18–63)	52.1% Male	1994–2001	Total bilirubin	Q1 0 to 10.2 μmol/L	Reference value	IS = 0.001 All stroke = 0.007	Increasing total bilirubin levels showed a decrease in hazard ratio of ischemic strokes and all strokes in males. These associations were not seen in hemorrhagic stroke or in female.
							Q2 10.3–15.3 μmol/L	Hazard ratio (HR) for ischemic stroke (IS) 0.80; HR for all stroke 0.86		
							Q3 15.4–22.1 μmol/L	HR for IS 0.72; HR for all stroke 0.81		
							Q4 22.2–34.2 μmol/L	HR for IS 0.66; HR for all stroke 0.74		
Perlstein et al. [36]	Cross-sectional examination	13,214	≥ 20	48.1% Male	1999–2004	Total bilirubin	1.71–39.3 μmol/L	OR for stroke 0.91 10% Reduced odds of adverse stroke outcomes (patients with history of stroke)	0.002 0.060	Higher serum total bilirubin level is associated with reduced stroke prevalence and improved stroke outcomes.
Kao et al. [37]	Retrospective cohort study	2235	70.5 ± 7.1 (60–84)	47.8% Male	1999–2002	Total bilirubin	Q1 ≤ 8.5 μmol/L Q2 8.6–10.2 μmol/L Q3 10.3–12.0 μmol/L Q4 12.1–22.2 μmol/L	OR for each standard deviation (SD) of total bilirubin 0.56	0.002	Higher serum total bilirubin levels were associated with lower likelihood of functional dependence in older adults.
Jørgensen et al. [38]	Retrospective cohort study	9804	≥ 55	57.0% Male	2003–2009	Total bilirubin	Q1 7.0 ± 1.0 μmol/L Q2 9.5 ± 0.5 μmol/L Q3 11.9 ± 0.8 μmol/L Q4 17.7 ± 4.9 μmol/L	HR = 1.00 (reference) HR = 0.80 HR = 0.73 HR = 0.77	< 0.001	Low levels of bilirubin were found to be associated with an increased risk of having a primary outcome event (non-fatal myocardial infarction, non-fatal stroke, resuscitated cardiac arrest, or cardiovascular death) in analyses adjusted for age, gender, and sibutramine treatment allocation.
		60	68.3 ± 11.9		2011–2013	Total bilirubin	10.1 ± 4.6 μmol/L	OR for total bilirubin 1.82	< 0.001	

Table 1 (continued)

Reference	Study design	*N*	Age	Sex	Study span	Type of bilirubin	Bilirubin level	Clinical endpoints	*p* value	Clinical outcomes
Li et al. [39]	Retrospective cohort study			68.3% Male		Direct bilirubin	3.2 ± 2.1 μmol/L	OR for direct bilirubin 2.83 OR for white blood cell count 0.35 OR for fasting glucose 0.34 OR for diastolic blood pressure 0.88	< 0.001 0.014 < 0.001 0.041	Serum level of heme oxygenase 1 (HO1) was higher in patients with stroke than transient ischemic attack (TIA), and serum levels of both total and direct bilirubin were lower in stroke than TIA. Serum direct bilirubin, total bilirubin, diastolic blood pressure, and fasting glucose were independent predictors of stroke.
Kimm et al. [40]	Retrospective cohort study	78,724	(30–89)	52.1% Male	1994–2001	Total bilirubin	Q1 0 to 10.2 μmol/L Q2 10.3–15.3 μmol/L Q3 15.4–22.1 μmol/L Q4 22.2–34.2 μmol/L	Reference value HR for IS 0.80; HR for all stroke 0.86 HR for IS 0.72; HR for all stroke 0.81 HR for IS 0.66; HR for all stroke 0.74	IS 0.001 All stroke 0.007	Increasing total bilirubin levels showed a decrease in hazard ratio of ischemic stroke and all stroke in male. These associations were not seen in hemorrhagic stroke or in female.
Luo et al. [41]	Retrospective cohort study	796	(15–92)	63.0% Male	2008–2012	Direct bilirubin Total bilirubin	Direct bilirubin for IS v. TIA (4.070 v. 4.702 μmol/L) Total bilirubin (16.147 v. 18.329 μmol/L)	OR for LACI: reference OR for TACI 6.99 OR for PACI 2.49 OR for POCI 2.41	> 0.001 > 0.001	Serum levels of direct and total bilirubin increased after AIS, which linked to the severity of stroke. Bilirubin levels are lower in TIA than in acute ischemic stroke (AIS). NIHSS and relative severity of AIS were associated with elevated bilirubin. The highest level of bilirubin was found in total anterior circulation.
Pineda et al. [32]	Retrospective cohort study	1046	67.5 ± 16.6	52.5% Male	2002–2007	Direct bilirubin	≥ 6.841 μmol/L ≤ 1.710 μmol/L	Higher NIHSS scores Lower NIHSS scores	0.001 Stroke severity; 0.034 poorer discharge outcome	Higher direct bilirubin levels were associated with greater stroke severity and poorer discharge outcome. Higher direct bilirubin levels had greater admission NIHSS scores compared to lower levels, but direct bilirubin is not independently related with discharge outcome. No independent relationship between total bilirubin versus stroke severity or outcome.
		1333			2005–2014	Total bilirubin	Q1 2.60–9.80 μmol/L		0.001	

Table 1 (continued)

Reference	Study design	*N*	Age	Sex	Study span	Type of bilirubin	Bilirubin level	Clinical endpoints	*p* value	Clinical outcomes
Tan et al. [42]	Prospective cohort study		65.5 ± 13.8 (18–93)	57.0% Male				Incidence of SCE 18.9%; LAA 31.3%; SAO 33.3%; SUE 31.9%		The incidence of SCE is linearly associated with AST and GGT. The incidence of SCE is not linearly associated with bilirubin. However, the incidence of SAO, LAA, and SUE decreased in a linear trend with an increase in bilirubin.
							Q2 9.90–13.10 μmol/L	Incidence of SCE 27.6%; LAA 26.4%; SAO 26.6%; SUE 24.2%		
							Q3 13.20–17.90 μmol/-L	Incidence of SCE 22.2%; LAA 21.3%; SAO 23.4%; SUE 23.5%		
							Q4 18.00–141.80 μmo-l/L	Incidence of SCE 31.3%; LAA 20.9%; SAO 16.7%; SUE 20.4%		
Liu et al. [43]	Cross-sectional study	1839	87.4 ± 4.0 (80–102)	100.0% Male	2014	Total bilirubin	Q1 < 11.1 μmol/L	Incidence of IS 46.4% OR reference	Incidence 0.039 OR 0.021	Higher total bilirubin was significantly associated with lower prevalence of major diabetic complication, including ischemic stroke, among senile diabetic patients. This association was independent of age and control status of diabetes.
							Q2 11.1–13.39 μmol/L	Incidence of IS 43.8% OR 0.90		
							Q3 ≥ 13.4 μmol/L	Incidence of IS 43.4% OR 0.82		
Kurzepa et al. [44]	Retrospective cohort study	43	71.9 ± 12.1	51.2% Male	Not men-tioned	Total bilirubin	D1 15.4 μmol/L D3 13.7 μmol/L D5 13.2 μmol/L D10 11.5 μmol/L	Not mentioned	< 0.001	Serum total bilirubin level evaluated during the acute phase of ischemic stroke proved to be a bad prognostic factor not only for early neurological status but also for late disability measured 3 months after stroke onset. Higher serum total bilirubin level during acute phase of stroke is associated with greater patients' disability evaluated three months after symptoms onset.
Lee et al. [45]	Retrospective cohort study	5599	Not men-tioned	66.8% Male	2004–2013	Total bilirubin	15.56 ± 5.99 μmol/L	Not mentioned	Not mentioned	The present study demonstrated a significant association between SNPs at UGT1 locus and bilirubin levels. However, genetic evidence based on Mendelian randomization approaches suggests no causal effect of bilirubin

Table 1 (continued)

Reference	Study design	*N*	Age	Sex	Study span	Type of bilirubin	Bilirubin level	Clinical endpoints	*p* value	Clinical outcomes
										levels on the development of stroke.
Kawamoto et al. [46]	Prospective cohort study	1511	Male 70 ± 9 Female 70 ± 8	41.9% Male	Not mentioned	Total bilirubin	Q1 in male 0.3–0.5 mg/dL Q1 in female 0.2–0.49 mg/dL Q2 in male 0.51–0.7 mg/dL Q2 in female 0.5–0.6 mg/dL Q3 in male 0.71–0.86 mg/dL Q3 in female 0.61–0.79 mg/dL Q4 in male 0.87–1.91 mg/dL Q4 in female 0.8–1.78 mg/dL	Mean abnormally high glycated hemoglobin (HbA1c) in males 5.85% Mean HbA1c in females 5.79% Mean HbA1c in males 5.83% Mean HbA1c of females 5.75% Mean HbA1c of males 5.78% Mean HbA1c of females 5.70% Mean HbA1c of males 5.66% Mean HbA1c of females 5.71%	Males < 0.05 Females < 0.05 Males < 0.05 Females < 0.05 Males < 0.02 Females < 0.03 Males < 0.01 Females < 0.03	HbA1c is significantly associated with oxidative stress and a heightened risk of cardiovascular disease. In both genders, HbA1c levels were significantly low in subjects with a high serum total bilirubin level. These findings demonstrate bilirubin's antioxidant effect in the body.
Schwertner et al. [47]	Review	NA	NA	NA	NA	Total bilirubin	Not mentioned	Not mentioned	Not mentioned	1.710 μmol/L increase in serum bilirubin is associated with 63% reduced risk of stroke. In categorized analysis, subjects with serum bilirubin greater than 1.710 μmol/L had a reduced risk of stroke by 48%.

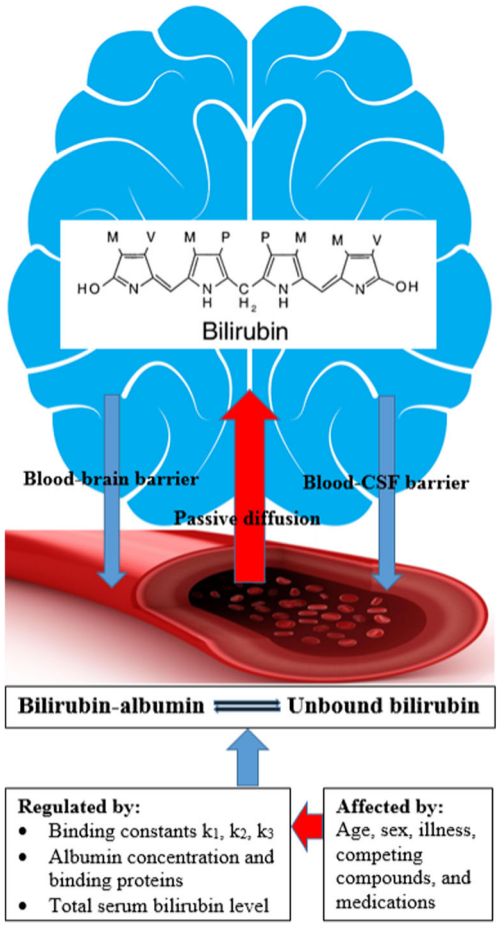

Fig. 1 Unbound, unconjugated form of bilirubin readily crossing the blood–brain barrier

power to scavenge some ROS to prevent further damage and to suppress the harmful series of reactions that would follow the initial infarct [60]. Likewise, through the suppression of NADPH oxidase activity, bilirubin is a powerful inhibitor of mitochondrial superoxide production [60]. Superoxide and reactive oxygen species collectively promote lipid peroxidation, foam cell formation, and the expression of pro-inflammatory cells in the vasculature [60]. This leads to endothelial dysfunction and facilitates the progression of atherogenesis, which ultimately leads to cerebral ischemia [33].

Bilirubin and Anti-Inflammation

The recruitment of pro-inflammatory cytokines coupled with dyslipidemia leads to atheroma formation that promotes thrombosis, which ultimately causes stroke. To better understand the role of bilirubin in anti-inflammation, it is imperative to understand the molecular mechanisms that cause attachment of leukocytes to endothelium [61]. Bilirubin affects the body's inflammatory pathways by inhibiting complement induction, modulating the activity of cytotoxic T-lymphocytes, and blocking the production of pro-inflammatory cytokines [59]. Complement induction is inhibited mainly at the C1 step of the classical pathway [62]. Bilirubin modulates the activity of cytotoxic T-lymphocytes by decreasing DNA synthesis, expressing Tac antigen (CD25), and expressing the transferrin receptor (CD71) [63]. Bilirubin also inhibits the production of pro-inflammatory cytokines to subdue the inflammatory response. Specifically, Wallner et al. found that elevated levels of bilirubin exerted immunomodulatory effects and decreased IL-6 and CRP levels [64]. These low serum CRP levels translate to anti-inflammatory effects from elevated bilirubin levels and can protect patients from cerebral ischemia.

Bilirubin and Lipid Metabolism

One of the most common etiologies of ischemic stroke is thrombus formation due to atherosclerosis. Low-density lipoprotein cholesterol (LDL-C) is a major contributor in plaque formation; its oxidative modification in the process of atherogenesis is inhibited by bilirubin's antioxidant and anti-inflammatory properties [65]. As bilirubin is lipophilic, it protects lipids from peroxidation and further prevents free radical formation and endothelial damage [66, 67]. In 2012, Boon et al. showed that total cholesterol and LDL levels were significantly reduced in patients with elevated bilirubin levels versus matched controls (0.83 and 0.91 mM, respectively) [35]. Thus, the anti-peroxidative roles of bilirubin are essential in slowing down and preventing atherogenesis in patients who suffer from ischemic stroke.

Bilirubin and Intercellular Adhesion

Intercellular communication is a process that governs and coordinates the ability of cells to respond to their environment in relation to development, tissue repair, mounting an immune response, and tissue homeostasis. Cellular adhesion molecules (CAMs) are proteins that facilitate callular adhesion and intercellular communication [68]. During atherosclerosis, changes in the endothelial environment result in the increased expression of adhesion molecules, which initiates the migration of inflammatory and immune cells into the arterial wall of a vessel [69].

Recent studies have shown that bilirubin inhibits the proliferation of vascular smooth muscle cells by impeding intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [59]. Specifically, unconjugated bilirubin (UCB) inhibits the migration of monocytes into the intima of the artery, thereby limiting an inflammatory response [70]. Furthermore, by limiting these steps in the intercellular adhesion process, bilirubin plays another important role in mitigating atherogenesis.

Bilirubin and Ischemic Stroke

The aforementioned antioxidative, anti-inflammatory, and cytoprotective properties of bilirubin have recently emerged as a feasible endogenous defense mechanism against ischemic stroke and as a potential bedside therapeutic option. Several clinical studies show the beneficial effects of bilirubin in ischemic heart disease; however, there is a need for more clinical research in the cerebral ischemia model. Thus, it is imperative to understand what has been done previously, the limitations of these studies, and how to improve them to conduct further clinical trials for translation of therapy to the bedside. Table 2 provides a current list of published clinical studies showing a relationship between bilirubin and ischemic stroke.

Relevant Clinical Studies with a Positive Relationship

Table 2 summarizes each of these papers. A study done in 2012 by Oda et al. showed that increasing total bilirubin levels were associated with a diminished prevalence of ischemic stroke in Japanese men and women [71]. The study data for men were represented in quintiles in relation to total bilirubin levels: 2.6–9.3 μmol/L, 9.4–12.7 μmol/L, 12.8–14.4 μmol/L, 14.5–17.8 μmol/L, and 17.–71.8 μmol/L. The study data for women were represented in tertiles: 0.9–9.3 μmol/L, 9.4–12.0 μmol/L, and 12.1–54.7 μmol/L; for both men and women, the first level was used as a reference. The prevalence and odds ratios (OR) for men were as follows: 3.3 and reference, 2.0 and 0.49, 2.3 and 0.65, 1.3 and 0.37, and 1.5 and 0.40 for each respective quintile. The prevalence and ORs for women were as follows: 2.4 and reference, 0.8 and 0.35, and 0.8 and 0.34 for each respective tertile. Thus, this cross-sectional cohort showed that higher total bilirubin levels were affiliated with less stroke prevalence in both men and women [71]. However, their study's limitations included a small sample size, limiting measurement to only total bilirubin, and not recording information about the type of stroke.

A study by Kim et al. in 2017 showed that a low serum total bilirubin level was associated with an increased risk of atherosclerosis [72]. The study consisted of 1128 subjects and looked at intracranial and extracranial arterial stenosis in relation to total bilirubin levels in men and women; prevalence

Table 2 List of bilirubin's antioxidative, anti-inflammatory, and cytoprotective properties during the atherogenic process

Vascular mechanism	Relation to bilirubin
Oxidative stress	Increases total antioxidant capacity
	Decreases LDL oxidation
	Decreases production of advanced glycation end-products
	Decreases production of superoxide via NADPH oxidase inhibition
Inflammation	Decreases production of pro-inflammatory cytokines
	Decreases high-sensitivity CRP levels
	Regulates T-regulatory cell differentiation
Serum lipids	Decreases LDL cholesterol
	Decreases triacylglycerols
	Increases HDL-cholesterol
Glucose metabolism	Preserves glucose homeostasis
	Increases insulin sensitivity
	Decreases insulin resistance
Blood pressure	Decreases blood pressure
Obesity	Lower bilirubin levels associated with greater obesity
Thrombosis	Decreases platelet activation
	Decreases mean platelet volume
	Increases activated partial thromboplastin time
Cellular adhesion	Decreases TNFα-induced expression of VCAM-1/ICAM-1/E selectin
	Decreases VCAM-1-mediated transendothelial leukocyte migration
Intracellular signaling	Decreases MAPK pathway
	Decreases NADPH oxidase pathway
	Decreases TNFα-stimulated NFκB translocation
Vascular dysfunction	Increases coronary microvascular function
	Increases aortic elastic properties
	Preserves coronary vasoreactivity
	Decreases arterial stiffness

was 13.0% and 11.3%, respectively. The study was set up into three models: model 1 adjusted for age and sex; model 2 adjusted for age, sex, hypertension, diabetes, hyperlipidemia, smoking, use of a statin, and glomerular filtration rate; and model 3 adjusted for the aforementioned in model 2 as well as hematocrit, platelet count, albumin, aspartate aminotransferase, and triglycerides. Each model was subcategorized into tertiles of total bilirubin levels (low, middle, and high). Results showed that serum total bilirubin levels were negatively associated with cerebral ischemia. Specifically, the prevalence of ECAS was significantly lower in the higher BR tertile groups ($p = 0.008$) [72]. This study's limitations included patients from an outpatient setting, measuring only total bilirubin levels, and not clearly defining whether low total bilirubin levels were a causative factor of atherosclerosis.

The National Health and Nutrition Examination Survey, conducted between 1999 and 2004, was one of the first studies to look at the cross-sectional correlation of total bilirubin levels with stroke prevalence and adverse stroke outcomes [36]. This study consisted of 13,214 patients and assessed the likelihood of stroke associated with a 1.71 μmol/L increase in total bilirubin level; tertiles of bilirubin were used. The study was able to conclude that a 1.71 μmol/L increment increase in total bilirubin level was associated with a 9% reduction in the odds of ischemic stroke (OR 0.91; 95% CI, 0.86–0.96). Further, the study also found there was a 24% and 44% reduction in the odds of stroke in the second and highest tertile of total bilirubin levels, respectively [36]. This study concluded that bilirubin levels were inversely associated with stroke prevalence and that higher total bilirubin levels improved stroke outcomes in patients with a history of stroke.

In 2012, Kao et al. investigated the correlation between serum total bilirubin levels and functional dependence in elderly patients (60 years and older) [37]. Their group measured the following five functional outcome measures: activities of daily living, instrumental activities of daily living, leisure and social activities, lower extremity mobility, and general physical activities. Functional dependence was classified as having difficulties with three or more of the five measures. Further, they used a multiple logistic regression to calculate the OR of functional dependence for 1 standard deviation increase in the total bilirubin level. Their group concluded that 1 standard deviation increment in the total bilirubin level correlated to a 35–44% decrease in functional dependence (OR 0.65, 95% CI, 0.54–0.78, $p < 0.001$) [37]. Thus, this study does an excellent job exploring the improvement of functional dependence after stroke in relation to elevated bilirubin levels.

A study done by Jorgensen et al. in 2014 discussed the Sibutramine Cardiovascular Outcomes trial, which looked at the relationship between total bilirubin levels and the risk of having a primary outcome event, including stroke [38]. The 9742 patients included in this study were divided into four quartiles in relation to total bilirubin levels at screening (bilirubin ≤ 8 μmol/L; > 8 and ≤ 10 μmol/L; > 10 and ≤ 13 μmol/L; > 13 μmol/L). Results showed a significantly ($p < 0.001$) decreased risk of stroke with increasing total bilirubin levels when analyzed as a continuous logarithmic transformed variable [38]. They were able to conclude that lower levels of bilirubin were associated with an increased probability of having a primary outcome event. Interestingly, they also found that weight loss led to an increase in bilirubin levels over a span of 4 weeks; this could be a topic of focus in the future.

In 2014, Li et al. studied 60 stroke patients and 50 TIA patients and analyzed the relationship between serum total bilirubin levels and the incidence of stroke [39]. The results of this study found that in stroke patients, the levels of total bilirubin and direct bilirubin were lower in comparison with patients who had a TIA ($p < 0.001$). Further, they also concluded that serum levels of direct bilirubin and total bilirubin were significant independent predictors of stroke ($p < 0.05$) [39]. This study did have limitations including a small sample size and the lack of variables involved in stroke prognosis. However, Li and his group did effectively show the importance of the protective benefits conferred by bilirubin in the setting of ischemic stroke.

In 2009, Kimm et al. looked at 78,724 patients to analyze if stroke incidence outcome was related to serum bilirubin concentrations [40]. Patients' serum bilirubin levels were divided into quintiles: 0–10.2, 10.3–15.3, 15.4–22.1, and 22.2–34.2 μmol/L. Results showed that the risk of all stroke types decreased as serum bilirubin levels increased (HR, 0.81; 95% CI, 0.68–0.97 in level 3; HR, 0.74; 95% CI, 0.58–0.94 in level 4; $p = 0.0071$) [40]. These findings suggest that lower bilirubin levels increase a patient's risk of stroke. Further, this study does an excellent job providing clinical evidence of the protective properties of bilirubin in the setting of stroke.

In 2017, Tan et al. examined the role of liver enzymes in the pathogenesis of stroke and its subtypes in 1333 patients [42]. Total patient bilirubin levels were divided into quartiles: 0–7.9, 8.0–11.3, 11.4–15.2, and 15.3–22.0 μmol/L. Results indicated that small artery occlusion, large artery atherosclerosis, and stroke of undetermined etiology decreased in a linear trend with an increase in bilirubin levels. The incidence rate for each stroke subtype is as follows: cardioembolic stroke: 18.9%, 27.6%, 22.2%, and 31.3%; large artery atherosclerosis: 31.1%, 26.4%, 21.3%, and 20.9%; small artery occlusion: 33.3%, 26.6%, 23.4%, and 16.7%; stroke of undetermined etiology: 31.9%, 24.2%, 23.5% and 20.4%, respectively, by each quartile; $p = 0.001$ [42]. These results show that lower bilirubin levels are associated with an increased incidence of all stroke subtypes described in this study. Further, this study does an excellent job identifying the anti-atherogenic properties of bilirubin in the setting of stroke.

Liu et al. looked at 1839 patients to determine if a patient's total bilirubin level altered the presence of major diabetic complications, which included stroke [43]. Total bilirubin levels

were divided into tertiles: 0–11.0, 11.1–13.3, and 13.4–25.2 μmol/L. Results of the study showed that the incidence of major diabetic complications, including stroke, decreased as serum bilirubin levels increased. Incidence for each respective tertile was as follows: 46.4%, 43.8%, and 43.4%; $p = 0.039$; ORs for each were as follows: reference, 0.90, and 0.82; $p = 0.021$ [43]. Thus, the results of their study indicated that lower bilirubin levels are associated with an increased incidence of stroke.

In 2008, Schwertner et al. conducted a meta-analysis that investigated total bilirubin and its possible protective effects and therapeutic applications [47]. The results of their study indicated that a 50% reduction in serum bilirubin was associated with a 47% rise in the odds of having a more severe disease; further, a 1 mg/dL increase in serum bilirubin level is associated with a 63% reduced risk of stroke [47]. This study was able to conclude, by looking at various retrospective and prospective studies, that elevated bilirubin concentrations conferred protection in the setting of stroke.

Relevant Clinical Study with a Negative Relationship

Each of these papers may be found in Table 2. In 2009, Kurzepa et al. looked at 43 patients, prospectively enrolled, who had an ischemic stroke at the time of admission and looked at their total bilirubin levels at 24 h and on day 3, 5, and 10 [44]. These levels were 15.4, 13.7, 13.2, and 11.5 μmol/L, respectively. The study team collected a Barthel Index score for each patient 3 months after the initial ischemic stroke to examine the effects of bilirubin on stroke outcomes. The Barthel Index is an ordinal score used to measure each patient's performance in their activities of daily living; a higher number is associated with greater independence. According to their results, there was a negative correlation between serum total bilirubin levels and the Barthel Index. A higher serum total bilirubin level during the acute phase of stroke was associated with a lower Barthel Index score (decreased functional independence) [44]. However, in the paper's discussion, they mentioned how the negative influence of higher levels of serum bilirubin and uric acid on patients' outcome could reflect the intensity of initial oxidative stress. This study also had a small sample size and thus could not represent the population accurately.

Bilirubin as a Marker of Oxidative Stress

In 2013, Luo et al. conducted a retrospective cohort study on 608 trial patients and 108 control patients to determine if direct bilirubin levels after a stroke are linked to stroke severity [41]. Patient direct bilirubin levels were divided into quartiles: 0–3.42, 3.43–5.13, 5.14–6.83, and ≥ 6.84 μmol/L. Patient total bilirubin levels were also divided into quartiles: 0–10.2, 10.3–15.3, 15.4–22.1, and ≥ 22.2 μmol/L. The team also used the National Institutes of Health Stroke Scale (NIHSS) score to objectively quantify the impairment produced by the stroke. Results showed serum levels of direct bilirubin and total bilirubin were significantly higher in the acute ischemic stroke (AIS) group versus the TIA group; p values were 0.043 and 0.078, respectively [41]. NIHSS scores also indicated a significant association with stroke severity and elevated direct bilirubin and total bilirubin levels [41]. This study demonstrated that direct bilirubin and total bilirubin levels were increased in the AIS group in response to oxidative stress and ultimately had profound impacts on outcomes. Thus, bilirubin can be used as an effective marker of oxidative stress.

Pineda et al., in 2008, prospectively looked at 743 patients who had an AIS and assessed whether the relation of serum bilirubin levels influenced clinical presentation and outcomes [32]. The group looked at direct bilirubin and total bilirubin levels on admission, assessed presenting stroke severity with the NIHSS score, and looked at functional outcomes at discharge using the mRS. Patients were split into the following two groups for direct bilirubin levels: ≤ 1.710 and ≥ 6.841 μmol/L. Results revealed that higher direct bilirubin levels were linked with greater stroke severity ($p = 0.001$) and poorer discharge outcome ($p = 0.034$), reflecting the intensity of the initial oxidative stress from the AIS [32]. The study goes on to indicate how this result was in accord with bilirubin being an effective marker of oxidative stress and cites several studies that concur with their finding.

Conclusions

Bilirubin has strong antioxidant, anti-inflammatory, cytoprotective properties that can be beneficial in the setting of cerebral ischemia, as seen in the aforementioned clinical studies. There are more reported studies that show a positive, beneficial relationship between bilirubin and ischemic stroke than there are negative ones. Kurzepa et al. showed that bilirubin was an ineffective prognostic factor in ischemic stroke; however, the group goes on to say that the initial intensity of oxidative stress could reflect the increased bilirubin levels to counter the oxidative damage that ensued from the initial insult [44]. This study was limited by a small enrollment size of 43 patients.

Collectively, most of these studies show an inverse relationship between serum bilirubin levels and stroke prevalence in patients. Of course, the aforementioned studies did have limitations. A majority of them only studied the effect of total bilirubin on ischemic stroke. Expanding this to compare total bilirubin, direct bilirubin, indirect bilirubin, and free bilirubin in relation to stroke prevalence should be a future endeavor to better understand bilirubin's protective effects [54, 73]. Also, these studies only looked at one type of stroke, ischemic stroke. It would be beneficial to expand the current research

to include subarachnoid hemorrhage, intracerebral hemorrhage, and traumatic brain injury to see the potential therapeutic benefits of bilirubin in these pathological states, as current treatment options for these events are limited as well.

Bilirubin's antioxidant, anti-inflammatory, and cytoprotective properties make it one of the strongest endogenous anti-atherogenic molecules in the body and an excellent marker of oxidative stress [74, 75]. The protective benefits of these properties have been extensively studied and shown in cardiovascular disease; however, the same extent of research has not yet been replicated in ischemic stroke. Thus, rigorous research is needed to provide evidence supporting the existing studies, expand on these studies, and facilitate the translation to bedside therapy for patients who suffer from acute ischemic stroke.

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Abbreviations *AIS,* acute ischemic stroke; *CHD,* coronary heart disease; *CRP,* C-reactive protein; *HDL,* high-density lipoprotein; *HO1,* heme oxygenase 1; *HR,* hazard ratio; *ICAM,* intracellular adhesion molecule; *IS,* ischemic stroke; *LAA,* large artery atherosclerosis; *LACI,* laclunar infarction; *LDL,* low-density lipoprotein; *MAPK,* mitrogen-activated protein kinase; *NADPH,* nicotinamide adenine dinucleotide phosphate; *NFκB,* nuclear factor kappa B cells; *OR,* odds ratio; *PACI,* partial anterior circulation infarction; *POCI,* posterior circulation infarction; *PTEN,* phosphatase and tensin homolog; *Q,* quartile; *SAO,* small-artery occlusion; *SCE,* cardioembolic stroke; *SD,* standard deviation; *SUE,* stroke of undetermined etiology; *SNP,* single-nucleotide polymorphisms; *TACI,* total anterior circulation infarction; *TIA,* transient ischemic attack; *TNF,* tumor necrosis factor; *UGT,* uridine diphosphate glucuronosyltransferase; *VCAM,* vascular cell adhesion protein; *GGT,* gamma-glutamyl transpeptidase; *UCB,* unconjugated bilirubin.

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