

Potential Beneficial Effects of Bilirubin Together with D-Penicillamine in the Treatment of Chronic Neurodegenerative Diseases

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Received: May 24, 2017; Published: July 12, 2017

Abstract

This review introduces to the possible molecular mechanisms of the neurodegenerative disorders. Firstly, it clears up the nature of these phenomenons, focusing on the cytotoxicity. The question arises: how to protect the brain from these pathological conditions? The authors' new concept addresses the medical necessity of chelation therapy (with D-Penicillamine - /D-PA/) and unconjugated bilirubin (UCB). The possible molecular mechanisms of the neuroprotection: (1) Both D-PA and UCB are able to modulate oxidative- and nitrosative stress (OS/NS); (2) As a carbonyl scavenger, DPA binds primarily to aldehydes in an irreversible manner; consequently, this drug inhibits their damaging effects and has also been shown to scavenge peroxynitrite. So, it alleviates lipid peroxidation (LP) of the membranes in the brain. The higher amounts of UCB also serve as a scavenger of lipid peroxides and peroxynitrite. (3) Although it seems that glutamate-mediated excitotoxicity is an important factor causing brain damage in the neonatal hyperbilirubinemia, D-PA can protect the brain from this dangerous phenomenon as a copper chelator and strong ROS/RNS inhibitor.

Keywords: Copper Overload; Oxidative, Nitrosative Stress; Lipid Peroxidation; Excitotoxicity; D-Penicillamine; Unconjugated Bilirubin

Abbreviations

ALS: Amyotrophic Lateral Sclerosis; AMPA: A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid; BG: Basal Ganglia; BBB: Blood-Brain-Barrier; BIND: Bilirubin-Induced Neurologic Dysfunction; BPD: Bronchopulmonary Dysplasia; CNS: Central Nervous System; D-PA: D-Penicillamine; IL-1, 23: Interleukins, INF- γ : Interferon Gamma; MAPT: Microtubule-Associated Protein Tau; MPTP: N-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; LP: Lipid Peroxidation; MPTP: N-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; NMDA: N-Methyl-D-Aspartate; NHBI: Neonatal Hyperbilirubinemia; ND: Neurodegenerative Disease; NS: Nitrosative Stress; OS: Oxidative Stress; ROP: Retinopathy of Prematurity; PUFAs: Polyunsaturated Fatty Acids; RBC: Red Blood Cells; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; TNF-alpha: Tumor Necrosis factor Alpha; UCB: Unconjugated Bilirubin; VCAM-1: Vascular Cell Adhesion Molecule; VEGF: Vascular Endothelial Growth Factor

Introduction

Unconjugated bilirubin (UCB) has long been considered as a cytotoxic waste product of heme catabolisms. However, during the last 30 years a large body of evidence indicated that UCB has several beneficial properties. It is much stronger antioxidant than many other agents: vitamin E, superoxide dismutase and catalase [1-4]. In addition, bilirubin also has cytoprotective and neuroprotective effects [5] and, such an endogenous molecule, is able to exert antiviral activity *in vitro* [6]. In our research works, which embrace more than 40 years [7,8], we have expounded that excessive metal (copper) accumulation in the nervous system may be toxic, inducing oxidative/nitrosative

stress (OS/NS), disrupting mitochondrial function, and impairing the activity of numerous enzymes. Copper excess may result in permanent injuries including severe neurodegenerative disorders (NDs). The immature and strikingly vulnerable neurons play an important role in the pathogenesis of bilirubin-induced neurologic dysfunction (BIND) as well. Our concept addresses the medical necessity of chelation therapy (with D-Penicillamine - /D-PA/) in the neonatal period as it is feasible that UCB molecule has a particular affinity to copper stored in basal ganglia (BG) of the neonatal brain, where copper-bilirubin complex can be formed [9]. Copper dyshomeostasis and OS have also been concerned in NDs such as Alzheimer's-, Parkinson's- or Menkes'-diseases, stroke, amyotrophic lateral sclerosis (ALS), and many others. These irreversible syndromes are related with a progressively aggravating lesions of neurons and injury of synaptic junctions in the central nervous system (CNS) [10]. The burden of NDs is growing permanently as the population ages, with enormous economic and human costs. This review focuses on the metal-induced oxidative/nitrosative stress and excitotoxicity in the most frequent NDs (see: above), and the potential beneficial effects of UCB together with D-PA.

Possible molecular mechanisms of the neurodegenerative disorders

The human brain is a unique organ with its biological complexity in the cranium. Although it adds up to only two-percent of total body mass, it consumes 20-percent of inhaled oxygen during respiration. Consequently, it needs high oxygen to control the accelerated oxidative metabolism. Moreover, the brain has among the highest levels of copper, as well as iron and zinc in the body [11]. These transition metals are essential micronutrients and play well-defined roles in cellular respiration, neurotransmitter production, pigment formation, peptide amidation, and connective tissue biosynthesis [12,13].

Genetic and environmental factors

As far as the molecular mechanisms are concerned, NDs are multifactorial disorders with associations of genetic and environmental factors [14,15]. The genetic background has a strong influence on the load of neurofibrillary tangles and the inflammatory pattern of activated microglia in the brain. The latter's immune response is a potent disease modifier of NDs. Consequently, the targeted immunomodulation rather than anti-inflammatory strategies may be a promising therapy for these diseases [16]. On the other hand, genetic predisposition for a ND is best predicted in the context of environmental exposures, both familial and sporadic forms of these conditions. Otherwise, familial forms represent only a minority of the cases (ranging from 5 to 10% of the total) [17]. Several causative genes for the familial forms have been discovered in recent years. The emerging connections between reactive oxygen species (ROS) and epigenetic mechanisms offer new perspective of research investigating NDs. In genetics of Alzheimer's, i.e. there are two types of genes that influence whether a person develops a disease: risk genes and deterministic genes [18]. (1) APOE-e4 is one of three common forms of the APOE gene and it certainly increases the risk of Alzheimer's. (2) Deterministic genes directly cause a disease, guaranteeing that anyone who inherits one will develop a disorder. These genes are estimated to be responsible for 5 percent of Alzheimer's.

In the past few years, since the discovery of Microtubule-associated protein tau (MAPT), large body of evidence emerged to demonstrate its mutations are important in the pathogenesis of neurodegeneration. The mutations of MAPT result in mutated tau losing its normal function. Further studies on the epigenetics of MAPT in neurodegeneration are essential. These studies may provide new and significant understandings of the etiology, and mechanism-based treatments of NDs [19]. Meanwhile, the "therapy" of these devastating diseases are single drug compounds or molecular and cellular strategies to interfere with the deleterious consequences of general cellular protein quality control, and the accumulation of toxic proteins and block neuronal cell death [20].

Brain Immune response and inflammation

It is clear that the brain can be considered as a site of active immune-surveillance. Recent evidence suggests an existence of bidirectional communication between the brain immune response and inflammation [21]. In addition, several studies have reported that lymphocytes are regularly patrolling the normal CNS in low number which can be increased upon recognition of familiar antigen and

initiation of inflammation at the site [22]. In NDs the resident immune competent cells such as microglia, neurons, astrocytes and oligodendrocytes, release inflammatory mediators to recruit more peripheral immune cells including lymphocytes leading to CNS inflammation [23-28]. During this process, endothelial cells of the blood-brain-barrier (BBB) generates various selectins and adhesion molecules that increase the migration of lymphocytes from the systemic circulation to the perivascular space [29-33]. Activated lymphocytes express chemokines receptors, integrins and selectins that help to interact with their respective ligands expressed on the surface of endothelial cells [34,35]. Activated lymphocytes also release various pro-inflammatory cytokines such as Interleukins (IL-1, IL-23), tumor necrosis factor alpha (TNF-alpha = Tumor Necrosis factor Alpha), interferon gamma (INF- γ) and chemokines including various neurotrophic factors which can contribute in the outcome of the CNS inflammation [36,37].

Metal dyshomeostasis [38]

Copper, zinc and iron are the three most abundant biometals in the human organism. In the past decade, there has been considerable interest in the metal dyshomeostasis (especially copper accumulation) that is a feature of several NDs. In addition, copper and iron are redox active metals and can generate ROS via Fenton and the Haber-Weiss reaction contributing in the OS/NS [39]. In this review, we focus mainly on copper which almost equally capable to generate ROS and RNS, because it is "evidence of abnormal copper transport and aberrant copper-protein interactions in numerous human neurological disorders supports the critical importance of this trace metal for proper neurodevelopment and neurological function" [40]. Copper toxicity increases exponentially over generations. Recently, the number of those children are growing considerably who have neurotoxic conditions such as autism, schizophrenia, attention deficit disorder, dyslexia and learning disabilities which can be related to the accumulation and transmission of excess copper from one generation to the next [41,42]. In addition, copper ions also activate several proangiogenic factors, for example: vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and IL-1, contributing to the development of Kaposi sarcoma and other malignant diseases [43].

Copper is a critical element for the normal brain functions, and the CNS is a major target of disorders of copper metabolism. The redox capacities of copper (particularly the free form) and its ability to generate free radicals are two important features in the process of neurodegeneration [44].

Oxidative stress

In a biological context, ROS have pivotal roles in cell signaling and homeostasis. However, under conditions of OS, ROS production is very high, resulting in damage of membrane lipids, proteins, and nucleic acids that may become irreversible, even cause cell death. Oxidative damage is occurred in the age-related diseases as well in a variety of pathological settings. The strategy which limits oxidant-induced tissue damage, called antioxidant defense mechanisms, is a complex network of endogenous and exogenous systems for scavenging of ROS. Binding of metal ions is also needed for up-regulation of exogenous/endogenous antioxidant defenses. The copper is the strongest redox-active metal which can generate excessive amounts of free radicals. Thus, we need such an antioxidants which have both metal-chelating and ROS/RNS-scavenging activity [10]. In the CNS, a high rate of oxidative metabolism takes place, and it is also true that the brain is more vulnerable to OS compared to other tissues. So, it seems reasonable that we need exogenous antioxidants which are effective in diminishing OS [45]. The BBB protects the neural tissue from harmful substances and toxins. It has the important function of maintaining brain homeostasis. At the same time, growing evidence suggests that ROS are key mediators of BBB breakdown and that they have been implicated in increased BBB permeability [46,47]. As mentioned above the presence of high concentrations of copper in the brain persuaded development of ROS most significantly in comparison with other metals [10]. For example, postmortem brain tissues from patients with NDs clearly display increased indices of ROS in affected brain regions [48]. Andersen in her comprehensive review has reported on evidence for OS in NDs and has explained how this relates to other cellular events. Interactions between these various components might be a cycle of events, of which OS is a major factor [49].

Nitrosative stress (NS)

Definition of this condition: “The reaction of body tissues to nitric oxide (NO), nitrous oxide or similar species at levels greater than can be neutralized” [50]. It is a state resulting from exposure to excessive levels of NO or the highly redox-active peroxynitrite produced following interaction of NO with superoxide anions. The NO plays a pivotal role in plants and mammals, including the human organism, as a negative or positive regulator of cell apoptosis. The cytotoxicity of NO has been studied in various tumour models, both *in vitro* and *in vivo* [51]. Thus, the Reactive Nitrogen Species (RNS)s are fundamental regulators of oxidative metabolism in the cell [52]. NO react rapidly with O₂ and with superoxide (O₂⁻) to generate a wide spectrum of RNSs that are highly damaging to cells [53,54]. Studies indicate that mitochondrial permeability transition and NS represent major factors in copper-induced toxicity in astrocytes, and RNSs can cause neuronal injuries [55,56].

Lipid peroxidation (LP)

LP occurs during the oxidative degradation of lipids. Initiation begins with ROS-induced hydrogen atom abstraction from polyunsaturated fatty acids (arachidonic-, linoleic-, eicosapentaenoic- and docosahexaenoic acid). Two damaging products of LP are 4-hydroxynonenal and acrolein. Overproduction of ROS is creating a continuous cycle of ion imbalance, Ca²⁺ buffering impairment, mitochondrial dysfunction, glutamate-induced excitotoxicity and microvascular disruption. NO, formed from mitochondrial nitric oxide synthase (NOS), in turn reacts with superoxide anion to produce the highly toxic peroxynitrite radical [57]. LP is one of the major sources of free radical-mediated injury: (1) the brain is greatly sensitive to OS (see: paragraph 2.); (2) and contains high levels of polyunsaturated fatty acids (PUFAs); (3) and high levels of redox transition metals. LP is one of the major sources of free radical attack to PUFAs leads to the formation of highly reactive electrophilic aldehydes [58]. Peroxidation of membrane lipids affects a variety of functions in the brain resulting in increased membrane rigidity, decreased activity of membrane-bound enzymes, impairment of membrane receptors and altered permeability [59,60]. Dysfunction in the lipid homeostasis in the CNS could be a dangerous risk factor for the various types of NDs which are marked by extensive neuronal apoptosis, gliosis, and pathological differentiation, proliferation, and development of neurons [61].

Excitotoxicity

Excitotoxicity is the critical process by which nervous cells are damaged and killed by the overactivations of receptors for the excitatory neurotransmitter glutamate, such as the N-methyl-D-aspartate (NMDA)- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor [62]. These glutamate receptors and ion channel protein found in neurons. Excitotoxins like NMDA and kainic acid, as well as pathologically high levels of glutamate, can cause excitotoxicity by allowing high levels of calcium ions to enter the cell. This process activates a number of enzymes, including phospholipases, endonucleases, and proteases such as calpain. Latter enzymes go on to damage cell structures such as components of the cytoskeleton, membrane, and DNA. Excitotoxicity may be involved in stroke, traumatic brain injury and several age-related, neurodegenerative diseases [63]. As glutamate is a major excitatory neurotransmitter in the CNS both in acute brain insults, such as ischaemia and traumatic injury, and in chronic neurodegenerative disorders such as ALS, multiple sclerosis, Parkinson’s disease and others [64]. It acts via two types of receptors: (1) ionotropic receptors - ligand gated ion channels; and (2) G-protein coupled, metatropic receptors; consequently, the glutamate receptors play a vital role in the mediation of excitatory synaptic transmission [65]. The Figure 1 shows the biomolecular mechanisms of copper-induced injuries (OS, NS, LP, excitotoxicity) in the brain.

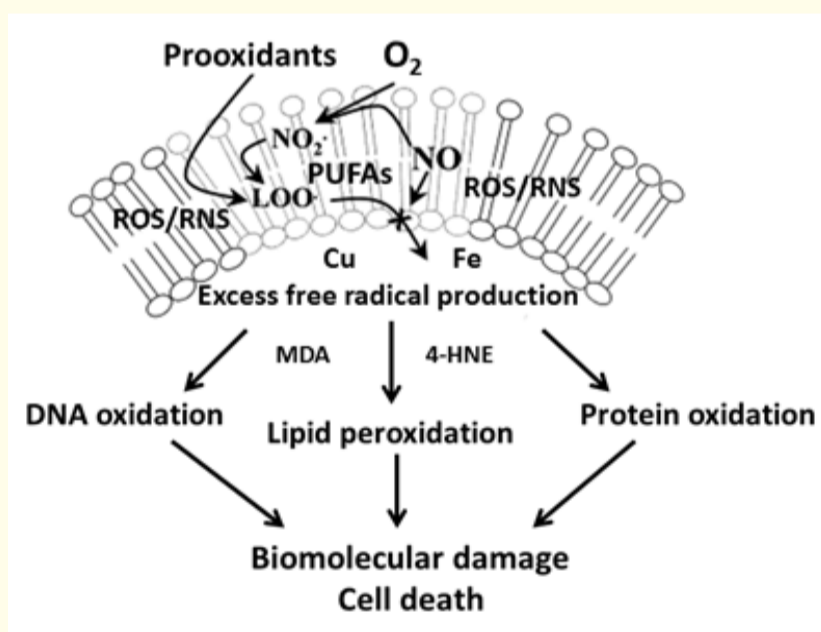


Figure 1: Molecular mechanisms of copper induced oxidative/nitrosative stress and cytotoxicity of the membranes in the brain (MDA: Malondialdehyde; 4-HNE: 4-Hydroxynonenal; PUFAs: Polyunsaturated Fatty Acids).

How to protect the brain against NDs?

It is obvious that the treatments have to serve the alleviation of phenomena, and their effects on the brain, summarized under the 2.1 - 2.7 dots.

Genetic and environmental factors

Neither UCB nor D-PA do seem to affect genes which contribute in the development of NDs. They are not able to control these processes; vice versa, there are several diseases determined by genes in which these substances have beneficial effects (Gilbert’s syndrome, Wilson’s disease and some other neurodegenerative disorders) [66]. Since its discovery [67], D-PA the most widely used copper chelator

in the treatment of Wilson's disease and has been shown to be effective for the treatment of copper toxicosis in dogs (Bedlington terriers) as well [68]. At the same time, the copper chelator D-PA does not attenuate MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced dopamine depletion in mice [69].

Brain Immune response and inflammation

Bilirubin as a powerful immunomodulatory agent

It is excellent from what is written above that UCB has multiple biological activities, including immunomodulatory properties. *In vivo*, treatment with UCB effectively suppressed experimental autoimmune encephalomyelitis [70]. Kristin and Christopher [71] recommended heme oxygenase, a rate limiting enzyme in heme degradation, for potential therapeutic applications. Other authors found connection between UCB concentrations and multiple sclerosis [72], and showed that bilirubin influences the expression of Fc receptors in macrophages [73]. They hypothesized that the bile pigment was capable of regulating immune functions due to its high lipophilia and its direct interaction with cell membranes. Free UCB diffuses through plasma membranes and acts on the immune system at various places, including disruption of antigen presentation, suppression of effector T cell responses, interception of the complement cascade, and promotion of regulatory T cell expansion [74].

UCB inhibits vascular cell adhesion molecule 1 (VCAM-1)-dependent cellular events suggesting that bile pigment blocks VCAM-1-dependent lymphocyte migration. These findings support a potential role for bilirubin as an endogenous immunomodulatory agent [75]. In addition, UCB, as an endogenous regulator of inflammation, acts by disrupting adhesion molecule-mediated leukocyte migration [76], and it is able to exert antiviral activity *in vitro* [6].

D-PA as an immunomodulatory agent

Improvement in joint and cartilage inflammation, pain and regression of rheumatoid nodules may be expected in patients [77]. This drug diminishes tissue damage by reduction of ROS. The area is confused by the fact that D-PA is capable of both increasing the production of ROS and quenching them, depending upon the experimental conditions [78]. In addition to the ability of D-PA to donate electrons for the sequential reduction of ROS, it may also form a stable compound with copper that can dismutate superoxide. This process therefore may contribute to the anti-inflammatory effect [79]. In spite of this its status in the treatment of rheumatoid arthritis (RA) is uncertain. The mode of action and the mechanisms of D-PA in toxicity are not well understood in RA. Consequently, recently safer and more effective analogues of D-PA have been produced and used in the clinical practice.

Antiviral effects of D-PA

High concentrations of D-PA inhibit the replication of several viruses in culture, including the human immunodeficiency virus [80-82]. This may be relevant to a hypothetical viral etiology of RA.

Metal dyshomeostasis

Complex formation of bilirubin and D-PA with copper

Adhikari, *et al.* have shown that UCB may protect mammals from copper poisoning [9]. Treatment of human copper overload disorders is based on the chelating and cupriuretic effect of several agents [83] and the impairment of copper absorption induced by oral zinc therapy. Although researchers have experienced significant improvement in the hepatic injury if chelation therapy has been started relatively early in the course of liver damage, many patients fail to have an adequate response to chelation therapy and eventually require liver transplantation to avoid death [84,85]. A similar lack of response to metal chelation has been demonstrated in advanced Indian childhood cirrhosis [86]. Copper forms complexes with bilirubin (H₂BR). These complexes are able to scavenge ROS and RNS. There were studied a series of Cu²⁺-bilirubin complexes to ascertain the nature of the binding between Cu²⁺ and bile pigment. Chelating agent, such

as D-PA reduces the amount of copper in the body, but it does not affect the increased levels of ROS caused by the copper [87]. That is why intensive investigations are going to find a much better chelating agents [88].

Oxidative and nitrosative stress

UCB can serve as a strong endogenous scavenger of both ROS and RNS [89], which provides a possible explanation for a low incidence of cardiovascular diseases and, to a certain extent, neurodegeneration in patients with Gilbert-Meulengracht syndrome [90]. Research of the last 3 decades indicated that individuals with higher levels of UCB may experience various health benefits (in atherosclerosis, demyelinating neuropathies, cancer and certain heart diseases). Studies have found higher levels of bilirubin in old people who are living with higher functional independence [91-93]. UCB can act as an important cytoprotector of tissues that are poorly equipped with antioxidant defense systems, including myocardium and nervous tissue [94,95]. The UCB levels in jaundiced and non-jaundiced pups exposed to 95% O₂ shows a negative correlation with lipid hydroperoxides at 3 days of exposure. Higher UCB concentrations resulted in lower lipid hydroperoxide levels [96]. In addition, UCB serves as a predictor of OS-mediated diseases [97,98]. To sum it up, high production of ROS/RNS from specific neurochemical reactions in the CNS with aging is dangerous for the human brain. For this reason, along with inflammation, OS seems to be one of the main inducers of neurodegeneration, causing excitotoxicity, neuronal loss, and axonal damage, ultimately being now considered a key element in the onset and progression of several NDs, including ALS [99].

D-PA alters both OS and NS

Our recently published case reports [100], together with other convincing cases which participated in the long-term (28 - 40 years) follow-up, suggested that DPA therapy of newborn infants may have significant neuroprotective effects in cases jeopardized by BIND or retinopathy of prematurity (ROP) [101]. This unexpected effect may be related to DPA capability to alter the nitric oxide (NO) system [102-105] and its antioxidant effects [106-108]. NO synthesized in the CNS produces a myriad of effects: it controls of blood flow, learning and memory, neurotransmitter release, gene expression, immune responsiveness, and cell survival. In the DPA metabolism of the human organism, the major products are the low molecular weight disulfides. *In vivo*, the oxidation of DPA may also important in the mode of its action through simultaneous reduction of ROS. Finally, we can say that DPA fulfills the criteria of a hybrid drug by its ability to modulate both oxidative stress and NO pathway and can be a neuroprotective agent in the pathophysiology of neurological dysfunction [109].

Lipid peroxidation

Mancuso, *et al.* [110] have found that bilirubin and biliverdin had greater inhibition of LP and protein oxidation in rat brain microsomes *in vitro* than vitamin-E. The higher amounts of UCB may serve as a scavenger of both peroxy radical and peroxy nitrite. These findings support the hypothesis of a major role of endogenous bile pigments as neuroprotective molecules in the brain. Malondialdehyde (MDA) is a metabolite commonly used as an indicator of LP. According to Fatani [111] the level of MDA was increased in stepwise manner in the hyperbilirubinaemia groups of neonates depending the UCB levels: the increases of UCB levels in neonates could be a response to greater lipid peroxide formation.

As far as D-PA is concerned Wade, *et al.* [112] have determined the concentration of lipid peroxides in the plasma and synovial fluid of patients suffering from RA. Contrary to previous reports by investigators using less specific methods, these authors were unable to demonstrate any increase in plasma levels of lipid peroxides in the rheumatoid patient. D-PA in the treatment of RA was associated with a significant reduction of lipid peroxide levels, suggesting that this drug may be effective as an oxygen radical scavenger in the joint cavity. These results give further support to the concept of oxygen-free radicals playing an important role in the pathogenesis of chronic inflammatory disorders [113]. Carbonyl scavengers (such as D-PA) [114] have been used with the aim of reducing the "aldehyde load" [115] and in several *in vivo* and *in vitro* studies have been investigated their effects on neuroprotection. D-PA binds primarily to aldehydes in an irreversible manner; consequently, this drug inhibits their damaging effects and has also been shown to scavenge peroxy nitrite as well. Acute D-PA administration has previously been shown to improve neurological recovery in the mouse concussive head injury model and to protect brain mitochondria [116].

Excitotoxicity

Silva, *et al.* [117] investigated the effect of UCB on glutamate uptake in cultured rat astrocytes examining the potential role of bilirubin in the astrocyte function and in the pathogenesis of UCB-related brain damage (BIND). Exposure of astrocytes for 15 min to various concentration of bilirubin and UCB/albumin molar ratio resulted in a significant decrease of glutamate uptake. These findings indicate that UCB impairs the glutamate transport and can play a potential role of astrocyte function in the BIND. Excitotoxicity was proposed as one of the major mechanisms of BIND [118].

It seems that glutamate-mediated excitotoxicity is an important factor causing brain damage in neonatal hyperbilirubinemia. So, drugs suppressing the over release of glutamate, may protect the brain against bilirubin-induced excitotoxicity [119]. This statement per se is connecting strongly with high level of UCB and it is not linking mild bilirubin level which can be used as potential therapeutic agent [120].

We were not able to find any article in the literature, accessible by us, about a direct inhibitory effect of D-PA on excitotoxicity. However, it is well-known that the ROS generation triggers glutamate-mediated excitotoxicity. D-PA is used as a copper chelator and strong ROS/RNS inhibitor for the treatment of Wilson's disease and rheumatoid arthritis; it is known to scavenge carbonyls. Previous literature has shown D-PA scavenging other toxic aldehyde by forming a thiazolidine compound with the aldehyde moiety [121-123].

Conclusion

We hope that our concept will help answer some of the unsolved questions and concerns occurred in the etiology and pathomechanisms not only of BIND but of various age-related neurodegenerative disorders as well. Until recently, UCB was considered as a mere by-product of heme degradation. In the last 3 decades, a growing body of evidence indicating that this "damned" substance is endowed with a strong antioxidant activity in many *in vitro* and *in vivo* experimental systems. Thus, widening the protective role of UCB to other reactive species originating within the cellular milieu. The beneficial neuropharmacological actions of metal-targeted (chelating) agent (D-PA) and UCB most likely arise from local metal redistribution rather than from massive metal removal [124-126].

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Volume 7 Issue 1 July 2017

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