

Copper-induced oxidative/nitrosative stress and excitotoxicity in the neonatal period: neuroprotection with D-Penicillamine

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Abstract

This review focuses on the possible molecular mechanisms of the neonatal brain injuries: copper-induced oxidative/nitrosative stress and excitotoxicity in the neonatal period. Firstly, it clears up the nature of these phenomena in newborn babies. The emerging question: how to protect the neonatal brain? The authors' new concept addresses the medical necessity of chelation therapy (with D-Penicillamine - /D-PA/) in the neonatal period. The possible molecular mechanisms of D-PA in the neuroprotection [1]. It's a hybrid drug in the neonatal period by its ability to modulate both oxidative stress and nitric oxide (NO) pathway [2]. As a carbonyl scavenger, D-PA binds primarily to aldehydes in an irreversible manner; consequently this drug inhibits their damaging effects and scavenges peroxynitrite as well. So, it alleviates lipid peroxidation of the membranes in the neonatal brain [3]. Chelation therapy in neonates restores the normal activity of enzymes participating in heme metabolism. Briefly, chelating agents facilitate heme synthesis and inhibit heme degradation. In other words, this drug as a chelating agent, boost or inhibit the immature enzyme systems to the adult level [4]. Since reactive oxygen/nitrogen species (ROS/RNS) generation triggers glutamate-mediated excitotoxicity, D-PA can also be used as a copper chelator and strong ROS/RNS inhibitor against this dangerous phenomenon.

Abbreviations: BG: Basal Ganglia, BBB: Blood-Brain-Barrier, BIND: Bilirubin-Induced Neurologic Dysfunction, BPD: Bronchopulmonary Dysplasia, CNS: Central Nervous System, D-PA: D-Penicillamine, NO: Nitric oxide, NOS: Inducible Nitric Oxide Synthase, LP: Lipid Peroxidation, NHBI: Neonatal Hyperbilirubinemia, ND: Neurodegenerative Disease, NS: Nitrosative stress, OS: oxidative stress, ROP: Retinopathy of Prematurity, RBC: Red Blood Cells, ROS: Reactive oxygen species, RNS: Reactive nitrogen species, VEGF: Vascular Endothelial Growth Factor

Introduction and aim

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 [1]. These transition metals are essential micronutrients and play well-defined roles in cellular respiration, neurotransmitter production, pigment formation, peptide amidation, and in the connective tissue biosynthesis [2]. In our recently published review article [3] we have expounded that excessive metal (copper) accumulation in the nervous system may be toxic, inducing oxidative stress (OS), disrupting mitochondrial function, and impairing the activity of numerous enzymes. Damage caused by copper excess may result in permanent injuries, including severe neurological/neurodegenerative disorders (NDs). The immature and strikingly vulnerable neurons play an important role in the pathogenesis of bilirubin-induced neurologic dysfunction (BIND) as well. The pathomechanisms of BIND have not been fully understood yet. Our concept addresses the medical necessity of chelation therapy (with D-Penicillamine - /D-PA/) in the neonatal period [4,5], as it is feasible

that unconjugated bilirubin (UCB) molecule has particular affinity to copper stored in basal ganglia (BG) of the neonatal brain, where copper-bilirubin complex can be formed [6]. Copper dyshomeostasis and OS have also been concerned in NDs such as Alzheimer, Amyotrophic lateral sclerosis or Menkes disease. These irreversible syndromes are related with a progressively aggravating lesions of neurons and injury of synaptic junctions in the central nervous system (CNS) [7]. This review focuses on the *copper-induced oxidative/nitrosative stress (OS/NS) and excitotoxicity in the neonatal period*. First of all it is necessary to clear up the nature of these phenomena especially in the newborn babies.

Possible molecular mechanisms of the neonatal brain injuries

Copper dyshomeostasis

Both copper excess, and copper deficiency are jeopardous, creating mineral imbalances. 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 [8,9]. *In the neonatal period* the human brain forms and develops over a long period, with

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neuron proliferation and migration. The blood-brain-barrier (BBB) does not work perfectly (immature) until the middle of the first year of life. The foetus exposed to toxic substances getting copper passively crosses the placenta with fetal levels of this metal often being higher than that of in the maternal blood. A recent report by the National Research Council found that 50% of all pregnancies in the US are now resulting in prenatal or postnatal mortality, significant birth defects, developmental neurological problems [10]. Moreover, the copper metabolism in Wilson's disease and in the newborn infants is strikingly similar: both have large quantities of copper in the liver (the fetal liver concentration is reported to be 16 times greater than that found in the adult [11]) and in the brain (mainly in the BG) which is contrasted by an unusually low ceruloplasmin level in the blood [12]. The copper almost equally capable to generate ROS and reactive nitrogen species (RNS) [13]. In addition, copper ions also activate several proangiogenic factors, for example: vascular endothelial growth factor (VEGF), basic fibroblast growth factor and interleukin-1, contributing to the development of retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD) in prematures [14].

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 occurs in the age-related diseases as well in a variety of pathological settings. It can also contribute to the aging process. The strategy which limits oxidant-induced tissue damage, called antioxidant defense mechanism, is a complex network of endogenous and exogenous systems for scavenging 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 [5]. In the CNS a high rate of oxidative metabolism takes place. At the same time, 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 [15]. The BBB protects the neural tissue from harmful substances and toxins. It has the important function of maintaining brain homeostasis. Growing evidence suggests that ROS are key mediators of BBB breakdown and that they have been implicated in increased BBB permeability [16,17]. *In the neonatal period* excessive production of ROS and other highly reactive free radicals are capable of causing functional and structural damage to cell components. A series of conditions in neonates may, at least partly, be caused by oxygen radicals, e.g. periventricular leukomalacia, intraventricular hemorrhage, BPD, ROP, necrotising enterocolitis, patent ductus arteriosus and, may be, bilirubin encephalopathy (BIND) [18-22]. Free radicals are generated by exposure of newborn infants, particularly prematures, to high oxygen concentrations in resuscitation and mechanical ventilation. As mentioned above the presence of high concentrations of copper in their brain persuaded development of ROS most significantly in comparison with other metals [5]. In addition, newborn infants, especially preterm babies, are endowed with an immature antioxidant defense: lower levels of plasma vitamin E, beta-carotene, sulphhydryl groups, plasma metal binding proteins such as ceruloplasmin and transferrin, as well as reduced activity of erythrocyte superoxide dismutase [23]. Consequently, they are prone to OS which may damage different organs (lung, brain, retina, and gastrointestinal tract), that will affect not only the survival

but also the quality of life of these infants.

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" [24]. It is a state resulting from exposure to excessive levels of NO or the highly redoxactive 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* [25]. Thus, the RNSs are fundamental regulators of oxidative metabolism in the cell [26]. NO reacts rapidly with O₂ and with superoxide radical (O₂⁻) to generate a wide spectrum of RNSs that are highly damaging to cells [27,28]. Studies indicate that mitochondrial permeability transition and NS represent major factors in copper-induced toxicity in astrocytes, and RNSs can cause neuronal injuries [29]. *In the neonatal period* several studies attribute an important role for inducible nitric oxide synthase (iNOS)-induced NS in periventricular leukomalacia and in necrotizing enterocolitis [30-32].

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 NOS, in turn reacts with superoxid anion to produce the highly toxic peroxynitrite radical [33].

In the neonatal period there are several conditions (hypoxia, hyperoxia, resuscitation, mechanical ventilation, phototherapy [34] and intracerebral hemorrhage [35] *et cet*), which set going the cascade of LP of unsaturated fatty acids leading to the formation of aldehydes as secondary LP products. These aldehydes can also act as messengers of ROS/RNS. Docosahexaenoic acid and arachidonic acid are important structural components of the CNS in the neonatal period. These fatty acids are transferred across the placenta, are present in human milk, and are accumulated in the brain during fetal and early postnatal period [36-39].

Excitotoxicity

Excitotoxicity is the pathological process by which nervous cells are damaged and killed by the overactivations of receptors for the excitatory neurotransmitter glutamate, such as *N*-methyl-D-aspartate (NMDA)- and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor [40]. 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 neurodegenerative diseases of the CNS such as multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, fibromyalgia, Parkinson's- and Huntington's disease. *In the neonatal*

period excitotoxicity is an important mechanism involved in perinatal brain injuries. Glutamate is the major excitatory neurotransmitter, and most neurons as well as many oligodendrocytes and astrocytes possess receptors for glutamate. Perinatal injuries caused by hypoxia-ischemia, stroke, hypoglycemia, kernicterus (or BIND), and trauma can disrupt synaptic function leading to accumulation of extracellular glutamate and excessive stimulation of these receptors [41,42].

How to protect the neonatal brain?

Despite major improvements in perinatal care over the last decades, the incidence of disabilities due to perinatal injuries has not decreased significantly even in the developed countries [43,44]. It is difficult to find such an intervention or drugs that are able to neutralize - separately or simultaneously - the outlined noxious phenomena [45]. Perinatal neuroprotection, however, is a major health care priority, and at the same time several questions remain actively debated particularly the problems of viability of the extremely small birth weight (<500 g) infants [46]. The feasibility of designing a strategy that prevents or treats brain damage without disrupting normal development will have to be evaluated. Therefore, there is a need to examine features which are potentially involved in multiple facets of neural insults [47,48] including BIND, ROP and other neuropathological conditions. In this review we discussed above the role of elevated copper in various molecular mechanisms, focusing on the newborn infants. As a strong copper chelator D-PA may be a potential neuroprotector for acquired disabilities in the neonatal period.

D-Penicillamine (D-PA): pharmacology and clinical uses

D-PA (β - β -dimethylcysteine) was first isolated as the amine, from the degradation products of penicillin by Abraham *et al.* in 1942 [49]. It does not have any antibiotic activity and so initially, interesting the compound arose only out of its position in the processes of degradation and synthesis of penicillin. Nevertheless, it subsequently has found an extensive use in medicine: in 1956 by Walshe' in the treatment of Wilson disease [50]. It has since been used or suggested for use in the treatment of cystinuria, Rheumatoid arthritis (RA), juvenile RA, palindromic rheumatism, scleroderma, primary biliary cirrhosis, alcohol detoxification, heavy metal removal, chronic active hepatitis, morphea, keloid, keratosis follicularis, and hyperviscosity syndrome. In addition, as a ligand, it has to apply in the preparation of radiopharmaceuticals for liver and kidney imaging [51]. Only D-PA is used in medicine, since the L isomer and the DL isomer (or racemate), are toxic (Figure 1).

D-Penicillamine in the neonatal period

D-PA was first recognized as a potential benefit for neonatal hyperbilirubinemia (NHBI) [4]. During this time there was a remarkably low incidence of ROP in the infants treated with this drug [52-54]. Later, our studies were replicated in other institutes in

Hungary, Poland, the USA, India and Mexico [55-57]. It is important to note that there was no intolerance or short- or long-term toxicity of the medication, in spite of the fact that in the newborn period D-PA was used 10-20 times higher doses than those in adult. In our article [58] and in a recently published book [59] we discussed the potential neuroprotective effects of D-PA in BIND and ROP. D-PA is a hybrid drug in the neonatal period by its ability to modulate both oxidative stress and NO pathway. Tataranno *et al.* [60] have summarized the new body of knowledge about the antioxidant drugs for neonatal brain injury. D-PA-therapy of newborn infants may also have significant neuroprotective effects in cases jeopardized by BIND or ROP. (The retina, ie., despite its peripheral location, is actually part of CNS [61,62]).

Possible molecular mechanisms of D-PA in the neuroprotection of neonatal brain

The possible neuroprotective effects of D-PA emerged, when we did not observe any serious damage in the course of a long-term follow-up of adults (28-40 years old), who suffered from acute encephalopathy in their neonatal period [63].

D-PA and the copper dyshomeostasis

Chelation of high copper levels with D-PA, which used routinely for treating Wilson disease, also decreased brain-copper content of prion-infected mice by 30% and increased the incubation period, supporting the idea that increased levels of brain copper promote encephalopathies [64]. D-PA is actually the drug most extensively used to treat copper overload [65,66]. *Angiogenesis* is a normal process in growth and development, as well as in wound healing. However, this is also a fundamental step in the transition of tumors from a dormant state to a malignant state, and in the development of various retinopathies. It is now recognized that the endothelial cells, by paracrine mechanisms, produces growth factors that stimulate the proliferation of blood vessels. The major targets of pharmacologic therapies are VEGF and basic fibroblast growth factor. Overall, angiogenesis can be viewed as the result of stimulatory and inhibitory peptides, proteases and endogenous inhibitors, and microenvironmental factors such as the level of oxygen or copper ion [67-69].

DPA alleviates OS and NS

These effects based on the capability of this drug to alter the NO system, and it is a strong antioxidant. Low molecular weight disulfides are the major products of D-PA metabolism in humans [70,71]. The oxidation of D-PA *in vivo* may also important in the mode of action of the drug through simultaneous reduction of the ROS and RNS. Consequently, D-PA fulfills the criteria of a hybrid drug in the neonatal period by its ability to modulate both oxidative stress and NO pathway, and can be a neuroprotective agent in the pathophysiology of neurologic dysfunction [72].

DPA and lipid peroxidation (LP)

Carbonyl scavengers [73] have been used with the aim of reducing the "aldehyde load" [74] and in several *in vivo* and *in vitro* studies have been investigated their effects on neuroprotection. The carbonyl scavenger D-PA binds primarily to aldehydes in an irreversible manner; consequently this drug inhibits their damaging effects and it also scavenges peroxy nitrite. Acute D-PA administration has previously been shown to improve neurological recovery in the mouse concussive head injury model and to protect brain mitochondria [75].



Figure 1. Structural formula of D-Penicillamine.

Table 1. Age-related differences in the effects of D-Penicillamine.

	Neonates	Adults
Hexobarbital		
sleeping time	shortened	no effect
Heme-oxygenase	inhibited	no effect
Cytochrom-P-450	increased	no effect
Catalase	increased	no effect
Peroxidases	increased	no effect
Radioprotection	significant	?
LD₅₀	> 4000 MG/KG (IP)	500 MG/KG (IV)

Age-related effects of D-PA

Paediatric patients display different pharmacokinetic and pharmacodynamic responses to drugs. This is why we can speak about developmental or age-related pharmacology [76]. In the Table 1 we demonstrate the results of our animal experiments regarding the age-related differences in effects of D-PA [77]. The high activity of heme oxygenase (HO) in the newborn could reflect the enzyme-inducing action of metals (primarily of Cu and Fe) derived from the breakdown of fetal erythrocytes [78]. Chelation therapy in neonates restores the normal activity of enzymes participating in heme metabolism. Briefly, chelating agents facilitate heme synthesis and inhibit heme degradation. In other words, D-PA as a chelating agent, boost or inhibit the immature enzyme systems to the adult level. Because those enzymes that play an important role in antioxidant defense and drug metabolism (peroxidases, catalase, cytochrome P-450) are heme proteins, it can be assumed that in preventing hyperbilirubinemia and OS/NS, the mechanism of action of D-PA is identical: the protection of biomembrans against lipid peroxidation [79].

D-PA and excitotoxicity

We did not find any article in the literature, accessible by us, that a direct inhibitory effects of D-PA on excitotoxicity would have been proved. 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 and it is known to scavenge carbonyls. Previous literature has shown penicillamine scavenging other toxic aldehyde by forming a thiazolidine compound with the aldehyde moiety [33,80,81].

Conclusion

We hope that our concept will help answer some of the unsolved questions and concerns occurred in the etiology and pathomechanisms of BIND and other neurodegenerative/neurodevelopmental disorders. The beneficial neuropharmacological actions of metal-targeted (chelating) agents most likely arise from local metal redistribution rather than from massive metal removal [3,82,83]. The chelation therapy for non-metal overload indications continues to be investigated. Our present article address the medical necessity of the use of a chelating agent (D-PA) in the prevention or treatment of neonatal brain injuries.

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