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D-Penicillamine Alleviates Oxidative Stress in Neonates by Its Influence on Copper Dyshomeostasis Due to Immaturity

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Abstract
Objective – *the aim of this article was to demonstrate the protection of biomembranes against lipid peroxidation, and highlight the role of D-Penicillamine (D-PA).*
Study Design *– The authors conducted a review searching the relevant literature of bilirubin and copper metabolisms, and of antioxidant effects of D-PA in the neonatal period.*
Results – *Unconjugated bilirubin has a special affinity for the globus pallidus, the hippocampus, and the subthalamic nucleus (basal ganglia). Furthermore, immaturity of the blood-brain barrier also contributes to the development of kernicterus. Homeostasis of metal ions usually involves a huge set of proteins which regulate the proper metal biology. Metal ions, especially copper and iron play very important roles in the pathogenesis of neurodegenerative diseases, including bilirubin-induced neurologic dysfunction (BIND), having impact on both protein structure (misfolding) and oxidative stress. D-PA by its ability to modulate both oxidative stress and nitric oxide (NO) pathway may have significant neuroprotective effects in cases jeopardized by BIND or retinopathy of prematurity (ROP).*
Interpretation – *The authors conclude that treatment with D-PA might result in a wide range of health benefits, improved quality of life and reduced healthcare costs and may help reduce complications in the neonatal period.*
Keywords: *Bilirubin-induced neurologic dysfunction; Reactive oxygen species; Copper dyshomeostasis;; Neurodegeneration; D-Penicillamine in the neonatal period*
Key points:
► *D-PA was first recognized as a potential benefit for neonatal hyperbilirubinemia. During this time there was a remarkably low incidence ROP in the infants treated with this drug. Later, our studies were replicated in other institutes in Hungary, Poland, the USA, India and Mexico.*
►*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.*
► *Metal ions, especially copper and iron play very important roles in the pathogenesis of neurodegenerative diseases, including BIND and ROP, having impact on both protein structure (misfolding) and oxidative stress.*
► *D-PA by its ability to modulate both oxidative stress and nitric oxide pathway may have significant neuroprotective effects in cases jeopardized by bilirubin encephalopathy or ROP*

I. Introduction

D-penicillamine (D-PA) was first recognized as a potential benefit for neonatal hyperbilirubinemia (NHBI). [1] During this time there was a remarkably low incidence of retinopathy of prematurity (ROP) in the infants treated with D-PA. [2] Later, our studies were replicated in other institutes in Hungary, Poland, the USA, India and Mexico. 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 Letter to the Editor [3] and in a book just published [4] we have discussed the potential neuroprotective effects of D-PA in BIND) and ROP. Now we will demonstrate the direct and indirect (endogenous) antioxidant effects of this drug.

II. Neonatal oxygen toxicity

In comparison with healthy adults, newborn infants have lower levels of plasma antioxidants such as vitamin E, beta-carotene, and sulphydryl groups, lower levels of plasma metal binding proteins including ceruloplasmin and transferrin, and reduced activity of erythrocyte superoxide dismutase. [5] Moreover, diseases in the neonatal period such as bronchopulmonary dysplasia, ROP, necrotizing enterocolitis, periventricular leukomalacia, and – according to our concept –, BIND are related to free radical damage. [6]

III. Mechanisms of action of D-PA in neonatal hyperbilirubinemia

The question arises, how does D-PA work in neonatal jaundice? The complete mechanism of action is still unknown, but some interesting pieces of information have unfolded over the last decades. An increased rate of bilirubin production and the limited ability to conjugate indirect bilirubin (UCB) during the neonatal period result in visible jaundice. In the reticuloendothelial system heme is oxidized by the enzyme heme oxigenase (HO). This is the initial and rate-limiting enzyme for heme degradation. Three crucial areas of bilirubin formation and excretion have been investigated in our laboratory: ►The lipid peroxidation of the red blood cell membrane and hemolysis [5-7] ► HO activity [8] and ►UDP-glucuronyltransferase activity [9] before and after D-PA treatment.

Lipid peroxidation has been considered to be a mechanism of membrane damage in a number of red blood cell disorders leading to hemolysis. The susceptibility of red cell lipids to autooxidation is about three times as high in the newborn as in adults. [7] *In vitro*, preincubation with D-PA resulted in a significant decrease of both the hemolysis and fluorescence of red cell lipid extracts. *In vivo*, pretreatment with D-PA has prevented the phenylhydrazine-induced lipid peroxidation in rats. Malondialdehyde is a product of lipid peroxidation resulting in disintegration and disruption of biologic membranes. The binding of D-PA to malondialdehyde may prevent this process. We measured, therefore, the 14C-labeled D-PA uptake and the malondialdehyde concentrations of adult and newborn red blood cell membranes *in vitro*. D-PA uptake of newborns' red blood cell membranes was significantly enhanced over that of adults [6-11].

Since heme metabolism is a crucial stage in bilirubin production, we examined the activity of HO, the initial and rate-limiting enzyme of heme degradation. The 3 days of D-PA treatment in the adult animals did not lead to any significant change in HO activity. In contrast, in the neonates a marked reduction in enzyme activity was observed following D-PA treatment. At the same time, the activity of UDP-glucuronyltransferase was measured in liver homogenates of newborn and adult rats. D-PA treatment could not cause any changes in enzyme activity [12]. The selective inhibition of HO-1 isoform is generally preferable. [13] Because those enzymes that play an important role in endogenous antioxidant defense and drug metabolism are heme proteins, it can be assumed that in preventing hyperbilirubinemia, ROP and oxygen toxicity, the mechanism of action of D-PA is identical: *the protection of biomembranes against lipid peroxidation caused by free radical.* [14]

Bilirubin production will be inhibited by decreased activity of HO. The age-related differences in the effect of D-PA concerning HO activity is supported by the experimental works of Maines and Kappas [15]. The high activity of HO in the newborn could reflect the enzyme-inducing action of metals derived from the breakdown of fetal erythrocytes. Chelation therapy in neonates restores the normal activity of enzymes participating in heme metabolism. Thus, the chelating agents facilitate heme synthesis and inhibit heme degradation.

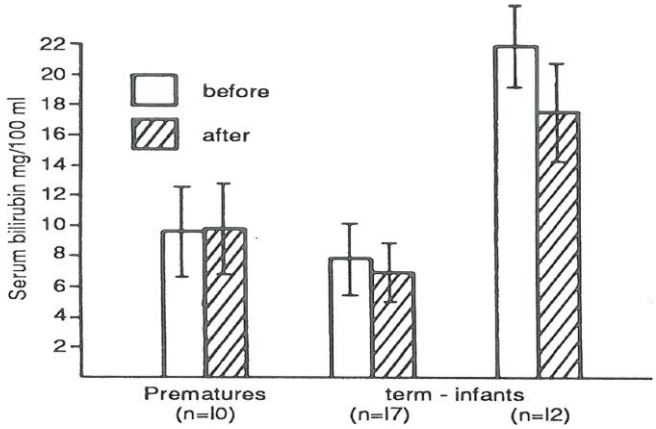

Fig. 1. Effect of a single dose of D-PA in 4-6 hours after IV administration

In the light of the foregoing, now we present our clinical observations**. Figure 1.** shows a single 100 mg/kg body weight intravenous dose of D-PA on bilirubin levels in premature and term infants, in 4-6 hours after the administration. A rapid decrease in the level was observed only in term infants with high bilirubin concentrations, but D-PA has not had any effect in prematures under 1500 g birth weight and neonates with low UCB values. A plausible explanation for this is that D-PA inhibits bilirubin formation but it does not cause any change in UDP-glucuronyltransferase activity. In cases with high TB, however, the marked decrease of UCB observed was due to enzyme induction by bilirubin itself, which had gradually increased during the previous days in term infants.

VI. Metal mediated oxidative injury in the neonatal period

Newborns, especially preterm infants, are particularly vulnerable to reactive oxygen species (ROS) because they exhibit accelerated production of free radical and limited antioxidant protection, which increases the susceptibility of rapidly growing tissues to damage. „Free radical-related diseases" of neonates promote cellular, tissue, and organ impairments. In 1988, Saugstad coined the phrase „oxygen radical disease in neonatology" to highlight the crucial role of ROS in a wide range of neonatal disorders. [16] There is now a large body of literature demonstrating that free or weakly bound iron and copper ions may exert their toxic action on basal ganglia (BG). In a way, metals may provide the link between protein misfold and aggregation, oxidative stress and the cascade of biochemical alterations, eventually leading to neuronal cell death. Predominantly the cellular content of copper determines copper-induced toxicity in brain astrocytes. [17]

Very wide-ranging studies have long been made on the possible biochemical transformations of UCB, which is formed during the decomposition of haemoglobin. Particular attention has been paid to its photochemical and redox reactions [18] but the relevant publications comprise only a very small proportion of those dealing with the molecular biochemistry of *UCB and metals interactions*. Bilirubin has a special affinity for the globus pallidus, the hippocampus, and the subthalamic nucleus (these brain regions are belonging to BG) because they are also target brain regions for divalent metal (Cu, Fe, Zn et cet.) accumulation. [19]

V. Neurodegeneration: a return to immaturity? [20]

This question certainly arouses the attention of neonatologists as *the immature and strikingly* ***vulnerable*** *neurons* play important role in the pathogenesis of BIND. The increased vulnerability of premature infants to brain damage may be due to a proneness of immature nerve cells to toxic stimulus. The developing neurons undergo programmed cell death, a necessary phenomenon for proper nervous system development. Following the developmental period, neurons mature and restrict the apoptotic pathway to permit long-term survival. On the basis of above described abundant research data and hypotheses, **according to our concept, the BIND** is a neurodegenerative disease (*ND) of immature brain caused by accumulation of free metals and UCB-Cu complex (as prooxidant) in the BG and other parts of CNS relevant to BIND*. The rate of formation of UCB-Cu complex when bilirubin extracts copper from copper–albumin complex, as obtained in a very exciting experiment, is 34.98 l mol^{-1} s^{-1}. At least 3 loci exist where UCB and copper can be "fusioned" in the neonatal period: (1) during hemolysis high UCB and copper level can be developed in the blood; (2) one albumin can bind one Cu^{++} in the primary binding site. At higher concentration of copper (if possible under certain conditions), loosely bound atoms, and can be very easily taken out by UCB. Bilirubin itself can displace loosely bound copper ions, which are electrostatically attached due to high negative charge on the surface of albumin; (3) in the basal ganglia (BG). [21] The main comorbidity is the ***hemolysis*** of neonatal blood red cells. During this process a great amount of heavy metals (mainly iron and copper) may circulate in free form in the bloodstream, and can pass through the blood brain barrier (BBB), finding entrance into the CNS as well. Understanding the differences between neonatal and adult erythrocytes is critical in the evaluation of perinatal erythrocyte disorders. The reason for the reduced RBC survival observed in newborns is not known, although there are many biochemical differences between adult and neonatal red blood cells (RBCs). [22-24] Increased oxidant sensitivity of newborn red cells and relative instability of fetal hemoglobin have been considered as possible causes for this shortened lifespan. In a chinese study, [25] the erythrocyte's copper content was significantly lower in the maternal blood than in the newborn cord blood. The compounds to be bound and transported by albumin are quite diverse and include bilirubin, fatty acids, metal ions and therapeutic agents. Bilirubin itself can displace metals (copper) from the albumin binding because UCB binds stronger to albumin than copper, in other words, copper **loosely bound** to albumin. Free or loosely bound, redox-active transition metal ions are potentially extremely pro-oxidant, having the ability to catalyze the formation of damaging and aggressive ROS from much more innocuous organic and inorganic species. In strictly biological terms the two most important such metals are iron and copper. [26,27] In fact, oxidative stress has been demonstrated to be a common link between several conditions such as Parkinson disease (PD), Alzheimer disease (AD), stroke, prion diseases and UCB encephalopathy, where it is involved in neuronal injury.

VI. Free copper and the oxidative stress in neonates

The basic role of metal ions in neurological pathologies is generally accepted, ― except for the case of BIND. Free copper ion in itself or binding to UCB and forming metal-bilirubin complex(es) involved in neurologic dysfunction, therefore they are important factors for whole brain damage processes in BIND. A significant portion of the toxicity of copper comes from its ability to accept and donate single electrons as it changes oxidation state. This increase in unmediated ROS is generally termed oxidative stress. When women become pregnant, their estrogen levels rise, greatly increasing the retention of copper in the body. This metal will pass through the placenta into the unborn child. So many children are being born with toxic levels of copper and other heavy metals which were stored in the mother's body **(BOX 1.)**

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, or otherwise chronically unhealthy babies.

BOX 1. Many children are being born with toxic levels of heavy metals [28]

Studies have also found heavy metals to deplete glutathione and bind to protein-bound sulfhydryl groups, resulting in inhibiting SH-containing enzymes and production of ROS such as superoxide ion, hydrogen peroxide, and hydroxyl radical. In addition, toxic metals exert part of their toxic effects by replacing essential metals such as zinc at their sites in enzymes. Here, it is noteworthy, how prominent similarities exist between Wilson's disease (WD) and neonates with kernicterus concerning *the copper metabolism, the neuropsychiatric manifestations and the hystopathological findings* **(BOX 2**.). In the neonatal period the ability of the liver to synthesize ceruloplasmin is not fully developed and adult levels of the protein are not found in the blood till about three months of age.

BOX 2

HIGH COPPER LEVEL IN THE BASAL GANGLIA

NEUROPSYCHIATRIC MANIFESTATIONS

WD: Movement disorders, tremors, involuntary movements, choreoathetosis, dysarthria, dystonia, personality changes, uncontrolled emotional outbursts. [29, 30]

Kernicterus: generalized dystonia, athetoid cerebral palsy, paralysis of upward gaze, sensori-neural hearing loss. BIND: impairment of audiologic, speech, and language processing as well as disturbances in visual-motor and cognitive functions associated with failure of fine neuromotor control (extrapyramidal signs). [31]

At AUTOPSY (both in WD and kernicterus):marked neuronal loss with demyelination and astrocytic replacement. [32, 33]

BOX 2. Common neuropsychiatric manifestations and pathological findings in WD and bilirubin encephalopathy

It is interesting therefore that the infant liver has a very much higher copper content than is found in the adult and a fall in concentration does not takes place until the ability to synthesize caeruloplasmin has fully developed. [34] In addition, our observations suggest that D-PA has important neuroprotective effects in cases jeopardized by BIND or ROP. These unexpected effects may be related to D-PA capability to alter the nitric oxide (NO) system. [35-39] NO synthesized in the CNS produces a myriad of effects. For example, it plays a role in the control of blood flow, learning and memory, neurotransmitter release, gene expression, immune responsiveness, and cell survival. It is also implicated in numerous pathologies such as Alzheimer disease (AD), Huntington's disease, and cerebral ischemia, and disorders of the BG caused by metals (in WD), bilirubin (in BIND) or other pathologic conditions (in Parkinsonism) [40].

D-PA is a hybrid drug in the neonatal period by its ability to modulate both oxidative stress and NO pathway. Tataranno et al. [41] have summarized the new body of knowledge about 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, which, despite its peripheral location, the retina or neural portion of the eye, is actually part of CNS. [42] These effects based on the capability of D-PA to alter the NO system, and it is a strong antioxidant. [43, 44] Low molecular weight disulfides are the major products of D-PA metabolism in humans. [45] The oxidation of D-PA *in vivo* may also important in the mode of action of the drug through simultaneous reduction of the ROS. So, we can say that 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. [46]

VII. Conclusion

We hope that our theory will help answer some of the unsolved questions and concerns ocurred in the etiology and pathomechanisms of BIND. The beneficial neuropharmacological actions of metal-targeted (chelating) agents most likely arise from local metal redistribution rather than from massive metal removal. [47] 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 treatment of NHBI and ROP.

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Abbrevations:

AD - Alzheimer disease; BG - Basal ganglia; BBB – Blood brain barrier; BIND - Bilirubin-induced neurologic dysfunction; CNS - Central nervous system; D-PA - D-Penicillamine; NO – nitric oxide; HO – Heme oxigenase; NHBI - Neonatal hyperbilirubinemia; ND – neurodegenerative disease; PD - Parkinson disease; ROP – retinopathy of prematurity; RBC – Red blood cells; ROS - Reactive oxygen species; TB - Total bilirubin level; UCB - unconjugated bilirubin; WD - Wilson disease

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