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THE EFFECT OF D-PENICILLAMINE IN THE DEVELOPING RAT CEREBELLUM*

*Murat Yurdakök MD***, Haluk Topaloğlu MD***, Rıfkı Hazıroğlu MD*****

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Copper deficiency produces a variety of clinical effects in animals and man which is not surprising in view of its known roles in the structure and function of a number of enzymes including cytochrome oxidase (electron transport), superoxide dismutase (free-radical detoxification), tyrosinase (melanin production), dopamine beta-hydroxylase (catecholamine production), lysyl oxidase (cross-linking of collagen and elastin), ceruloplasmin (ferroxidase, transport), and unidentified copper enzyme (cross-linking of disulphide bonds of keratin)[1,2]. Copper deficiency in grazing animals is responsible for the condition termed enzootic neonatal ataxia in lambs, and copper deficiency resulting in neurological abnormalities in neonates has been experimentally produced in laboratory animals[3].

The simularities between Menkes' syndrome and the copper deficient rat includes slow growth, abnormal behavior, a decrease in myelin, a reduction in cytochrome oxidase and presumably less brain copper[3,4]. In this study we evaluate the effects of D-penicillamine, a well-known copper chelator, in the cerebellar maturation of the offspring of pregnant rats.

Material and Methods

Albino pregnant rats with a mean weight of 250 g (ranging from 220 to 300 g) were used in this study. Their gestational ages were determined by the presence of a vaginal plug on the first day of gestation, and abdominal palpation after the tenth day.

Ten animals received D-penicillamine (DPA) in their drinking water in a daily dose of 300 mg (low-dose) or 400 mg (high-dose) during their last six days of gestation and four rats were selected as controls and received no drug. On the 20-21th day of gestation, the fetuses were delivered by cesarian section in four animals of the

* From the Department of Pediatrics, Hacettepe University Faculty of Medicine, Ankara.

** Associate Professor of Pediatrics, Hacettepe University Faculty of Medicine.

*** Instructor in Pediatrics, Hacettepe University Institute of Child Health.

**** Veterinary Pathologist, Ankara University Faculty of Veterinary Medicine, Ankara.

study group (two high-dose and two low-dose) and two of the controls. The remaining two animals received high-dose DPA during their gestation, and two controls were given a normal diet which contained no DPA for three weeks after delivery. At the end of this period, their offspring were sacrificed and evaluated in the same manner as the newborn animals.

The animals were weighed and then killed by decapitation. The cerebellum was separated from the rest of the brain and bisected along the transverse line across the widest diameter. After weighing, the cerebellar tissue was embedded in paraffin blocks stained with hemotoxylin-eosin and examined by means of light microscopy.

A new scoring system was developed to determine the maturation and degeneration of the cerebellum which was based on the thickness of the granular layer, number and disarrangement of Purkinje cells and the heterotopic granular cells in the molecular layer (Table I, Figs. 1-4).

The results were evaluated statistically according to Student's *t* test or the Kruskall-Wallis analysis.

TABLE I: Staging System for Cerebellar Degenerative Changes

Criteria	Score*
1. Reducing thickness of granular layer	0, +, ++, +++
2. Reduced in number of Purkinje cells	0, +, ++, +++
3. Disarrangement of Purkinje cells and heterotophic granular cells in the molecular layer	0, +, ++, +++

* Score was given "from no change to severe pathological changes" as 0, +, ++, +++

** The scores in positive values for three scores was summed arithmetically and the calculated value was used to evaluate the staging as follows:

Total score	Stage
0 - 2	0
3 - 5	1
6 - 8	2
⩾ 9	3

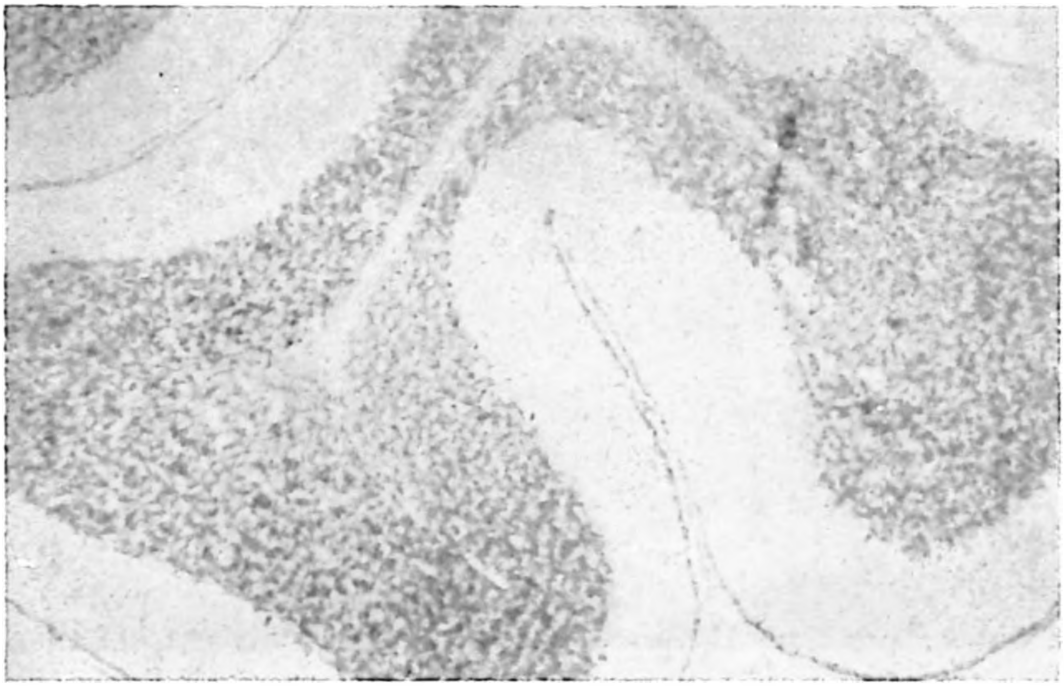

Fig. 1: Normal cerebellum (Stage 0).

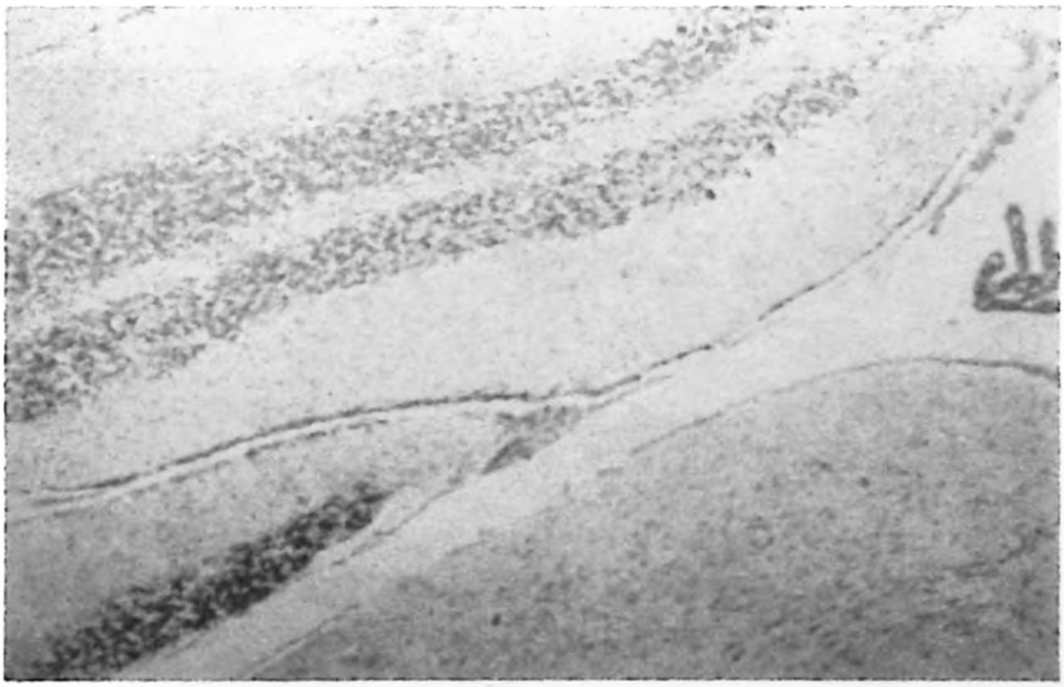

Fig. 2: Moderately cerebellar degeneration (Stage 2).

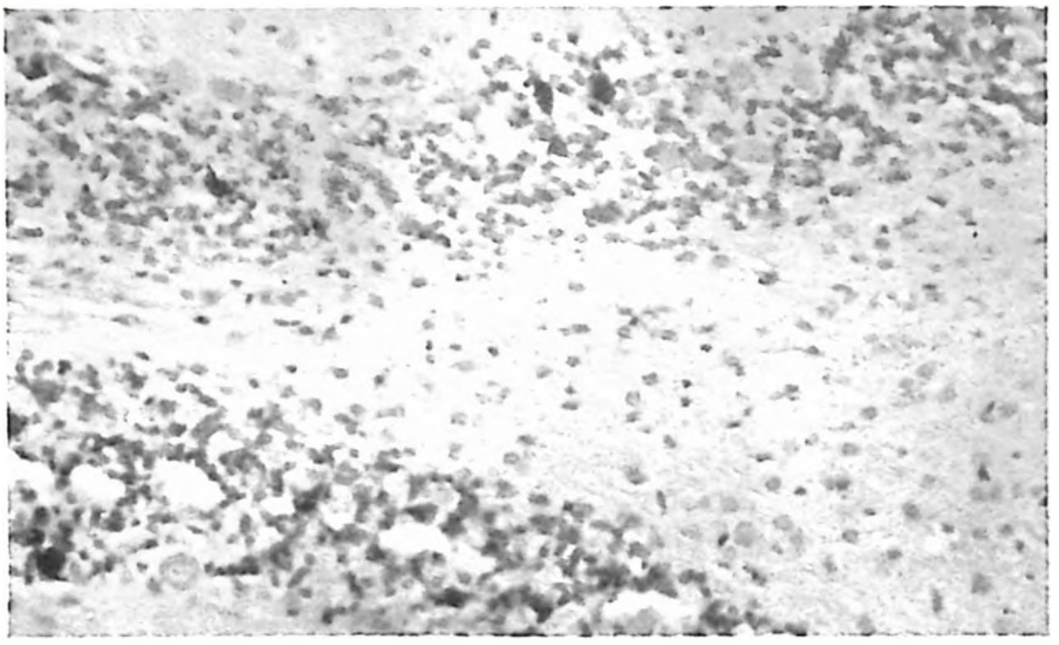

Fig. 3: Histological appearance in high magnification in Stage 2: Reducing in the thickness of granular layer and number of Purkinje cells, disarrangement of Purkinje cells, and heterotupic granular cells in the molecular layer.

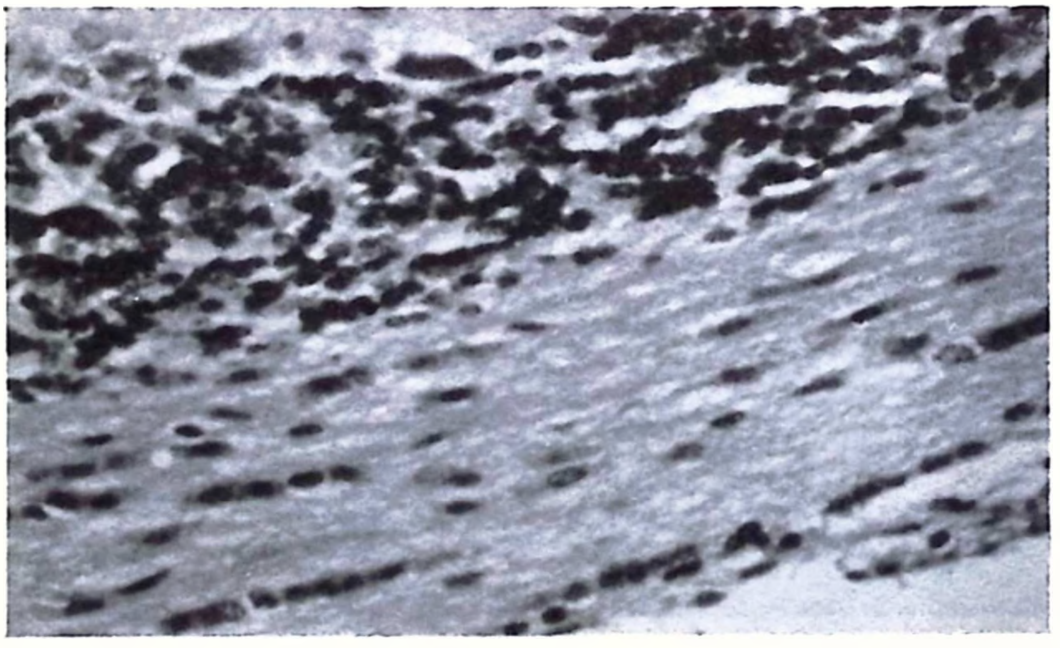

Fig. 4: Severe cerebellar degeneration (Stage 3): Reducing in the thickness of granular layer and marked loss of Purkinje cells.

TABLE II: Cerebellar Findings in the Offspring of Rats

	At birth			At the third week	
	Group I	Group II	Group III	Group IV	Group V
D-penicillamine treatment during gestation	No	Low-dose	High-dose	No	High-dose
Maternal values*					
Serum copper (µg/dl)	95 and 89	83 and 84	71 and 78	92 and 88	93 and 85
Serum zinc (µg/dl)	108 and 113	111 and 114	106 and 113	112 and 114	118 and 113
Serum ceruloplasmin (IU)	41 and 46	36 and 38	31 and 29	44 and 41	47 and 44
Body weight (mg)	9537 ± 390 (n: 7)	8378 ± 483 (n: 10)	4897 ± 579 (n: 8)	13034 ± 303 (n: 8)	1225 ± 418 (n: 7)
CW/BW (%)**	0.36 ± 0.04 (n: 6)	0.35 ± 0.05 (n: 10)	0.27 ± 0.03 (n: 7)	1.05 ± 0.04 (n: 8)	1.03 ± 0.03 (n: 7)
Score for cerebellar degenerative changes***	0 (n: 6)	0.4 ± 0.1 (n: 10)	2.5 ± 0.3 (n: 7)	0 (n: 8)	0 (n: 7)

* At the end of gestation, or at the end of the third week after delivery.
** Cerebellar weight / Body weight (mg) X 100
*** According to Table I.
Statistical comparisons:
Body weight: I-II, I-III, II-III $p < 0.001$, IV-V $p > 0.05$.
CW / BW : I-III, II-III $p < 0.001$, I-II, IV-V $p > 0.05$.
Score : I-III, II-III $p < 0.001$, I-II $p < 0.05$, IV-V $p > 0.05$.

Results

Pups of the DPA treated mothers showed a greater decrease in body weight. Compared to the controls their hair was short, sparse, easily pulled out and of coarse texture. They were less active in general, but became hyperirritable when stimulated. Pups of the DPA-treated mothers also showed an appreciable decrease in the size of their cerebellum. Histological examination revealed a highly significant decrease in myelination, reduction in the thickness of the molecular layer and the number of Purkinje cells. It was observed that heterotopic granular and Purkinje cells in the molecular layer were much more pronounced in these animals (Table II). A postnatal DPA-free diet given to the lactating mothers appeared to have a significant effect in largely reversing the hypomyelination and other pathological findings (Table II).

Discussion

Menkes' syndrome is a sex-linked recessive disorder in male infants, which results in growth retardation, "steely" or kinky hair (pili torti), depigmentation of the skin, and focal cerebral and cerebellar degeneration[4]. Numerous lines of evidence suggest that Menkes' disease is derived from an intracellular defect of copper utilization, which may sequester copper in an abnormal form. The distribution of copper in various tissues of these patients is abnormal, with intestine and kidney having elevated concentrations, and brain and liver being depleted. Copper-dependent enzymes may not be synthesized in tissues from these patients even when tissue copper is elevated[2].

In most patients Menkes' syndrome has not been diagnosed before six to eight weeks of age, when developmental failure and other clinical features become apparent, and low copper levels are found. But early postnatal diagnosis of Menkes' disease may be possible, since hypothermia, lethargy and hyperbilirubinemia as well as the characteristic hair, may be present in the neonatal period. Parenteral copper therapy does not seem to prevent cerebral damage, even when initiated in neonates. Postnatal therapeutic failures may be due to difficulties in maintaining high circulating levels of copper in the first months of life and/or to irreversible cellular damage[1,5]. Copper deficiency may also be seen in very low birth-weight preterm infants who are being fed with parenteral or artificial nutrition[6].

The result of this study which is contrary to a previous study[3] carried out on rats demonstrates that even though copper replacement was begun before the onset of myelination in the cerebellum, whose myelination and neuroglial proliferation occurs primarily postnatally in the first three weeks (at a much earlier developmental stage than that of the human infant), it did completely restore the rate of cerebellum growth and myelin deposition to normal.

DPA is an amino acid, dimethylcysteine penicillin derivative. It chelates metals, particularly copper and was introduced as therapy for hepatolenticular degeneration (Wilson's disease) 30 years ago, because it is stable, extremely soluble and is excreted in the urine. DPA is a teratogenic drug. It has been shown that feeding DPA to pregnant rats throughout gestation causes fetal resorption and congenital abnormalities. Although DPA may cause a decrease in the copper and zinc levels in maternal and fetal tissues, the addition of copper to a DPA-containing diet significantly reduced the frequency of malformed fetuses, supporting the hypothesis that DPA teratogenicity is primarily due to induced copper deficiency[7]. Thus, we can conclude that the result obtained in this study was due to copper deficiency rather than the DPA treatment.

Summary

The effect of D-penicillamine (DPA), a copper chelator, on the cerebellar maturation of the offspring of pregnant rats is evaluated. Pups of the mothers which received DPA in a daily dose of 300-400 mg during their last six days of gestation, showed an appreciable decrease in body and cerebellum weights, and cerebellar degenerative changes on histological examination. A postnatal diet DPA-free given to lactating mothers appeared to have a significant effect in reversing the cerebellar degenerative changes in their offspring.

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