Chloroquine-Enhanced Cerebellovascular Changes in Nutritionally Imbalanced Chicks

Vernon W. Fischer, Ph. D.
Department of Anatomy, Saint Louis University School of Medicine

James S. Nelson, M. D.
Departments of Pathology and Pediatrics, Washington University School of Medicine

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Summary. Young, vitamin E-depleted chicks, fed an encephalopathy-inducing diet, containing linoleic acid, were injected with chloroquine. The effects of this drug on previously demonstrated cerebrovascular alterations, present in the preclinical stage of the nutritional encephalopathy (NE), were observed. Chloroquine administered to chicks on the experimental diet markedly accentuated the early cerebrovascular changes associated with NE, but prevented the ultimate clinical onset of the disease. Intensification of the abnormalities included enhanced autofluorescence, increased reactivity for acid phosphatase, and progressive accumulation of dark bodies within the vascular endothelium; the material within the endothelial cytoplasm stained positively for polar phospholipids. It is suggested that chloroquine may cause these effects through stabilization of lysosomal membranes, controlling movement of hydrolytic enzymes. An alternate mode of action may be stimulation of fatty acid incorporation into phospholipids leading to accelerated regeneration of subcellular membranes, with diminished availability of fatty acids, unprotected by antioxidants and thus susceptible to peroxidative breakdown.

Key words: Vitamin E Deficiency — Nutritional Encephalopathy — Fatty Acid Metabolism — Lysosomal Membranes.

Introduction

Nutritional encephalopathy, an acute disease of the avian central nervous system, is inducible with regularity by feeding chicks a diet lacking in a-tocopherol, and augmented with linoleic acid, a polyunsaturated fatty acid (Dam et al., 1958). We have demonstrated the progressive accumulation of acid phosphatase-positive, electron-dense, autofluorescent cytoplasmic bodies, selectively, within the CNS capillary endothelium of chicks, fed such a diet (Fischer and Nelson, 1973).

The present communication describes the changes observed in cerebellar vessels of vitamin E-depleted chicks subjected to repeated administration of a 4-amino-quinoline (chloroquine), an agent believed to exert a stabilizing effect on lysosomal membranes.

Materials and Methods

Newly hatched chicks were divided into experimental and control series. Twenty-four chicks in the experimental group were fed an encephalopathy-inducing diet (EID), lacking in tocopherol and containing 8% of linoleic acid (Machlin and Gordon, 1960). Previous experiments demonstrated the effectiveness of this diet in inducing nutritional encephalopathy.
(NE) in all our animals within 28 days. The control group of seven chicks received a normal starter feed (Startena).

At four days of age both groups were initially injected subcutaneously twice daily with 1 mg/100 g (body weight) of 4-amino-quinoline, chloroquine diphosphate. The initial dose was maintained during the first week, and was subsequently increased by 1 mg/100 g every week, until the fourth week.

A third group of eight chicks, fed the EID in order to test its efficacy, was not injected with the drug. These birds were observed for clinical signs associated with the onset of NE; the presence of the disease was confirmed with the light microscope.

Comparative examinations of chloroquine-treated and untreated chicks were carried out on days 15, 18 and 21; subsequently, treated birds were examined at intervals until day 69. All animals were sacrificed by decapitation; blocks of cerebellum were studied using the following methods:

**Electron Microscopy**

One-millimeter cubes of tissue were fixed by immersion in 3% glutaraldehyde in Sorensen's phosphate buffer with post-fixation in Millonig's osmium tetroxide and subsequent embedment in Epon-Araldite; sections stained with uranyl acetate and lead citrate were viewed in a Philips 300 electron microscope. Thin slivers of tissue were also used to study the ultrastructural localization of acid phosphatase, according to the method of Kreutzberg and Hager (1966).

**Histochemistry**

Sagittal slices were quenched in liquid nitrogen and unstained cryostat sections, 14 µ thick, were examined with a fluorescence microscope, using a BG 12 excitation filter and a 530 mµ emission filter. Adjacent frozen sections were incubated according to Burstone's (1958) method for acid phosphatase. Formalin-calcium-fixed frozen sections were stained with the OTAN and Elleder's methods for phospholipids (Adams, 1965; Elleder and Lojda, 1973), Sudan Black "B" for neutral fats, and Nile Blue sulfate for fatty acids.

**Light Microscopy**

Fixation in 10% neutral buffered formalin, followed by routine paraffin blocking and histological staining techniques, including H.-E., PAS, Prussian blue, bromphenol blue, and carbol fuchsin.

**Results**

The chloroquine-treated chicks on the EID survived beyond 22 days of age without exhibiting signs of nutritional encephalopathy (NE). In contrast, all of the untreated birds on the EID, comprising the third group of animals, exhibited the clinical signs and histopathologic alterations associated with NE (Young and Tureen, 1966), and did not survive beyond 22 days.

By day 15 chloroquine-treated chicks on the EID showed marked intensification of abnormalities present in the brain capillaries of untreated chicks in the incipient stage of NE. The details of these changes during chloroquine treatment are described in following paragraphs.

**Fluorescence Microscopy**

Yellow autofluorescent material delineated the cerebellar microvascular bed of EID-fed, chloroquine-treated chicks by day 15. The material's random spacing, irregular contours and intramural localization matched the characteristics of the vascular fluorescence previously observed in untreated chicks; however, the intensity of the fluorescence was enhanced considerably (Fig. 1a and b). Long-term administration of chloroquine resulted in diminished spaces between fluo-
rescent deposits, so that intense fluorescence was noticeable almost along the entire capillary wall, with the exception of occasional non-fluorescent, nuclear regions (Fig. 2). Fluorescence was also observed in larger vessels in the cerebellar white matter, in the cytoplasm of neurons of cerebellar nuclei and, minimally, in Purkinje cells.

The control group of chloroquine-injected chicks on normal starter feed did not exhibit fluorescence on day 15. To a minimal degree it began to be seen by day 21 in vascular walls and within the cytoplasm of Purkinje cells and neurons in the corpus cerebelli. Prolonged exposure to chloroquine (51 days) intensified the fluorescence of these neurons and, in addition, revealed strongly fluorescing cells in the molecular layer of the cerebellar folia.

**Acid Phosphatase**

The amount of reaction product deposited in the vascular endothelium of chloroquine-treated EID-fed chicks was increased in comparison to chicks given only the EID (Fig. 3a and b). The degree of increase paralleled the degree of intensification of vascular fluorescence (Fig. 4).

Chloroquine-treated chicks on normal starter feed, in contrast, showed only minimal vascular enzyme products, even after 6 weeks of injections; these animals, however, showed a marked acid phosphatase reaction of parenchymal cells and processes within the granule cell and molecular layers of cerebellar folia.

No enzymatic reaction occurred in sections exposed to an inhibitor for acid phosphatase (0.01 m Na-fluoride), or incubated in a medium without substrate.

**Electron Microscopy**

Coincident with the changes described above, injected EID-fed chicks showed an increase in size and number of dark bodies within the cytoplasm of cerebellar endothelial cells. These bodies varied in size, contour, degree of osmiophilia, and membrane delimitation (Fig. 5a and b). Following prolonged treatment with chloroquine, the luminal portion of the endothelial cytoplasm became packed with these dark bodies (Fig. 6); intermittent cytoplasmic protuberances composed of large aggregates of bodies narrowed the vascular lumen; with higher magnification the presence of amorphous debris within the dark bodies was noticeable (Fig. 7).

Acid phosphatase reaction occurred mainly in the interstices between or, at times, on adjacent dark bodies in endothelial cells of EID-fed chicks injected with chloroquine (Fig. 8).

Endothelial cells of injected controls, on the other hand, contained only a modest number of dark bodies, even after prolonged chloroquine administration.

Control and experimental groups receiving chloroquine showed progressive accumulation of lamellated, membranous structures in the cytoplasm of Purkinje and granule cells, in neurons of the cerebellar nuclei, and in cells of the molecular layer (Fig. 9); occasionally a few examples of these structures were also seen in the cytoplasm of endothelial cells. These lamellated bodies, morphologically distinct from the endothelial dark bodies, and unrelated in development to dietary intake, have been described by others (Dukes et al., 1971; Read and Bay, 1971).
Fig. 1a. 15-day-old chick, EID-fed, untreated; cerebellar vasculature outlined by autofluorescence; frozen section, unstained, ×70 (arrow points to vessel in folial white matter)

Fig. 1b. Similar to Fig. 1a, but chloroquine-treated; note increased vascular autofluorescence

Fig. 2. 55-day-old chick, EID-fed, chloroquine-treated; progressive intensification of autofluorescence within blood vessels; frozen section, unstained, ×70

Fig. 3a. 15-day-old chick, EID-fed, untreated; acid phosphatase reaction within capillaries of molecular layer, and to a lesser degree within Purkinje and granule cells; frozen section, ×70

Fig. 3b. Similar to Fig. 3a, but chloroquine-treated; increased vascular and parenchymal deposition of enzymatic reaction products

Fig. 4. 55-day-old chick, EID-fed, chloroquine-treated; note intense enzymatic reaction within vasculature and parenchymal cells of cerebellar folium; chloroquine-treated chicks on
Cerebellovascular Chloroquine Changes in Chicks

Light Microscopy

Cerebellar endothelial cells conspicuous by a granulated cytoplasm were noted in H.-E.-stained sections of EID-fed chicks, following extended treatment with chloroquine (Fig. 10). Special histological staining methods revealed that the cytoplasmic material was stained only by the Elleder (Fig. 11), OTAN, Sudan black “B” and Nile blue sulfate methods.

Discussion

The onset of NE was inhibited in chicks fed the encephalopathy-inducing diet, if the animals were exposed to constant treatment with chloroquine, at precisely measured dosage levels; inadequate amounts resulted in the occurrence of NE, while overdosing led to stupor and, at times, to death of the chicks. The deaths probably resulted from the toxic effects of chloroquine, which are known to include depression of myocardial excitability and vasomotor function to the point of circulatory collapse and respiratory paralysis.

Our studies demonstrate chloroquine markedly intensifies the cerebrovascular alterations associated with the preclinical phase of NE (Fischer and Nelson, 1973). These alterations represent selective activation of the lysosomal system in cerebellar capillary endothelium in response to excessive intake of linoleic acid. In view of the fact that chloroquine in vitro has the capacity of selective concentration within, and functional modification of a specific subcellular organelle, the lysosome (Allison and Young, 1964), our previous supposition on the involvement of the lysosomal system in the preclinical stage of NE is supported by the present study.

It is clear that the endothelial changes alone cannot be responsible for the subsequent development of edema, hemorrhage, and necrosis which characterize the acute lesions of NE, since these alterations are also seen in parts of the CNS which only occasionally are sites of the acute lesion, e.g., the cerebrum; while chloroquine accentuates strikingly some of these changes we have found to be associated with NE, it also prevents its ultimate development. The mechanisms involved in either case are uncertain.

A wide variety of unrelated disorders are greatly ameliorated by chloroquine administration (Knox and Owens, 1966). Among its multifarious modes of action, the following postulates may provide a plausible basis for an understanding of our findings:

1. stabilization of lysosomal membranes, controlling entry and/or release of hydrolytic enzymes;
2. stimulation of fatty acid incorporation into phospholipids, leading to accelerated regeneration of peroxidatively deranged subcellular membranes, or

normal, commercial feed exhibit an equally intense cellular, but only a minimal vascular, enzymatic reactivity; acid phosphatase, frozen section, \( \times 70 \)

Fig. 5a. 21-day-old chick, EID-fed, untreated; note electron-dense bodies within capillary endothelium; electron micrograph, orig. mag. \( \times 4000 \)

Fig. 5b. Similar to Fig. 5a, but chloroquine-treated
Fig. 6. 55-day-old chick, EID-fed, chloroquine-treated; note accumulation of dense bodies within endothelial cells markedly narrowing vascular lumen; electron micrograph, orig. mag. ×3000

Fig. 7. Endothelial cell, EID-fed, chloroquine-treated chick; close-up of dense bodies (arrows), containing amorphous debris; electron micrograph, orig. mag. ×12000

Fig. 8. 41-day-old chick, EID-fed, chloroquine-treated; acid phosphatase reaction of dense body aggregate within endothelial cell cytoplasm; electron micrograph, orig. mag. ×6800

Fig. 9. 21-day-old chick, on normal, commercial feed, chloroquine-treated; granule cell layer; arrows point to lamellated structures within granule cell cytoplasm; electron micrograph, orig. mag. ×4500

Fig. 10. 34-day-old chick, EID-fed, chloroquine-treated; note tinctorial distinction of blood vessels in molecular layer; formalin-fixed paraffin section. H.-E., ×70

Fig. 11. 36-day-old chick, EID-fed, chloroquine-treated; blood vessel in molecular layer; frozen section, Elleder's procedure for polar phospholipids, ×280
causing diminished availability within the cell of large amounts of fatty acid, unprotected by antioxidants.

The widely held, though still disputed, suggestion that one of chloroquine’s therapeutic effects occurs through stabilization of lysosomal membranes may provide an explanation of the intensification of the vascular alterations which we have observed in chloroquine-treated chicks. A consequence of lysosomal stabilization, it is believed, is the inhibition of lysosomal enzyme release (Weissmann, 1966). In an extension of this view, it has been proposed that chloroquine also decreases the entry of newly formed enzymes into lysosomes (Abraham et al., 1968). This proposition of a twin effect of chloroquine’s stabilizing capacity is consistent with our findings that lysosomes are increased in size and contain partially digested material; the ineffective disposal mechanism of these lysosomes may perhaps be due to inadequate supplies of lytic enzymes to these organelles; the therapeutic effect of inhibiting the onset of NE, on the other hand, may be based in part on the blockage of lysosomal spillage of deleterious hydrolytic enzymes, since there is considerable evidence that the integrity of cellular membranes in a vitamin E-depleted environment is impaired.

An alternative explanation for the action of chloroquine is suggested by observations concerning the effect of the drug on the metabolism of phospholipids. Studies with labelled chloroquine have shown that incorporation of certain fatty acids into phospholipids can be accelerated in chloroquine-exposed cells (Gutierrez, 1966). Uptake of linoleic acid, available in our chicks in excessive amounts from dietary sources, may well be increased by chloroquine, leading to increased rates of phospholipid synthesis and membrane formation, possibly compensating for the loss of peroxidatively damaged membranes. Further, the sequestration of degenerating membranes within stabilized lysosomes would reduce the presence of large amounts of a fatty acid, unprotected by antioxidants, within the cell, and may be a contributing factor in preventing the initiation of a peroxidative breakdown of linoleic acid affecting subcellular membranes. Support for this speculation may be derived from our histochemical studies which indicate the accumulation of polar phospholipids within the cerebellar endothelial cells of EID-fed, chloroquine-treated chicks.

Such a supposition suggests that the viability of subcellular organelles in our chicks may be dependent on the delicate balance between membranous peroxidative derangement and chloroquine-stimulated synthesis.

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