EFFECT OF A SINGLE, LARGE DOSE INJECTION OF PYRITHIAMIN ON THE THIAMIN DEFICIENCY OF THE MOUSE

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Accepted April 28, 1982

Abstract—The effect of pyrithiamin on thiamin deficient mice was studied clinically and pathologically. Thiamin deficiency was produced in the mice fed with a thiamin deficient diet by a single injection of pyrithiamin (10 mg/kg, s.c.) on day 0 to produce a mild illness and on day 14 to produce a severe disorder. Clinically, the mild illness showed only Wooley-White sign and histologically, it was characterized by edema in the thalamus, mammillary bodies, and pontine tegmentum. The severe illness showed convulsion and multiple hemorrhages in the same area. This pyrithiamin-induced thiamin deficient animal model can also be useful for research on thiamin deficient encephalopathy.

Numerous biochemical and/or pathological studies of thiamin deficiency have been reported (1–5). Peters (1) demonstrated that acute thiamin deficiency in pigeons was associated with accumulation of lactic acid and other metabolites in the central nervous system and, more rapidly, in the brain stem. Evidence has been accumulated for the non-coenzymatic role of thiamin in nervous tissue. Fox and Duppel (6) have shown that thiamin diphosphate and especially thiamin triphosphate prevent a low exponential decline of the peak of sodium and potassium currents in the node of Ranvier.

Pyrithiamin, an antagonist of thiamin (7), has been useful for the purpose of making a thiamin-deficient animal model of Wernicke-Korsakoff Syndrome in man (8). This chemical effectively inhibits thiamin in nervous tissue (9) and rapidly produces neurological signs and pathological lesions in the experimental animals (10). In the experimental diet-induced thiamin deficiency, slow progression of the illness causes malnutrition which makes it difficult to evaluate the direct effects of thiamin deficiency on nervous tissue. The rapid course of pyrithiamin-induced thiamin deficiency makes this evaluation easier.

Thus, it is pertinent to use pyrithiamin to study the correlation between clinical signs and morphological changes in the developing stage of thiamin deficiency. Although other investigators (3, 10) have used small amounts of pyrithiamin by daily injections, no studies on the effects of a single injection of pyrithiamin at high doses in conjunction with a thiamin deficient diet have been reported. This report deals with clinical signs and historigic and morphologic changes in the brains of mildly and severely thiamin deficient mice aggravated by a single injection of a large dose of pyrithiamin.

MATERIALS AND METHODS

Materials: Pyrithiamin hydrobromide was obtained from the Sigma Chemical Co., St. Louis, MO. The injection solution contained
0.4% pyrithiamin in physiological saline.

The low thiamin test diet was obtained from Teklad Test Diets, Madison, WI.

Throughout the experiment, the animals used were adult, male, outbred Swiss mice, weighing 30 to 35 g. (Charles River, Wilmington, MA).

Procedures to produce thiamin deficiency:
In group 1, ten mice were fed with the thiamin deficient diet (TDD) from day 0 and received s.c. one injection of pyrithiamin (PT) (10 mg/kg, s.c.) on day 0. In group 2, ten mice were also fed with TDD from day 0 and received the same dose of pyrithiamin by a single s.c. injection as group 1 on day 14 (Table 1). Five control mice were fed with a regular diet and received no injection of pyrithiamin. Body weights of the mice in all groups were recorded daily. Mice were sacrificed by direct intracardiac perfusion 3 days after the first appearance of Wooley-White sign (11) which is vibration or rapid jerking and spinning movement of the entire body when the animal was held by its tail.

Fixation of the brain: The fixative which contained glutaraldehyde (1.25%) and paraformaldehyde (1%) in 0.1 M phosphate buffer at pH 7.4 was perfused intracardially by a perfusion pump (Harvard). After removal from the skull, the brains were kept in the same fixative for three days. Then the fixative was replaced with 10% formalin in 0.1 M phosphate buffer at pH 7.4 for JB-4. Some brains were used for TEM.

JB-4 embedding, sectioning and staining (12): The brains were cut coronally and the slices containing the thalamus, mamillary body, and pontine tegmentum were taken for JB-4 embedding.

After rinsing with 0.1 M phosphate buffer, the blocks were dehydrated in 30, 50, 70, 90, and 95% ethanol and three times in 100% ethanol, 10 min in each solution, and then in propylene oxide for 10 min. These blocks were pre-embedded with solution A (mixture of glycol methacrylate, 2-butoxyethanol, and benzoyl peroxide) of JB-4 and catalyst in plastic embedding molds for 4 hr at 23°C. The blocks were sectioned at 3.5 μm thickness with a DuPont, Sorvall, JB-4 Microtome and stained with hematoxilin and eosin solution.

Preparation of the tissue for transmission electron microscopy (TEM): After post-

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Table 1. Experimental plan and clinical changes

| Group 1 | Group 2 |
|---------|---------|
| Start TDD feeding | Start TDD feeding |
| Days | Days |
| PT injection | PT injection |
| 7 | 14 15 16 17 18 19 20 |
| 10 11 12 13 14 15 16 | The first +++WWS appear |

PT: pyrithiamin
TDD: thiamin deficient diet
+++WWS: distinctive Wooley-White sign
fixation with 1% OsO₄ for 1 hr, the 1 mm/brain blocks from the thalamus, mammillary body and pontine tegmentum were rinsed with 0.1 M phosphate buffer containing sucrose and dehydrated in a series of graded ethanol concentration (30–100%), 10 min in each concentration. Then these blocks were embedded in EPON 812. From 500 to 1000 Å-thick ultrathin sections were cut with a LKB Ultratome and stained with uranyl acetate and lead citrate. These sections were examined under a JEM-100s transmission electron microscope.

RESULTS

Clinical findings: The mice in group 1 and 2 developed transient anorexia and continuously slight weight loss after the single pyrithiamin injection on both day 0 and day 14. In group 1, the mice showed distinctive Wooley-White sign (++WWS) and slight tremor from day 10 through 16. In group 2, tremor was noted from day 15 through 20. Wooley-White signs appeared for 3–5 days in group 1, but in group 2, they were discontinuous and transitional to more severe convulsion. As shown in Table 2, the rate of body weight loss was 21 and 36%, and the duration of ++WWS was 3.3 days and 1.5 days, respectively. In the preliminary experiment, the mice did not eat or drink and died within 72 hr after convulsion started. In this experiment, all the experimental mice were sacrificed by intracardiac perfusion 3 days after Wooley-White sign became positive. Control mice were also sacrificed on the same days as those in group 1 or 2.

Gross observation: The whole brains were dipped into fixative for 24 hr and examined under a dissecting microscope. In group 1, only one out of ten mice showed very slight hemorrhage in the thalamus and one showed hemorrhage in the mammillary bodies. The others did not show any hemorrhages or other abnormalities. In group 2, all the mice showed serious hemorrhages in the olfactory bulb, thalamus, mammillary body, pontine tegmentum, and sometimes in the deep white matter of the cerebellum. Particularly in the mammillary body, the most extensive hemorrhages were observed. Petechial hemorrhage was mostly observed in the thalamus. The hemorrhage in the olfactory bulb that was seen both on the surface and inside reflected the severity of other hemorrhages in the thalamus, mammillary body and pontine tegmentum; the severe hemorrhage on the surface of the olfactory bulb was often correlated with severe hemorrhage of other areas of the brain. In group 2, eight out of ten mice showed remarkable enlargement of the fourth ventricle which was sometimes associated with an enlarged third ventricle (Table 3).

Light microscopic observation: The sections containing the thalamus, mammillary body and pontine tegmentum were examined. In group 1, edema of the astrocytes was seen in one side or both sides of the thalamus, mammillary body and pontine tegmentum.

|       | Date of the first ++WWS appearance (day) | Duration of ++WWS (days) | Date of the first convulsion appearance (day) | Rate of body weight loss (%) |
|-------|----------------------------------------|--------------------------|-----------------------------------------------|----------------------------|
| Group 1 | 13.3                                   | 3.3                      | —                                             | 21                         |
| Group 2 | 17.2                                   | 1.5                      | 20.5                                          | 36                         |

++WWS: distinctive Wooley-White sign
Table 3. Gross observations of hemorrhages and enlargement of the fourth ventricle and neurological signs

| Hemorrhage* | Olfactory bulb | Thalamus | Mammillary body | Pontine tegmentum | Enlargement of fourth ventricle* | Neurological signs** |
|-------------|----------------|----------|-----------------|-------------------|---------------------------------|---------------------|
| -           | -              | -        | -               | -                 | -                               | ++                  |
| -           | -              | -        | -               | -                 | -                               | ++                  |
| -           | -              | -        | -               | -                 | -                               | ++                  |
| -           | -              | -        | -               | -                 | -                               | +, + +              |
| Group 1     | -              | -        | -               | -                 | -                               | +, + +              |
| -           | -              | +        | -               | -                 | ±                               | ++                  |
| -           | -              | -        | +               | -                 | ±                               | ++                  |
| -           | -              | -        | -               | -                 | -                               | +++                 |
| -           | -              | -        | -               | -                 | -                               | +++                 |
| Group 2     | +              | -        | +               | +                 | +                               | +++                 |
| +           | +              | +        | +               | +                 | +                               | +++                 |
| ++          | +              | +        | +               | ++                | +                               | +++                 |
| +           | +              | +        | +               | +                 | +                               | +++                 |
| +           | +              | +        | +               | +                 | +                               | +++                 |
| ++          | +              | +        | +               | +                 | +                               | +++                 |

*: -, +, and ++ showing the degrees of hemorrhagic change or enlarging change. **: + showing slightly positive Wooley-White sign, ++ showing distinctly positive Wooley-White sign, and +++ showing convulsion or tonic seizure of the entire body.

(Fig. 1). In addition to abnormal astrocytes, some nerve cells showed edema in these areas. These edematous lesions were not associated with hemorrhage except in one mouse. The space of the fourth ventricle was normal, and there were no abnormalities in the ependymal cells. In group 2, edema was more severe. Petechial hemorrhages were accompanied by astrocytic edema in their vicinity (Figs. 2-4). The fourth ventricle was enlarged, and the ependymal cell layer appeared wavy and thickened in some parts. Immediately under the ependymal cell layer, spongious swelling was often observed (Fig. 4).

Transmission electron microscopic observation: TEM observations were made on the brains which were judged "mild" by light microscopic examinations of the JB-4 sections. In the mildly edematous lesion, the most common change was the edematous swelling of the astrocytes (Fig. 5). Within the cytoplasm of the astrocyte, mitochondria were decreased in number, but the remaining ones showed intact structures. Edematous change was also observed in some nerve cells; it occurred particularly in the sub-plasmalemmal cytoplasm (Fig. 6). Some nuclei and cytoplasmic organelles remained intact. Neuropils were sometimes swollen and completely degenerated processes were also observed.

DISCUSSION

Although a number of ultrastructural studies (13-16) have been made on the lesions of the vestibular nucleus and adjacent areas of the tegmentum of the rats with diet-
Fig. 1-4. H & E stained plastic JB-4 sections of mice.

Fig. 1. Edema of the astrocytes (arrows) in the mild lesion. Mammillary body. (group 1). ×100.

Fig. 2. Hemorrhages (arrows) including ring type hemorrhage in the severe lesion. Thalamus. ×100.

Fig. 3. Petechial hemorrhages (large arrows) and edema of the astrocytes (small arrows) in the severe lesion. Mammillary body. ×160.

Fig. 4. Hemorrhage (arrow) associated with edematous lesion in the severe lesion. Pontine tegmentum. ×43.
Figs. 5 and 6. Transmission electron microscopic views of the mammillary body in mildly ill mice (group 1)

Fig. 5. Marked swelling of astrocytes. Mitochondria remain normal. N: nucleus of the astrocyte. ×10000.

Fig. 6. Cytoplasmic edema of the nerve cell closely associated with a completely degenerated process. N: nucleus of the nerve cell, dp: degenerated process. ×12000.
induced thiamin deficiency, only a few studies were reported on pyrithiamin-induced thiamin deficiency (4, 10). Furthermore, there are no reports which deal with lesions of thiamin deficiency induced by a single injection of a large dose of pyrithiamin.

In this experiment, there was a marked clinical difference between group 1 and 2. Group 1 mice, injected with 10 mg/kg pyrithiamin on day 0, showed a positive Wooley-White sign (++WWS) continuously for several days, but never developed tonic convulsion. On the other hand, group 2 mice, injected on day 14 with the same dose of pyrithiamin as that to the group 1 mice, also showed ++WWS within a couple of days after injection. However, in this group, Wooley-White sign was of short duration and was followed by a state of frequent convulsion that continued until death. What is the cause of this difference? This result may be confirmed by the reports of DeCaro et al. (16) and/or Rindi and Perri (17). They found that administration of pyrithiamin to rats caused a decrease of the thiamin contents and an increase of the pyrithiamin contents of all the tissues, particularly of the brain. The results of these two investigators provided two important suggestions to this experiment on the action of pyrithiamin on the mice treated with a low thiamin diet. First, one injection of a large dose of pyrithiamin on day 0 may affect some regions of the neuronal system mildly and continuously for ten days. This is because of the fact that pyrithiamin on day 0 caused neurological signs as early as day 10, while with a thiamin deficient diet alone, it takes 4 or 5 weeks for mice to develop the same neurological signs (11). Second, pyrithiamin injection on day 14 is more effective than that on day 0 because it causes very severe neurological signs which rapidly progressed to lethal convulsions within a couple of days. From these points of view and other reports (10, 14, 15) in which a change of the blood-brain-barrier was shown in thiamin deficiency, it is considered that pyrithiamin may pass through the blood-brain-barrier more easily when the barrier has been changed by diet-induced thiamin deficiency. Once pyrithiamin is allowed to enter the brain, it may occupy the site normally occupied by thiamin and inhibit the function of thiamin as shown in Cooper's experiments (9, 18) which suggest that thiamin phosphates in the cell membrane may regulate pump activity as reflected by cation-activated ATPase; and the inhibition of the ATPase activity by pyrithiamin may correlate with a destruction or displacement of bound thiamin from the preparation. In our previous experiments (19, 20), pyrithiamin-induced thiamin deficient rats showed changes in the myelinated axons and Schwann cells of the sciatic nerve in a short term, and it was suggested that pyrithiamin might directly affect the nervous system and that the dysfunction of the sciatic nerve in pyrithiamin-induced thiamin deficiency might originate from the central nervous system. From these data, it seems possible to speculate that pyrithiamin-induced thiamin deficiency occurs through a different mechanism from diet-induced chronic thiamin deficiency; systemic nutritional malfunction occurs in the latter, but it is absent in the former.

To produce pyrithiamin-induced encephalopathy in mice after a very short treatment time, daily injections of small amounts of pyrithiamin have recently been used (10). It has been demonstrated that this method has produced more severe pathological change, including massive hemorrhagic lesions, than actual Wernicke's encephalopathy in man. However, this method has yielded lesions located with closer topographic similarity to Wernicke's encephalopathy than those in diet-induced thiamin deficient encephalopathy in which only the
pontine tegmentum is damaged (21). In group 1 and 2 of this experiment, the thalamus and mammillary body as well as the pontine tegmentum are frequently damaged. These areas are commonly involved in human Wernicke’s encephalopathy (8).

Wernicke’s encephalopathy is a disorder of thiamin deficiency manifested by mental confusion, ataxia, ophthalmoplegia and coma. It is usually associated with alcoholism. Korsakoff’s psychosis, an amnestic disorder, is regarded as the psychotic component of Wernicke’s disease. The effect of administration of thiamin to the patient is dramatic. In human Wernicke’s disease, the striking feature is the selective localization of the lesion in the central nervous system, especially in the paraventricular parts of the medial and dorsal nuclei, in the anterior medial nuclei, pulvinar of the thalamus, in the mammillary body, in the periaqueductal region, in the floor of the fourth ventricle, particularly in the dorsal motor nuclei of the vagus and vestibular nuclei, and in the anterior lobe of the cerebellar vermis. The most frequent site for such a lesion is the mammillary body. In addition, striking atrophy of brain parenchyma and enlargement of the lateral and the third ventricle with decreased brain weight has been reported (8). The extent of changes in Wernicke-Korsakoff syndrome in man differs from case to case depending upon the severity and the stage of progressive necrosis with multiple petechial hemorrhages and hyper-plastic vasculature. In mild cases, hemorrhage is a rather infrequent manifestation (8).

Group 1 results and group 2 results of this experiment closely resemble a mild case and an acute case of Wernicke-Korsakoff disease, respectively. It is indicated that this pyritiamin-induced thiamin deficient animal model can be useful for the research of thiamin deficient encephalopathy, since it is easy and rapid to produce encephalopathy, and the transition of the neurological stage from Wooley-White sign positive condition to a lethal convulsive state is very clearly recognizable. The rapid transition of the lesion from edema to hemorrhage may reflect the developing damage of the membrane-transport mechanism by the pyritiamin-induced thiamin deficiency.

Concerning changes of the vascular permeability, Robertson and Manz (14) demonstrated by using fluorescent dye-labeled albumin that the protein permeability was altered only when necrosis and hemorrhage supervened in the early lesions of acute dietary thiamin deficiency in rats. They observed that the cerebral edema in the thiamin deficient lesion was biphasic. It might occur initially in the absence of a change of vascular permeability and perhaps as the result of inhibition of active transport related to the thiamin deficient state. In contrast, the late edema might be the result of a gross breakdown of barrier function to protein with enhanced pinocytotic transport and extracellular accumulation of the marker between the neuropil (15).

In the convulsive mice of this experiment, the fourth ventricle showed an enlargement and the ependymal cell layer was wavy looking. These changes may not be only due to dysfunction of the brain parenchyma, but also due to the effect of thiamin deficiency on the ependyma and subependyma which are reported to have a transport function between the cerebro-spinal fluid and brain parenchyma (22).

Conceivably, the following postulation can be made: a large dose of pyritiamin may displace thiamin in the membrane and cause the dysfunction of the membrane transport mechanism which is reflected as edema and/or hemorrhage. Consequently, the damage of the blood-brain-barrier may correlate with convulsion because the time of occurrence was coincident. Furthermore, since in our
preliminary experiment (unpublished data) the uptake of glucose was increased in the area showing a small hemorrhage, the hemorrhage in the specific area may induce convulsion by a stimulating effect on the blood itself as a physiological activator in thiamin deficiency.

Acknowledgements: We are grateful to Dr. I. Watanabe and Dr. C. Anderson for pertinent discussions. This work was supported in part by a Grant from the Department of Pathology of the Kansas University Medical Center.

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