JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. **24**, 497–500 (2004)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jat.1007

Pyridoxine (Vitamin B6) Neurotoxicity: Enhancement by Protein-deficient Diet

Seymour Levine* and Arthur Saltzman
Nathan S. Kline Institute for Psychiatric Research, Orangeburg, New York 10962, USA

Key words: pyridoxine; B6-vitamers; peripheral neuropathy; sensory neuropathy; protein nutrition.

Large doses of pyridoxine cause injury to the primary sensory neurons in trigeminal and dorsal root ganglia of animals and patients subjected to megavitamin therapy. The increased hazard to subjects with reduced renal excretory function has been explored previously. In the present work, the neurotoxicity of pyridoxine for rats was found to be increased by dietary protein deficiency. A mere 3 or 7 days of pretreatment with either of two protein-deficient diets were sufficient to accelerate and intensify the clinical neurological signs and histological lesions from pyridoxine injections. These results are caused, at least in part, by loss of body weight, decreased protein binding in serum and decreased consumption of water and decreased volume of urine, which reduce the urinary losses of the toxicant. The vitamers related to pyridoxine (pyridoxal, pyridoxamine) and the coenzyme (pyridoxal 5-phosphate) did not cause clinical signs or lesions similar to those produced by pyridoxine even when injected in maximum tolerated doses. Neither a protein-deficient diet nor bilateral nephrectomy changed the results with the vitamers. Copyright © 2004 John Wiley & Sons, Ltd.

INTRODUCTION

Although the water-soluble B-vitamins are often considered to be non-toxic, there are many reports of neurotoxicity from large doses of pyridoxine (vitamin B6) prescribed for the treatment of various diseases ('megavitamin' therapy). Pyridoxine is especially toxic to the peripheral nervous system (Antopol and Tarlov, 1942; Krinke *et al.*, 1985; Windebank *et al.*, 1985; Xu *et al.*, 1989; Jortner, 2000). This aspect of the vitamin has special interest because it is not known if its toxicity is related to one or more of its numerous physiological functions or to a still unknown metabolite of the original molecule or of the other vitamers to which it may be converted *in vivo*. In addition, large doses of pyridoxine cause severe damage to primary sensory neurons and nerves that mimic axonal reaction with central chromatolysis. This feature makes it a very useful model for neurotoxicology research.

Recently, we reported that reduced renal excretion of pyridoxine could contribute to the development of neurotoxicity in rats (Levine and Saltzman, 2002). In the course of that work, we noticed that food deprivation could influence the toxic effects of large doses of this vitamin. In the present work we have explored the effects of various types and durations of dietary restriction on the neurotoxicity of pyridoxine.

Pyridoxine is interconvertible *in vivo* with the vitamers pyridoxal and pyridoxamine (McCormick and Chen, 1999). In order to find the mechanism for the neurotoxicity of pyridoxine, it was important to determine if these vitamers caused similar clinical signs and histological lesions as the parent vitamin.

* Correspondence to: S. Levine, Nathan S. Kline Institute for Psychiatric Research, Orangeburg, New York 10962, USA.
E-mail: levines@nki.rfmh.org

MATERIALS AND METHODS

Lewis rats of both sexes, bred in this laboratory, were kept in plastic cages in groups of four with hardwood litter and were fed Rodent Diet 5001 (PMI Feeds, St Louis, MO) *ad libitum* until they weighed 150–250 g. At that time, metal girds were inserted in the cages to reduce coprophagy, and dietary restrictions were imposed. Water was always freely available and lights were on from 6 a.m. to 6 p.m. The dietary restrictions were:

(i) The usual diet (5001) was provided but only every other day.
(ii) A semi-synthetic, protein-free diet (based on AIN 76A) (Dyets, Bethlehem, PA) was freely available every day as the sole nutrient.
(iii) Sucrose cubes (Domino Dots, Domino Sugar Corp., New York) were freely available every day as the sole nutrient.

Each experiment included a control group in which no dietary restrictions were imposed. On the third or seventh day after the start of dietary restrictions the rats were treated with pyridoxine hydrochloride (Sigma Chemical Company): 600 mg kg^{-1} dissolved in distilled water at 60 mg ml^{-1} or dissolved in a solution of 25 mg ml^{-1} sodium bicarbonate. This additive reduced the acidity of the hydrochloride and thereby avoided the mild peritoneal irritation caused by the hydrochloride. Alternatively, an equimolar amount of the free pyridoxine base (500 mg kg^{-1}) was dissolved at 50 mg ml^{-1} in water. A preliminary experiment

Received 29 April 2003
Revised 16 June 2003
Accepted 31 December 2003

showed that all three preparations caused identical neurotoxicity. The dose was 10 ml kg^{-1} body weight injected intraperitoneally in the morning and again in the afternoon on three or four consecutive days. Dietary restrictions were continued during the treatment period.

The experiments were evaluated by clinical signs: 1+, slight weakness or minimal splaying of the hindlimbs; 2+, moderate weakness and splaying of hindlimbs and slight weakness and splaying of forelimbs; 3+, severe weakness and immobility or lateral recumbency. Hindlimb extension was impaired, as described by Jolicoeur *et al.* (1979), but crossed legs and curled toes were not observed.

One day after the last treatment, the rats were anesthetized with CO_2 and exsanguinated through the inferior vena cava. Serum was analyzed in a Roche-Hitachi model 747–100 analyzer. The skull was opened, the brain removed and the trigeminal nerves and ganglia were fixed *in situ* with Bouin's fluid. The skulls were decalcified and coronal sections taken at two levels through the ganglia. Paraffin-embedded blocks were cut at 5 μm and stained with hematoxylin and eosin or cresyl violet for Nissl bodies. Histological lesions of the large trigeminal neurons were loss of Nissl bodies (central chromatolysis), peripheral displacement of nuclei and irregular or indented nuclear membranes, as described by others (Antopol and Tarlov, 1942; Krinke *et al.*, 1985; Windebank *et al.*, 1985; Xu *et al.*, 1989; Jortner, 2000). When these changes were present in all large neurons, the lesions were designated as severe. In moderate lesions, chromatolysis and nuclear displacement were common but nuclear deformation was rare. In mild lesions, nuclear displacement was less common and chromatolysis was less severe. Evaluations were done at 440 × magnification, on randomized slides, without knowledge of the treatment.

Experiments on the vitamers were done as described above, but in addition anephric rats were utilized as described previously (Levine and Saltzman, 1997).

RESULTS

Control rats fed their usual diet of 5001 pellets without restriction, studied 1 day after 3 days of pyridoxine injections i.p., and no clinical signs, had only minimal histological lesions in the trigeminal ganglia. Even 4 days of pyridoxine treatment caused only slight (or no) clinical signs (Table 1) but the ganglia had lesions, albeit mild.

Consumption of the usual diet restricted to every other day, starting 3 or 7 days before pyridoxine treatment and continuing to the end, did not enhance the neurotoxicity (Table 1).

In contrast, rats fed the protein-free semisynthetic diet, or only sucrose cubes, starting 3 or 7 days before pyridoxine and continuing through the treatment period, already had mild neurological signs after 3 days of pyridoxine injections that progressed to severe signs after the fourth day of treatment (Table 1). Their histological lesions in trigeminal ganglia were moderate after 3 days and severe after 4 days of treatment. In three additional experiments (not included in Table 1) two groups of rats were given only half the usual dose for the four daily pyridoxine injections i.p., or they were given the full dose by oral gavage instead of i.p. In all these experiments the sucrose-fed rats had more severe signs and/or lesions than the control rats on the unrestricted diet. Thus, enhancement by dietary restriction was not limited to the particular pyridoxine dose and route used for the experiments recorded in Table 1.

Table 1—Pyridoxine neurotoxicity is enhanced by prior dietary restrictions

Dietary restriction (and start time)[a]	Clinical signs, average	
	After 3 days of i.p. pyridoxine[b]	After 4 days of i.p. pyridoxine[b]
Usual diet, no restriction	0 (n = 4)	0.8 (n = 25)
Usual diet q.o.d.[c]		
Day 3		0.6 (n = 8)
Day 7		0.0 (n = 4)
Protein-free diet		
Day 3		2.0 (n = 4)
Day 7	0.5 (n = 4)	3.0 (n = 4)
Sucrose diet		
Day 3	0.8 (n = 10)	2.5 (n = 12)
Day 7	0.8 (n = 10)	3.0 (n = 4)

[a] Start time: number of days before first pyridoxine injection when the diet was started. Diet was continued until the end of the experiment.
[b] 600 mg kg^{-1} in morning and again in afternoon.
[c] q.o.d.: every other day.

Rats pretreated with sucrose as the sole nutrient might have been subjected to vitamin deficiency during the pretreatment period while they were losing weight. In an additional experiment (not included in Table 1), this potential lack was avoided by twice-daily subcutaneous injections of a multivitamin preparation during the pretreatment period. Sucrose-fed rats given the additional multivitamin pretreatment responded in the same way as sucrose-fed rats from Table 1 to the subsequent challenge with 4 days of the usual dose of pyridoxine. In accordance with the experiments in Table 1, they all developed severe clinical signs, in contrast to the lack of signs in the control rats fed their usual diet who also had been pretreated with vitamins. Therefore, the vitamin status during the preliminary period of dietary restriction had no influence on the subsequent neurotoxicity of large doses of pyridoxine.

Relation of body weight changes to pyridoxine neurotoxicity

Rats fed the sucrose diet for 7 days before inception of pyridoxine injections had lost 27 g (average) of body weight during that period (16% of initial weight). Rats fed sucrose for only 3 days had lost only 21 g (average 12% of initial body weight). Rats in both of these sucrose-fed groups lost an additional 20 g during 4 days of pyridoxine treatment (12% of initial body weight). Rats on the protein-free diet also lost considerable body weight during the preliminary 7 days and during the treatment period. All these rats developed clinical signs and severe lesions after the pyridoxine treatments. The control rats fed their usual diet gained rather than lost weight before the injections started and they lost only 5 g after 4 days of treatment with pyridoxine. They did not develop clinical signs.

Rats fed their usual diet every other day for 7 days before treatment lost much less weight, only 5 g (average), and those fed every other day for only 3 days managed to gain a small amount of weight (2 g) before pyridoxine treatment. They did not develop clinical signs. Therefore, in all

Table 2—Toxicity of pyridoxine and vitamers

Vitamer	Pretreatment[a]	Non-lethal dose (mmol/kg^{-1})[b]	Clinical signs and histological lesions
Pyridoxine·HCl	None	5.9	Mild
	Sucrose diet	2.3–5.9	Severe
	Bilateral nephrectomy	0.30–0.73	Severe
Pyridoxal·HCl	None	1.8–2.4	0
	Sucrose diet	1.5–1.8	0
	Bilateral nephrectomy	0.4–1.5	0
Pyridoxal 5-phosphate	None	1.2–2.9	0
	Sucrose diet	0.6–1.7	0
	Bilateral nephrectomy	0.6	0
Pyridoxamine·2HCl	None	3.5–11.6	0
	Sucrose diet	0.3–11.6	0
	Bilateral nephrectomy	0.3–2.3	0

[a] Bilateral nephrectomies were preceded by the sucrose diet, as described.
[b] Doses listed were injected on four successive days without mortality. Where a range of doses is indicated, 2–7 levels were tested, each level in 2–4 rats. Doses are specified as mmol kg^{-1} to facilitate comparisons. Larger doses of pyridoxal·HCl and pyridoxal 5-phosphate were tested but are not included in the table because they were lethal after one, two or three daily doses.

the experiments loss of body weight correlated well with enhanced neurotoxicity of pyridoxine.

Analysis of serum after 3 or 4 days of pyridoxine treatment did not reveal any differences between sucrose-fed rats and 5001-fed controls in levels of glucose, urea, creatinine, electrolytes or enzymes. However, total protein levels were lower in the sucrose-fed rats (4.80 g dl^{-1}, SD = 0.43, n = 18) than in the 5001-fed controls (5.40 g dl^{-1}, SD = 0.33, n = 13), as could be expected.

The vitamers

The vitamer pyridoxal and the coenzyme pyridoxal 5-phosphate were more toxic than pyridoxine. They did not produce clinical signs or lesions in ganglia similar to those produced by pyridoxine even though maximum tolerated doses were injected (Table 2). The vitamer pyridoxamine was somewhat less toxic, which made it possible to study effects after doses that matched or exceeded the doses of pyridoxine. Nevertheless, the results were negative. Pretreatment with the sucrose diet as described above, or preparation with bilateral nephrectomy as described previously, did not elicit signs or lesions in trigeminal ganglia (Table 2).

DISCUSSION

Large doses of pyridoxine were required in order to demonstrate neurotoxicity. These doses are relevant to megavitamin therapy of patients with various diseases, some of whom have also developed neurotoxicity.

Dietary restriction has profound consequences for the organism and its response to toxicants (Levin *et al.*, 1993). There are many examples of increased susceptibility to neurotoxicants (cyanide, quinacrine, DDT, lead, ergot, etc.) caused by dietary factors, most often low protein content (Holck, 1949). Pyridoxine is an essential nutrient but in high doses it is a neurotoxicant that is as disabling or deadly as any other. In this work, we found that a few days of dietary restriction were enough to cause considerable enhancement of pyridoxine's neurotoxicity. A diet of sucrose without any other nutrient, and a diet that provided all nutrients except protein, were both effective provided that they were instituted before the large doses of pyridoxine were injected. These two very different types of food deprivation caused loss of body weight. In contrast, a normal diet fed only every other day was not effective and caused hardly any weight loss.

During dietary deprivation, loss of body fat, liver mass and gastrointestinal tract (with contents) exceed losses of other tissues (Peters and Boyd, 1966; Levin *et al.*, 1993). Neural tissue is protected from weight loss, so it becomes, relatively speaking, a larger part of the organism and presumably accumulates a larger amount of the neurotoxin. These considerations may be pertinent to the enhanced neurotoxicity of pyridoxine, but diminished thirst and oliguria during food restriction are probably involved also. Pyridoxine is normally excreted in the urine, and removal of the kidneys increased its toxicity (Levine and Saltzman, 2002). Diminished drinking and oliguria with reduced renal losses could enhance the vitamin's toxicity. In a preliminary experiment both weight loss and oliguria were induced simultaneously by depriving rats of drinking water during the 4 days of high-dose pyridoxine treatment. As in the previous experiments, this procedure increased the clinical signs and histological lesions compared with control rats having free access to water.

Diminished serum proteins in dietary-restricted rats could lead to reduced protein binding of pyridoxine or a metabolite. Free pyridoxine is more likely to be toxic than protein-bound pyridoxine. Reduced renal excretion and reduced protein binding combined with an increase in the proportion of neural tissue in the body could explain, at least in part, the effects of dietary restrictions in our experiments. However, other (nutritional) factors have not been excluded.

Feeding sucrose should not be thought of as equivalent to starvation. During starvation, body proteins are catabolized to provide amino acids for conversion to glucose ('gluconeogenesis'). Feeding sucrose avoids the need for gluconeogenesis and thereby conserves body protein. Rats consume sucrose readily and will survive many weeks before a vitamin deficiency (vitamin A) develops (Richter, 1941). Furthermore, our experiment

with prophylactic multivitamin therapy proved that the effects of preliminary sucrose feeding were not due to vitamin deficiency. Preventing gluconeogenesis with a sucrose diet has proved especially valuable in prolonging the life of uremic rats (Levine and Saltzman, 1997, 2000).

Pyridoxine is converted after injection into pyridoxal-5-phosphate, which is the active coenzyme for many enzymes, including amino acid decarboxylase, transaminases, racemase, etc. It is not known if the neurotoxicity of large doses of pyridoxine is caused by the unaltered pyridoxine molecule, by the vitamers or the active coenzyme, by intermediates involved in the conversion to coenzyme or by some unknown derivative. This unsolved problem is important because of the continued, contemporary therapeutic use of large doses of pyridoxine (McCarty, 2000; Lerner *et al.*, 2001). The information on nutritional and renal aspects of pyridoxine neurotoxicity in this and our previous report may facilitate research to identify the active neurotoxic molecule. It is important to be aware that diets, drugs or procedures that reduce renal function or cause loss of body weight can be responsible for a non-specific increase in the neurotoxicity of pyridoxine, which could cause confusion in the search for the active metabolite.

Contributing to this last issue is a study by Windebank (1985), who found almost equal toxicity to cultures of dorsal root ganglia neurons from pyridoxine as from the other B6-vitamers (pyridoxal and pyridoxamine). This observation tended to link pyridoxine toxicity to its coenzyme function. In support of such a linkage, the three vitamers are known to be interconvertible through the activity of pyridoxine oxidase and pyridoxine kinase, yielding the active coenzyme pyridoxal 5-phosphate (McCormick and Chen, 1999).

The importance of the present work is that we have demonstrated that the question of neurotoxicity by the B6-vitamers *in vivo* was not settled by the *in vitro* experiments, which found no differences among them (Windebank, 1985). Further study must include assays of each of the vitamers in serum and in the target neural tissues. The incentive for such a study is the possibility that the pyridoxine molecule itself, or a presently unsuspected metabolite, might be responsible for the neurotoxicity.

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