The vitamin B6 paradox: Supplementation with high concentrations of pyridoxine leads to decreased vitamin B6 function

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ABSTRACT

Vitamin B6 is a water-soluble vitamin that functions as a coenzyme in many reactions involved in amino acid, carbohydrates and lipid metabolism. Since 2014, > 50 cases of sensory neuronal pain due to vitamin B6 supplementation were reported. Up to now, the mechanism of this toxicity is enigmatic and the contribution of the various B6 vitamers to this toxicity is largely unknown.

In the present study, the neurotoxicity of the different forms of vitamin B6 is tested on SHSY5Y and CaCo-2 cells. Cells were exposed to pyridoxine, pyridoxamine, pyridoxal, pyridoxal-5-phosphate or pyridoxamine-5-phosphate for 24 h, after which cell viability was measured using the MTT assay. The expression of Bax and caspase-8 was tested after the 24 h exposure. The effect of the vitamers on two pyridoxal-5-phosphate dependent enzymes was also tested.

Pyridoxine induced cell death in a concentration-dependent way in SHSY5Y cells. The other vitamers did not affect cell viability. Pyridoxine significantly increased the expression of Bax and caspase-8. Moreover, both pyridoxal-5-phosphate dependent enzymes were inhibited by pyridoxine.

In conclusion, the present study indicates that the neuropathy observed after taking a relatively high dose of vitamin B6 supplements is due to pyridoxine. The inactive form pyridoxine competitively inhibits the active pyridoxal-5′-phosphate. Consequently, symptoms of vitamin B6 supplementation are similar to those of vitamin B6 deficiency.

1. Introduction

Vitamin B6 is a water-soluble vitamin found in e.g. meat, poultry, fish, legumes, bananas, nuts and cereals (Ueland et al., 2015). Vitamin B6 functions as a coenzyme in many reactions that are involved in amino acid, carbohydrates and lipid metabolism (Eliot and Kirsch, 2004). Additionally, vitamin B6 also plays a role in neuronal signaling through the synthesis of neurotransmitters (Percudani and Peracchi, 2009). The major forms of vitamin B6 are: pyridoxine, pyridoxal, pyridoxamine and their phosphorylated derivatives pyridoxine 5′-phosphate, pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate (Ueland et al., 2015).

Vitamin B6 is mainly present in our diet as pyridoxine, pyridoxal and pyridoxamine. After passive absorption in the intestine, the major part of the vitamin is converted in the liver to pyridoxal-phosphate (Merrill et al., 1978; Zuo et al., 2015). After hydrolysis to pyridoxal by alkaline phosphatases, pyridoxal-phosphate becomes available for

every cell in our body. Within the cells, pyridoxal is phosphorylated by pyridoxal kinase to pyridoxal-phosphate (Albersen et al., 2015). Most of the pyridoxal in excess of tissue requirements is oxidized by the liver to 4-pyridoxic acid (4-PA), which is the major degradation product of vitamin B6 in the urine. The metabolic routes of vitamin B6 are shown in Fig. 1.

Vitamin B6 deficiency may arise from a too low intake, malabsorption, or due to drugs that inhibit enzymes involved in pyridoxal-phosphate metabolism. Low levels of vitamin B6 are found in elderly, individuals with traumatic femoral fractures, and in alcoholics (di Salvo et al., 2012). Vitamin B6 deficiency has been related to increased risk of cardiovascular diseases and polyneuropathy, which is the general term for damage to peripheral nerves, causing symptoms as weakness, numbness and pain in the extremeties (Spinneker et al., 2007). In these cases, vitamin B6 supplements are advised (Kashanian et al., 2007; Wyatt et al., 1999; Bendich, 2000). In the US, about 28%–36% of the general population uses supplements containing vitamin B6 (Bailey
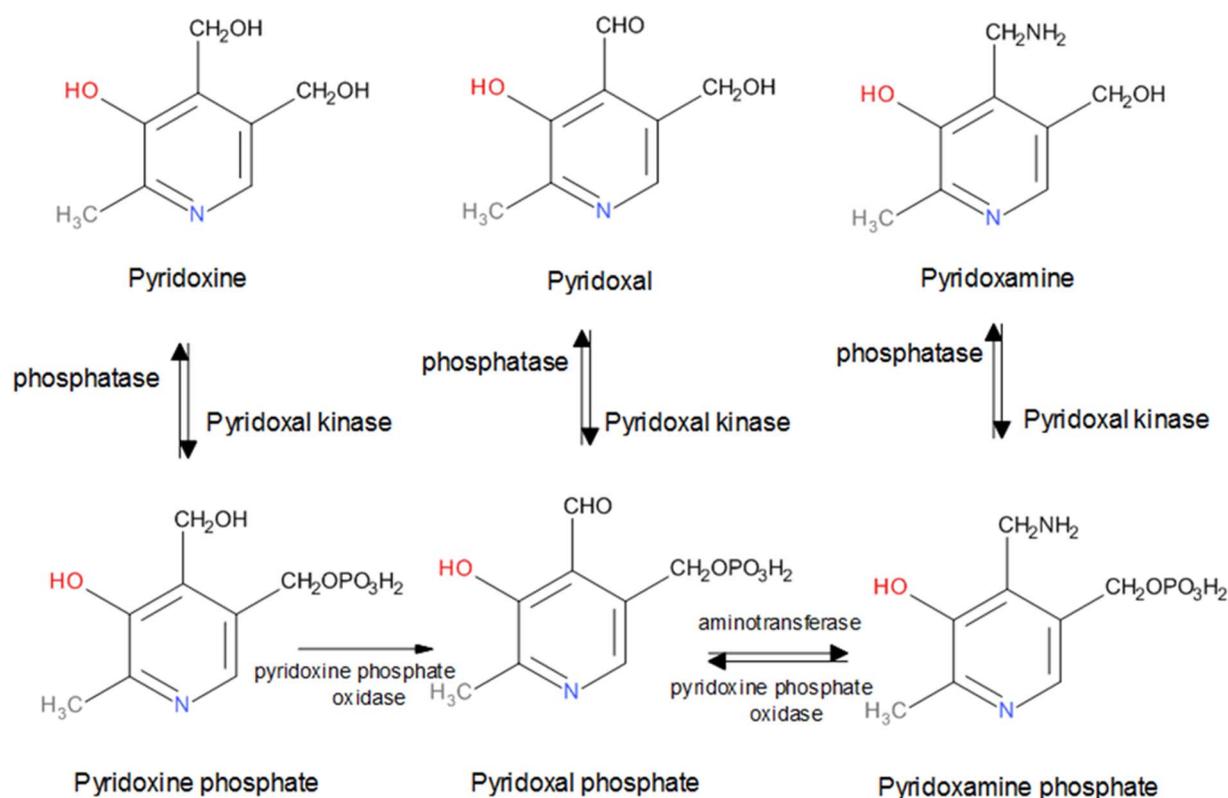
Fig. 1. Vitamin B6 metabolism.

et al., 2011; Morris et al., 2008). These supplements are most often taken by children up to 6 years and by adults over 51 years of age. Most vitamin B6 supplements contain pyridoxine ranging from 25 to 100 mg/tablet.

Recently, the European Food Safety Authority (EFSA) has established an upper limit (UL) of 25 mg/day. This UL was based on neurological complaints observed after taking 50 mg of pyridoxine per day. This is 4 times lower than the previous UL of 100 mg/day of USDA and other authorities. However, since vitamin B6 is considered to be relatively safe, doses of over 2000-fold the recommended dietary allowance of 1.4–2.1 mg/day (depending on sex and age) are used in some circumstances (Simpson et al., 2011).

Paradoxically, supplementation of vitamin B6 has also been shown to lead to polyneuropathy. Already in 1987, a case of polyneuropathy after supplementation of a high dose of vitamin B6 was reported (Schaumburg et al., 1983). In the years that followed, more cases on vitamin B6 toxicity were reported. Recently, The Netherlands Pharmacovigilance Centre Lareb, which collects and analyses reports of adverse reactions of medicines and vaccines, published a report concerning the side effects of vitamin B6 supplements. Since 2014, > 50 cases of sensory neuronal pain due to vitamin B6 were reported to Lareb (Lareb, 2015). The dose of vitamin B6 differed from 1.5–100 mg. In these cases, high plasma levels of pyridoxal-phosphate between 183 nM and 4338 nM were found. Plasma levels of pyridoxine were not determined. Up to now, the mechanism of this toxicity is enigmatic and the contribution of the various B6 vitamers to this toxicity is largely unknown. Our hypothesis is that pyridoxine is the vitamer mainly responsible for the vitamin B6 induced toxicity. Pyridoxine, which is not the active form, is ultimately converted into pyridoxal-phosphate, which is the active form. High levels of pyridoxine are thought to inhibit pyridoxal-phosphate -dependent enzymes by competing with the bioactive vitamer, pyridoxal-phosphate.

In the present study, the neurotoxicity of the different forms of vitamin B6 is tested on neuroblastoma cells (SHSY5Y). A second cell line (CaCo-2) was used to determine if the effects of vitamin B6 are specific

for neuronal cells. In line with our hypothesis, pyridoxine showed the highest toxicity. The paradox that the vitamin B6 pyridoxine vitamer in high concentrations inhibits vitamin B6 dependent processes was confirmed using vitamin B6 dependent enzymes tyrosine decarboxylase and alanine transaminase.

2. Materials and Methods

2.1. Chemicals

L-alanine, α-ketoglutarate, alanine transaminase, lactate dehydrogenase (LDH),NADH, pyridoxine, pyridoxal, pyridoxamine dihydrochloride, pyridoxal-phosphate hydrate, pyridoxamine -phosphate, Toluene, tyrosine decarboxylase apoenzyme (1.1 U/mg) from Streptococcus faecalis, L-tyrosine and picrylsulfonic acid were all obtained from Sigma (St. Louis, USA).

2.2. Tyrosine decarboxylase enzyme activity

Tyrosine decarboxylase activity was measured according to the tyrosine decarboxylase enzyme activity assay. In this assay, tyrosine is converted into tyramine by the enzyme tyrosine decarboxylase and the co-enzyme pyridoxal-phosphate. At first, tyrosine (0–100 μM) was added to a tube containing tyrosine decarboxylase (1.1 U/mL) with pyridoxal-phosphate and acetate buffer (pH 5.5). This mixture was incubated for 10 min at 37 °C. Then, 1 mL of K_2CO_3 (1 M) was added to the tube, which was followed by the addition of 1 mL of TNBS (10 mM). Finally, 2 mL of toluene was added, after which the tube was vortexed for 20 s. After centrifugation for 5 min at 2000 RPM, the enzyme activity was determined by measuring the absorbance at 340 nm using a spectrophotometer. In order to determine the effect of the different forms of vitamin B6, the enzyme was first incubated together with the vitamer of vitamin B6 (at 5 μM) for 2 min. Then, the same protocol was followed as described above.

2.3. Alanine transaminase activity

Alanine transaminase activity was measured according to the alanine transaminase activity assay. In short, alanine transaminase catalyzes the transfer of an aminogroup from alanine to α-ketoglutarate using pyridoxal-phosphate as a coenzyme. Glutamate and pyruvate are the products that are formed in this reaction. The formation of pyruvate is then spectrophotometrically followed during its conversion to lactate by LDH in the presence of NADH. In this reaction, NAD is formed which is monitored at 340 nm. After adding alanine transaminase (1 U/mL) to the reaction tube containing alanine (0.5 mM), α-ketoglutarate (15 mM), LDH (1.25 U/mL), NADH (0.18 mM) and Tris buffer (100 mM; pH 7.5), the reaction was started and followed during 5 min. The effect of pyridoxine on this enzymatic reaction was tested by following the same protocol as described above including the addition of 5 μM pyridoxine in the reaction tube.

2.4. Cell culture

Human neuroblastoma (SHSY5Y) cells were cultured in $75 \, cm^3$ cell culture flasks (Corning® Flask, Corning Incorporate, NY, USA) in DMEM:F122 (Invitrogen), supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen), 1% L-glutamine and 1% penicillin streptomycin (P/S; Invitrogen) at 37 °C in a 5% CO_2 and 20% O_2 atmosphere. Media was renewed twice weekly and cultures were split when 90% confluence was reached. Cells were differentiated by exposing them to 3% medium containing 10 μM retinoic acid for 5 days. Differentiation medium consisted of 3% growing medium (which was prepared by using complete growth medium and serum-free medium) and 10 μM retinoic acid. After differentiation, cells were seeded 1 day prior to treatment and maintained at 37 °C in a 5% CO_2 atmosphere. CaCo-2 cells were cultured in $75 \, cm^3$ cell culture under standard conditions. Dulbecco's modified Eagle medium (DMEM) with the addition of 10% FBS, 1% of non-essential amino acids, 1% Na-pyruvate and 1% of penicillin–-streptomycin.

2.5. Cell treatment

For the cell viability assays, both differentiated SHSY5Y and CaCo-2 cells were seeded in 96-wells plates at a density of 25.000 cells/well. After one day, cells were exposed to pyridoxine (0–5 μM), pyridoxamine (0–500 μM), pyridoxal (0–500 μM), pyridoxal-phosphate (0–500 μM) or pyridoxamine-phosphate (0–500 μM) for 24 h in normal culture medium. In order to determine if pyridoxal-phosphate could inhibit pyridoxine-induced toxicity, all cells were exposed to 1 μM of pyridoxine and an increasing concentration of pyridoxal-phosphate (0–100 uM). Untreated cells were used as controls in all experiments. For gene expression experiments, both SHSY5Y and CaCo-2 cells were seeded in 6-wells plate at a density of 500.000 cells/well After 24 h, cells were exposed to pyridoxine (0–1 μM), pyridoxamine (0–50 μM), pyridoxal (0–50 μM), pyridoxal-phosphate (0–50 μM) or pyridoxamine-phosphate (0–50 μM) for 24 h. Treatment of the cells with Triton-X100 was used as a positive control for cell death.

2.6. MTT assay

After the 24 h incubation, medium was removed and cells were washed with PBS. Then, 200 μl of MTT solution (0.5 mg/mL) was added and cells were incubated for 1 h at 37 °C. After this incubation, the wells were washed with PBS, after which the formazan crystals were dissolved in 200 μl DMSO. Cells were incubated for 30 min after which the absorbance at 540 nm was measured spectrophotometrically using a microplate reader. Relative cell viability is expressed as a percentage relative to untreated cells.

2.7. RNA isolation

After removing the medium, 500 μl of Qiazol was added to each well. After incubation of 5 min at room temperature, the suspension was transferred into an Eppendorf tube. Next, 100 μl of chloroform was added in the tube. The tube was vigorously shaken for 15 s. Then, samples were centrifuged for 10 min at 12,000 RPM (4 °C). From this point, samples were held on ice. The aqueous phase was transferred to a new tube without disturbing the interphase. To this phase, 250 μl of isopropanol was added and the samples were incubated for 10 min at room temperature. Next, samples were centrifuged for 10 min at 12,000 RPM at 4 °C, after which the supernatant was poured out. Pellets were washed twice by adding 500 μl of 70% ethanol, mixing the samples and centrifuging them for 3 min at 12,000 RPM at 4 °C, respectively. After pouring of the supernatant, pellets were washed one last time with 500 μl of 100% ethanol. Samples were centrifuged for 5 min and supernatant was poured out. After air drying the pellets, RNA was dissolved in 30 μl RNase free water by passing through pipette tip several times. The quality and quantity of RNA was determined using the nanodrop (Thermo Scientific Nanodrop 1000 spectrophotometer, Isogen Life Science, De Meern, The Netherlands).

2.8. PCR

One microgram of RNA was converted into complementary DNA (cDNA) by using iScript cDNA synthesis kit (Biorad, Veenendaal, The Netherlands). Sensimix SYBR & Fluorescein kit (Bioline, Alphen aan de Rijn, The Netherlands) was used to perform quantitative RT-PCR measuring BAX (FW: 5′-GAGAGGTCTTTTTCCGAGTGG-3'and RV: 5′-CCTTGAGCACCAGTTTGCTG-3′) and caspase-8 (FW: 5′-CATCCA GTCACTTTGCCAGA-3′ and RV: 5′-GCATCTGTTTCCCCATGTTT-3′). GAPDH (FW: 5′-CGCTCTCTGCTCCTCCTGTT-3′ and RV: 5′-CCATGGTGTCTGAGCGATGT-3′) was applied as housekeeping gene. Finally, the $2 - \Delta\Delta CT$ method was used to determine relative gene expression.

2.9. Statistical analysis

All results were shown as mean ± SEM. Data were analysed compared to (untreated) controls using an unpaired *t*-test. Results were considered statistically significant when $P < 0.05$, in which *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

3. Results

3.1. Cell viability after exposure to the vitamin B6 vitamers

MTT assays were performed to measure vitamin B6-induced cytotoxicity in SHSY5Y cells after 24 h incubation. Of the vitamers, pyridoxine induced a significant amount of cell death at 5 μM (Fig. 2). At this concentration, the other vitamers (pyridoxamine, pyridoxal and their phosphorylated forms) did not increase cell death. Pyridoxine was found to significantly increase cell death also in lower concentrations (Fig. 2 insert). Triton-X100 was used as a positive control.

Already at a concentration of 200 nM, cell death increased to 17%. After doubling the concentration to 400 nM, cell death increased to 30%. Only at very high concentrations (up to 500 μM), pyridoxal-phosphate tended to increase cell death (data not shown) although this did not reach statistical significance. In CaCo-2-cells, pyridoxine in various concentrations did not affect the cell viability after 24 h (Supplementary data Fig. 1).

3.2. Expression of apoptosis genes after exposure to different vitamers of vitamin B6

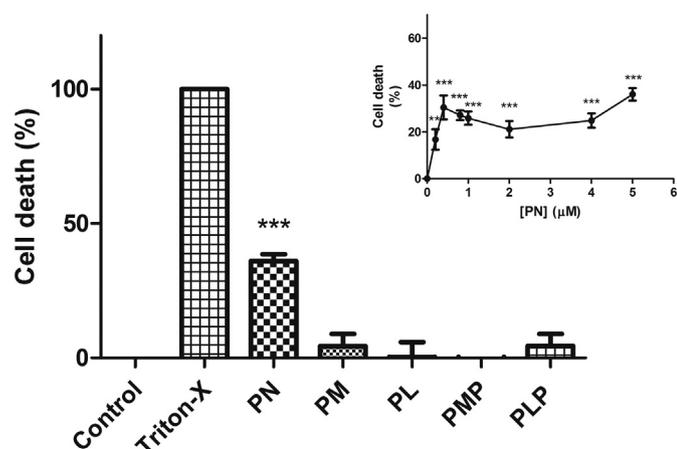Gene expression levels of two apoptosis genes (BAX and Caspase-8)

Fig. 2. Cell viability of SHSY5Y cells in the presence of the 5 different vitamin B6 vitamers at a concentration of 5 μM was measured with the MTT assay. Cells were exposed to these vitamers (pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), pyridoxamine-phosphate (PMP) and pyridoxal-phosphate (PLP)) for 24 h. Untreated cells were used as controls and results obtained with the vitamin B6 vitamers were compared to controls (which was set at 0%). Triton-X100 was used as a positive control. The effect of pyridoxine (PN) was also determined in a concentration-dependent manner (0–5 μM), which is shown in Fig. 1 insert. Data are shown as means ± SEM ($n = 4$). Differences were considered to be statistically significant when $P < 0.05$. **$P < 0.01$; ***$P < 0.001$.

were measured in SHSY5Y and CaCo-2 cells after incubation with the different vitamers. Untreated cells were used as control condition, which was set to 1. In SHSY5Y cells, pyridoxine increased the gene expression levels of BAX and caspase-8 in a concentration-dependent way (Fig. 3A and B). At a concentration of 1 μM, the increase in expression of BAX and caspase-8 was significant. The expression of the two genes was not changed by pyridoxal, pyridoxamine, pyridoxal-phosphate and pyridoxamine-phosphate at a concentration of 10 μM (Fig. 3C and D). Even at 50 μM of pyridoxal, pyridoxamine, pyridoxal-phosphate and pyridoxamine-phosphate, the expression of the apoptosis genes remained unchanged (Supplementary data Fig. 2).

In contrast to SHSY5Y cells, pyridoxine did not change the expression of BAX and caspase-8 in CaCo-2 cells (Fig. 4). In these cells, the different vitamers of vitamin B6 (at 10 μM) did not change the expression of BAX and caspase-8. Even at 50 μM, the expression of these genes was not changed (Supplementary data Fig. 3).

3.3. Protection against pyridoxine toxicity by pyridoxal-phosphate

In order to determine whether pyridoxine toxicity might be caused by competing with the bioactive vitamer pyridoxal-phosphate, SHSY5Y cells were exposed to both pyridoxine and pyridoxal-phosphate for 24 h and cell viability was measured using the MTT assay. Pyridoxine (1 μM) significantly increased cell death (Fig. 5). Pyridoxal-phosphate already at a concentration of 0.3 μM protected against cell death induced by 1 μM pyridoxine, indicating that cell death is dependent on competition of pyridoxine with pyridoxal-phosphate.

3.4. Pyridoxal-phosphate -dependent enzyme activities

The effect of the different vitamers (at 5 μM) on the enzyme activity of tyrosine decarboxylase was determined (Fig. 6A). At a tyrosine concentration of 10 μM, pyridoxamine-phosphate did not show any effect on the enzyme activity. Pyridoxamine only slightly decreased the enzyme activity, while pyridoxal and pyridoxal-phosphate even tended to increase the enzyme activity. Interestingly, pyridoxine decreased the enzyme activity with approximately 65%. A tenfold Increase in the substrate concentration of tyrosine reduced the inhibitory effect of pyridoxine, suggesting that the inhibition by pyridoxine is competitive. Besides tyrosine decarboxylase, pyridoxine (at 5 μM) also inhibited the

activity of alanine transaminase by 40% (Fig. 6B).

4. Discussion

In the last decades, various cases of vitamin B6 induced polyneuropathy have been reported (Schaumburg et al., 1983; Morra et al., 1993; Dalton and Dalton, 1987). People chronically taking vitamin B6 supplements reported complaints such as pain in the extremities and muscle weakness. These complaints were reversible as they faded away after stopping the supplementation. Most often, these complaints were seen when taking mega doses (> 50 mg/day) of vitamin B6 for a longer period of time. However, recently, Lareb reported cases in which lower doses (2 mg/day) of vitamin B6 gave the same complaints (Lareb, 2015). Up to now, the mechanism of vitamin B6 induced polyneuropathy is still unknown, making it difficult to give well substantiated advices.

Six different vitamers of vitamin B6 exist, namely pyridoxine, pyridoxal, pyridoxamine and their phosphorylated derivatives pyridoxine-phosphate, pyridoxal-phosphate and pyridoxamine-phosphate. Pyridoxal-phosphate is the most active form of vitamin B6. Pyridoxal-phosphate is a well-known coenzyme in a wide variety of enzymatic reactions. Of these vitamers, pyridoxine is most commonly taken as a food supplement. Since vitamin B6 is considered to be safe, supplements with high doses of pyridoxine are on the market. Once taken up, pyridoxine is converted into pyridoxal-phosphate in several steps, of which phosphorylation by pyridoxal kinase to pyridoxal-phosphate is the most critical one. All the supplements that caused adverse effects contained pyridoxine. Therefore, the focus was on pyridoxine. We hypothesized that pyridoxine is the vitamer responsible for the neurotoxic effects, by competing with the active form pyridoxal-phosphate.

Our study indeed showed that pyridoxine significantly increases cell death in the neuronal cell line SHSY5Y after a 24 h exposure. At high concentrations, the concentrations at some points failed to give the expected increases in toxicity. Nevertheless, at relative low concentrations up to 400 nM, which corresponds to the concentrations found in vivo, the toxic effects are significant. These concentrations were selected based on plasma levels that were found in several studies (Ubbink et al., 1987; Bisp et al., 2002; van der Ham et al., 2012). In contrast, the other vitamers did not affect cell viability. Additionally, pyridoxal-phosphate was able to prevent pyridoxine induced cell death, indicating that pyridoxine toxicity is caused by competition with pyridoxal-phosphate, which is in line with our hypothesis. The toxic effects of pyridoxine furthermore seem to be neuronal specific, since pyridoxine did not induce cell death in the intestinal cell line CaCo-2. In diabetes neuronal cell death is the major cause of polyneuropathy indicating that this is also involved in the neuropathy observed in vitamin B6 toxicity (Vincent et al., 2002). Besides cell death, axonopathy also causes polyneuropathy. Axonopathy is probably also involved in vitamin B6 toxicity as high levels of vitamin B6 have also been linked to demyelination of nerves (Castagnet et al., 2010). Demyelination is a more subtle toxic effect meaning that this toxicity of vitamin B6 is expected to occur at even lower concentrations than the concentrations that were needed for cell death. Together with the demyelinating effects, this could lead to polyneuropathy.

Remarkably, neuropathy is seen in vitamin B6 toxicity as well as vitamin B6 deficiency. In the present study, the effect of the inactive pyridoxine on the active pyridoxal-phosphate was tested with two enzymes involving pyridoxal-phosphate as a coenzyme, namely tyrosine decarboxylase and alanine aminotransferase. Tyrosine decarboxylase catalyzes the decarboxylation of tyrosine to produce tyramine. Alanine aminotransferase catalyzes the transfer of the amino group of L-alanine to α-ketoglutarate. The activity of both enzymes was shown to be competitively inhibited by pyridoxine. The other vitamers either increased the enzyme activities or did not affect the enzyme activities. It was concluded that pyridoxine indeed competes with the active pyridoxal-phosphate resulting in the inhibition of vitamin B6 dependent
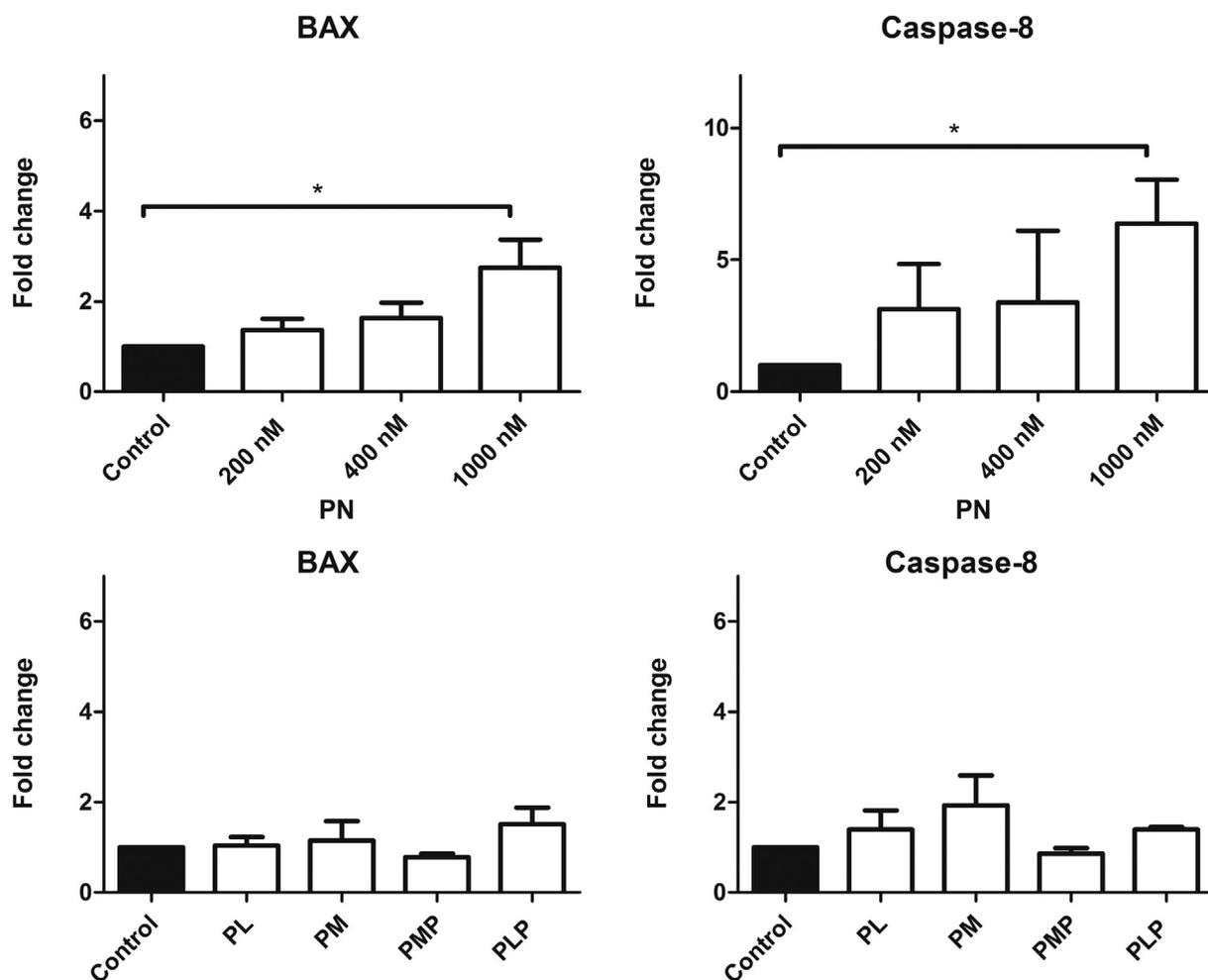
Fig. 3. Gene expression data of two apoptosis genes (BAX and Caspase-8) after exposure of SHSY5Y cells to the 5 different vitamin B6 vitamers (pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), pyridoxamine-phosphate (PMP) and pyridoxal-phosphate (PLP)). The effect of pyridoxine (PN; 0.2–1 μM) on the expression of BAX and caspase-8 is shown in panels (A) and (B). The effects of the other vitamers at a concentration of 10 μM are shown in panels (C) and (D). Untreated cells were used as controls and results obtained with the vitamin B6 vitamers were compared to controls (which was set at 1). Data are shown as means ± SEM ($n = 3$). Differences were considered to be statistically significant when $P < 0.05$. *$P < 0.05$.

enzymes. In vitamin B6 deficiency, enzymes depending on pyridoxal-phosphate (the bioactive vitamer) as a cofactor will be (partially) inhibited. Vitamin B6 supplementation mostly concerns pyridoxine, which is converted by pyridoxal kinase and pyridoxine phosphate oxidase into pyridoxal-phosphate. At high doses, pyridoxine saturates these enzymes. As a consequence, the inactive vitamer pyridoxine accumulates. Indeed, high levels of pyridoxine (up to 18 μM) have been found in the cerebrospinal fluid (CSF) and (up to 200 nM) in plasma of two persons taking pyridoxine supplements (Ubbink et al., 1987; Bisp et al., 2002; van der Ham et al., 2012). 200 nM of pyridoxine already induced cell death in our study, furthermore underlining the relevance of our findings. At such high concentration, pyridoxine effectively competes with pyridoxal-phosphate resulting in the inhibition of vitamin B6 dependent enzymes. Hence, this explains that these effects will be paradoxically similar to that of a deficiency in vitamin B6.

One of the pathways in which pyridoxal-phosphate is essential is the kynurenine pathway (Ueland et al., 2015; Schwarcz, 2004; Midttun et al., 2011). In this pathway, the amino acid tryptophan is metabolized to nicotinamide. Intermediate products formed are quinolinic acid, kynurenine, 3-hydroxykynurenine (3 − HK) and 3-hydroxyanthranilic acid (3-HAA) (Ueland et al., 2015). 3-HK is converted into xanthurenic acid and 3-HAA by two pyridoxal-phosphate -dependent enzymes. 3-HK is a known neurotoxic compound which is related with central nervous system diseases (Schwarz et al., 2013). The kynurenine pathway has been linked to several central nervous system diseases, such as

Huntington's disease and Alzheimer's disease (Stoy et al., 2005; Bonda et al., 2010). Significantly increased 3-HK levels were found in brain tissues of Huntington's disease patients.

It has previously been shown that exposure of SHSY5Y cells to relatively high levels of 3-HK increased cell death (Jeong et al., 2004). Addition of 3-HK to striatal neuronal cell cultures caused increased cell death via apoptosis (Smith et al., 2007). In this process, hydrogen peroxide and hydroxyl radical formation play an important role, since the antioxidant enzyme catalase reduced 3-HK induced cell death. In the present study, the expression of Bax and caspase-8 was used to monitor apoptosis in the neuronal cell line. Of the vitamers, only pyridoxine increased the expression of Bax and caspase-8. This suggests that accumulation of 3-HK, caused by inhibition of the pyridoxal-phosphate dependent metabolism of 3-HK by pyridoxine may play a role in the polyneuropathy observed during vitamin B6 toxicity.

Our results indicate that vitamin B6 could lead to polyneuropathy by inducing cell death after a 24 h exposure. Although cell death is shown to contribute to polyneuropathy in diabetic patients, it would be interesting to include more subtle endpoints, such as impairment of neuronal functioning and neuronal length of for instance human iPSC-derived sensory neurons, which are also relevant for sensory neuronal pain. Moreover, we studied a relatively short exposure while vitamin B6 is used chronically. Due to these limitations, despite the clear and prominent toxicity observed, our study will probably underestimate vitamin B6 toxicity.
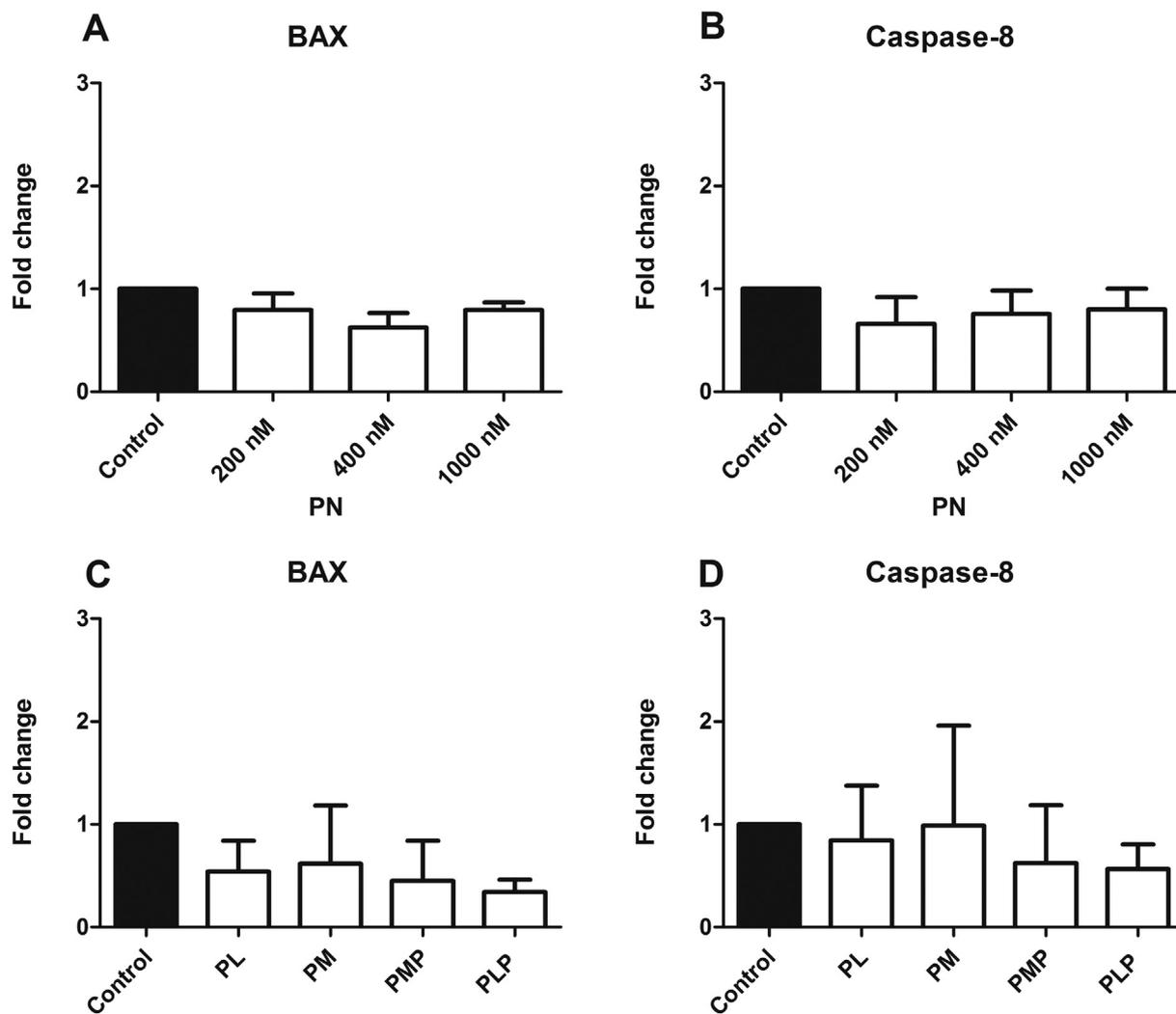
Fig. 4. Gene expression data of two apoptosis genes (BAX and Caspase-8) after exposure of CaCo-2 cells to the 5 different vitamin B6 vitamers (pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), pyridoxamine-phosphate (PMP) and pyridoxal-phosphate (PLP)). The effect of pyridoxine (PN; 0.2–1 μM) on the expression of BAX and caspase-8 is shown in panels (A) and (B). The effects of the other vitamers at a concentration of 10 μM are shown in panels (C) and (D). Untreated cells were used as controls and results obtained with the vitamin B6 vitamers were compared to controls (which was set at 1). Data are shown as means ± SEM ($n = 3$).
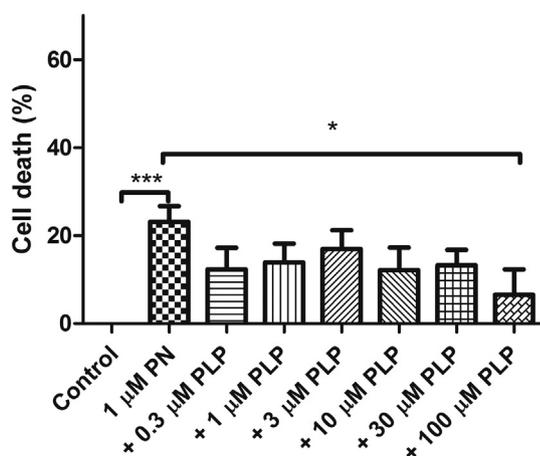

Fig. 5. Cell viability of SHSY5Y cells in the presence of was measured by using the MTT assay. Cells were exposed to these 1 μ of pyridoxine (PN) together with increasing concentrations of pyridoxal-phosphate (PLP) (0–100 μM) for 24 h. Untreated cells were used as controls and results obtained were compared to controls (which was set at 0%). Data are shown as means ± SEM ($n = 4$). Differences were considered to be statistically significant when $P < 0.05$. *$P < 0.05$; ***$P < 0.001$.

In conclusion, the present study strongly indicates that the neuropathy observed after taking a relatively high dose of vitamin B6 supplements is due to the vitamer that is used in the supplements, namely pyridoxine. The inactive form pyridoxine competitively inhibits the active pyridoxal-phosphate. As a consequence, the paradox arises that the symptoms of vitamin B6 supplementation are similar to those of vitamin B6 deficiency. Vitamin B6 supplements are used by a large number of people. The safety of vitamin B6 is debated and recently EFSA has lowered the upper limit for vitamin B6. The question is whether lowering the safe dose for vitamin B6 is the solution. Remarkably, even at relatively low dose, vitamin B6 supplementation has given rise to complaints. Our study indicates that the toxicity of vitamin B6 is not only determined by the dose, but by the vitamer in which it is taken. Perhaps it might be better to replace pyridoxine by pyridoxal or pyridoxal-phosphate as vitamin B6 supplements, which are much less toxic. In this way, the vitamin B6 paradox may potentially be prevented.
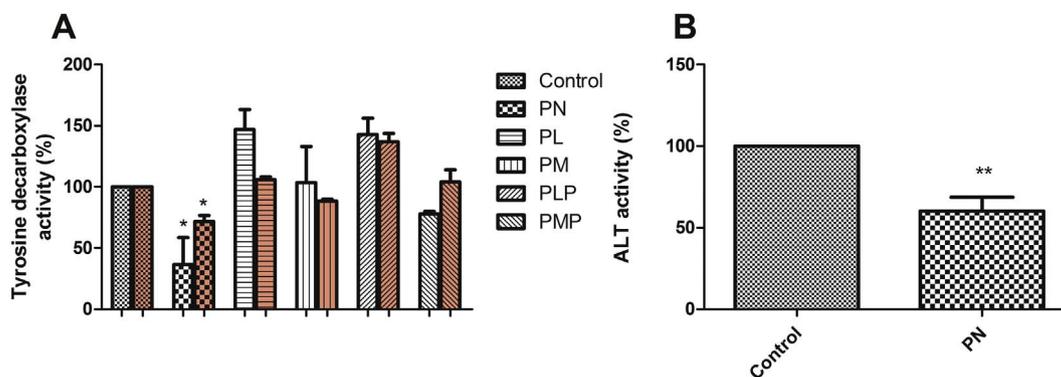
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Fig. 6. The effect of the different vitamin B6 vitamers (pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), pyridoxamine-phosphate (PMP) and pyridoxal-phosphate (PLP)) at a concentration of 5 μM at the pyridoxal-phosphate -dependent enzyme tyrosine decarboxylase using two different substrate concentrations (10 (white bars) and 100 μM (red bars)) is shown in (A). The effect of pyridoxine (PN; 5 μM) at a second pyridoxal-phosphate -dependent enzyme (alanine aminotransferase) is shown in (B). Data are shown as means ± SEM ($n = 3$). Differences were considered to be statistically significant when $P < 0.05$. *$P < 0.05$; **$P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tiv.2017.07.009.

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