Effects of excess vitamin B_6 intake on cerebral cortex neurons in rat: an ultrastructural study

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Abstract: The aim of this study was to investigate whether excess of vitamin B_6 leads to ultrastructural changes in cerebral cortex of forty-eight healthy albino rats which were included in the study. Saline solution was injected to to the control groups (CG-10, n=12 for 10 days; CG-15, n=12 for 15 days; CG-20, n=12 for 20 days). The three experimental groups (EG-10, n=12; EG-15, n=12; EG-20, n=12) were treated with 5 mg/kg vitamin B_6 daily for 10 days (EG-10), 15 days (EG-15) and 20 days (EG-20). Brain tissues were prepared by glutaraldehyde-osmium tetroxide double fixation for ultrastructural analysis. No significant changes were observed in the control groups. The ultrastructural analysis revealed that the numbers of damaged mitochondria, lipofuscin granules and vacuoles were significantly higher in all the experimental groups than in the control groups (p<0.05). However, synaptic density was significantly decreased in the experimental groups as compared to the control groups (p<0.05). The results suggest that the excess of vitamin B_6 intake causes damage to the cerebral cortex due to cellular intoxication and decreased synaptic density. Thus, careful attention should be paid to the time and dose of vitamin B_6 recommended for patients who are supplemented with this vitamin.

Key words: Vitamin B_6 - Cerebral cortex - Neuron - Rat - Ultrastructure

Introduction

It has been known that malnutrition causes developmental impairments in the central nervous system (CNS). Due to nutritional deficiencies in basic elements, cellular differentiation can not be completed. Cognitive dysfunction due to deficiency of some nutritive factors has been reported; including pellagra syndrome and depression, irritability, confusion and disorientation resulting from niacin deficiency, and Wernicke-Korsakoff syndrome resulting from thiamine deficiency. It has been reported that vitamin B_6 deficiency negatively affects brain development in rodents [26, 34], and causes changes in memory efficiency in rats [8, 18, 19].

Although the effects of malnutrition on CNS are well documented [26, 31], discussion on the basic mechanisms still continues [7, 12, 14, 25, 29, 33]. Neocortex cells showed ultrastructural abnormalities due to vitamin B_6 deficiency [27]. Moreover, there is evidence for accelerated aging of neurons. Partial dendritic loss of neurons and dysfunctions of the immune system are related to malnutrition status [5]. The structural changes associated with maternal vitamin B_6 deficiency have been reported in developing brain regions. Vitamin B_6 restriction during pregnancy periods has been suggested as a risk factor for synaptogenesis and neural differentiation [16, 17].

Previous studies indicated metabolic effects of high dietary intake of B_6 and revealed substrate-cofactor interaction between dietary histidine or tryptophan and B_6. Therefore, pyridoxine caused a clear interaction between substrate and coenzyme. The precursors influence brain metabolism of histamine and serotonin [23]. Growing evidence shows that the putative monoamine neurotransmitters, such as dopamine (DA), norepinephrine (NE), serotonin (5-HT) and gamma-aminobutyric acid (GABA) are formed through decarboxylation of precursor amino-acid derivatives. Pyridoxine plays an important role in the metabolism of neurotransmitters in the nervous system as a crucial enzymatic co-factor [20, 29].

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Experiments on excess of vitamin B_6 have been inadequate up to now. Many studies dealing with administration of different daily doses of excess vitamin B_6 have suggested that the excess of this vitamin affects the brain and serum concentrations of some amino acids and cortical serotonin receptors [3, 4, 24, 25, 30]. In a study of Xu *et al.* [37], it was shown that excess vitamin B_6 caused a neuropathy with necrosis of dorsal root ganglion sensory neurons, which was accompanied by the breakdown of peripheral and sensory axons. Moreover, it has been suggested that excess vitamin B_6 causes altered startle behavior in rats due to changes in the central nervous system [28].

The limited number of studies [3, 4, 8, 28, 29] on high dose vitamin B_6 administration led us to investigate the effects of excess vitamin B_6 on the fine structure of blood-brain barrier and brain cortex cells. Therefore, the aim of this study was to investigate whether high dose vitamin B_6 affects the ultrastructure of the brain motor cortex neurons, and to compare the control and experimental groups in a time-dependent manner.

Materials and methods

Experiments. The study protocol was approved by the Akdeniz University Animal Care Center. Since there is a difference in nerve conduction velocity between males and females [9] we only used healthy male albino rats; obtained from the animal care center of Medical Faculty of Akdeniz University. Animals were housed in groups, 4 rats per group, in stainless steel cages under standard condition (24±2°C and 50±5% humidity) with a 12 h light-dark cycle [22]. Twelve rats were used for each of three control groups (CG-10, CG-15, CG-20; total=36) and for each of three experimental groups (EG-10, EG-15, EG-20, total=36). Thus, 72 Swiss Albino rats weighing 200-250 g were used for the study. The National Research Council (NRC) recommends a dose of 7 mg/kg diet for vitamin B_6, dissolved in physiological saline solution but higher doses were administered in previous studies [28-30]. The 5 mg/kg daily dose chosen in this study is similar to that applied in previous studies [2, 32]. Vitamin B_6 at a dose of 5 mg/kg/day was injected intraperitoneally to the experimental groups (EG-10, EG-15 and EG-20 for 10, 15 and 20 days, respectively). Physiological saline solution was injected for 10, 15 and 20 days to the control groups, CG-10, CG-15, CG-20, respectively. Treatments of all groups, including daily doses and time periods are summarized in Table 1.

Ultrastructural analysis. Following cardiac puncture, the aorta was catheterized and the central nervous system was perfused with phosphate buffered 2.5% glutaraldehyde (pH 7.4; 0.08 M). The glutaraldehyde-osmium tetroxide double fixation method was applied [1] in order to examine the perfused tissue samples using transmission electron microscopy (TEM). The tissue samples were fixed in 1% osmium tetroxide solution, prepared in phosphate buffered (pH 7.4) isotonic solution at 4°C for one hour. Following dehydration they were embedded in Vestopal. The semithin (1 μm) and thin sections (40-60 nm) were cut by an LKB III ultramicrotome. Thin sections were contrasted with uranyl acetate (5 g uranyl acetate in 100 ml methanol) and Reynolds's lead citrate solution (1.76 g sodium citrate, 1.33 g lead nitrate, 50 ml distilled water and 8 ml NaOH) [10]. Semithin sections were stained with toluidine blue. The thin sections were examined under JEM 100 C and Leo 906 electron microscopes.

Table 1. Treatments of control (CG) and experimental (EG) groups of rats

Group	Treatment duration (days)	Daily i.p. injection
EG-10 (n=12)	10	5 mg/kg vitamin B_6
CG-10 (n=12)	10	5 mg/kg 0.9% NaCl
EG-15 (n=12)	15	5 mg/kg vitamin B_6
CG-15 (n=12)	15	5 mg/kg 0.9% NaCl
EG-20 (n=12)	20	5 mg/kg vitamin B_6
CG-20 (n=12)	20	5 mg/kg 0.9% NaCl

Quantitative analysis. For evaluation of the fine structure of cerebral cortex cells, transmission electron microscopic (TEM) micrographs were standardized under the same magnification (×2,500). Thirty-two electron micrographs (8 from CGs and from each EGs) were randomly selected, and evaluated for; (1) total numbers of perikaryons (26 pyramidal neurons from CG and from each EGs; total: for CG = 26; for EGs=78) and their subcellular components: (2) damaged mitochondria, (3) lipofuscin pigment granules, (4) unmyelinated axon sections, (5) vacuoles in neuropil, and (6) synapses in neuropil. Following the quantification, the electron micrographs were arranged according to experimental groups, respectively. The quantification method was applied according to our recent detailed publication [11].

Statistical analysis. The results of ultrastructural scoring were normally distributed (as tested by Kolmogorov-Smirnov test) throughout the experiment days. Analysis of variance (ANOVA) and the Tukey test were carried out for statistical analysis and pairwise multiple comparisons. Statistical calculations were performed using Sigmastat for Windows, version 2.0 (Jandel Scientific Corporation, San Rafael, CA, USA).

Results

By light microscopy, no significant differences were detected between groups CG-10, CG-15 and CG-20 when they were compared to each other. The morphological appearance of perikaryons and processes of the neurons in the cortical layers of motor areas of the brain were histologically normal. Electron microscopy revealed changes in the vitamin B_6-treated groups.

Control group

The architecture of nerve cells and fibers inthe motor cortex in the control group was normal. There were no abnormalities either in the perikaryons, axons and dendritic processes or in the neuropil with a very high synaptic density (Fig. 1a). All cell components were clearly visible. Euchromatic nucleus, active Golgi complex, and most Nissl bodies with granular endoplasmic reticulum cisternae (GER) were widespread as seen in the normal structure (Fig. 1a). The ultrastructure of the barrier between vessels and brain tissue was also in normal condition. Myelinated and unmyelinated axons
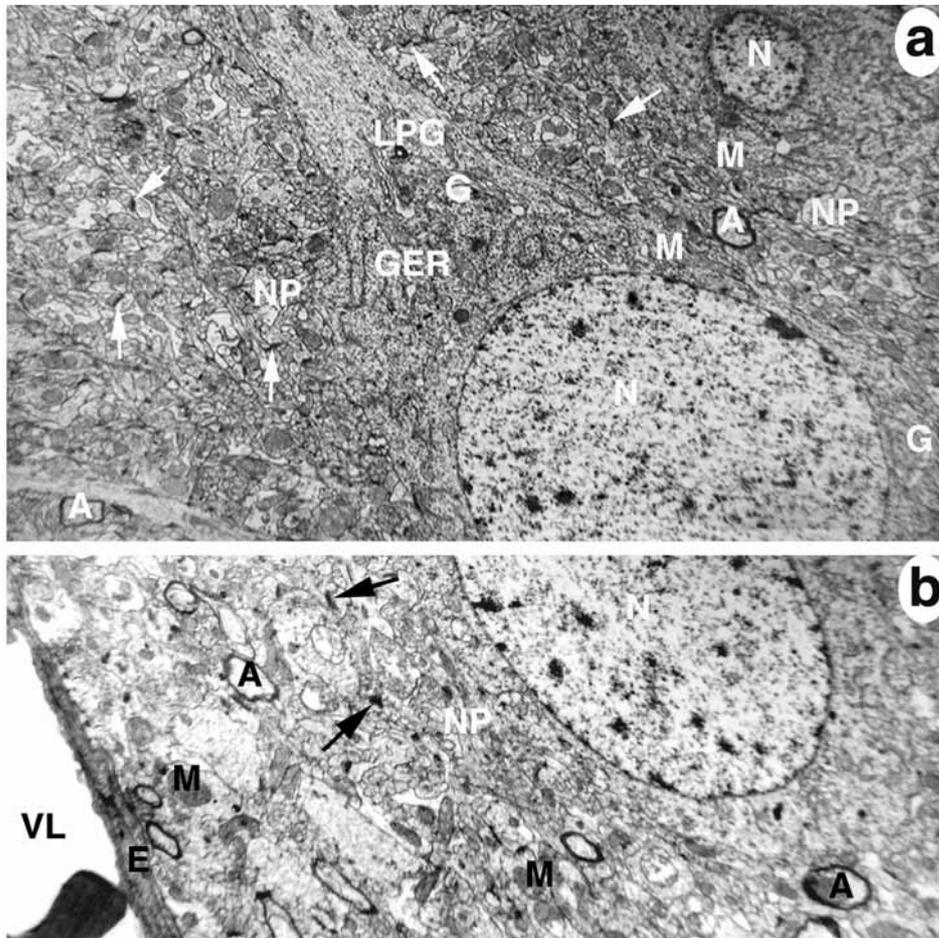
Fig. 1. (a) A perikaryon of pyramidal neuron with axonal hillock and euchromatic nucleus (N), and neuropil (NP) of rat from the control group. Subcellular components of perikaryon and neuropil with synaptic junctions (arrows) are clearly seen. Granular endoplasmic reticulum (GER), Golgi complex (G), mitochondria (M), lipofuscin granules (LPG), myelinated axon (A), and synaptic junctions (arrows) are shown. **(b)** The fine structure of blood-brain barrier of rat from the control group. Vascular lumen with endothelium (E) and perivascular membrane consisting of glial processes and basement membrane show normal organization. A perikaryon with euchromatic nucleus (N) and neuropil with myelinated and unmyelinated axons (A) are also seen. × 6 500.

and dendritic structures with many synaptic contacts were observed. Moreover, no cellular and/or neuropil damage was observed in perivascular areas (Fig. 1b).

Experimental group-10 (EG-10; 5 mg/kg vitamin B₆ administered for 10 days)

Neuron structure was generally normal. The most significant morphological features were the activity of nucleoli and nuclear shape deformation. Moreover, increased cytoplasmic density and vacuolization, dilated Golgi complex and partial loss of cristae in some mitochondria were observed (Fig. 2). Astrocytic projections had various dimensions and shapes. Interestingly , the unmyelinated axon sections showed smooth surfaced vesicles and vacuoles of different size and shape (Fig. 2). However, the myelinated axons were normal, with many synapses.

Experimental group-15 (EG-15; 5 mg/kg vitamin B₆ administered for 15 days)

In this group, the ultrastructural modifications were more evident in the motor cortex when compared to

those observed in EG-10. Neuronal and neuropil damage was a general feature in this experimental group. Shrunken heterochromatic nuclei with electron dense nucleoplasm and irregular nucleoli were observed. The structural abnormalities of the cytoplasm included irregular surfaces with cytoplasmic fragments, and damaged cell organelles (Fig. 3a). Excessive dilatation of the Golgi complexes and the presence of lipofuscin pigment granules as well as multivesicular bodies around Golgi complex regions were noticeable. The most interesting ultrastructural findings in this group were an increased degree of mitochondrial damage and excessively increased lipofuscin pigment granules in the neuroplasm (Fig. 3b). Although not widespread, local loosening of myelin sheaths was observed. Neuropil structures showed structural damage, atrophy of cellular processes, loosening of neuropil tissue architecture with many vacuoles, edematous areas and very rare synaptic junctions (Fig. 3b).

Experimental group-20 (EG-20; 5 mg/kg vitamin B₆ administered for 20 days)

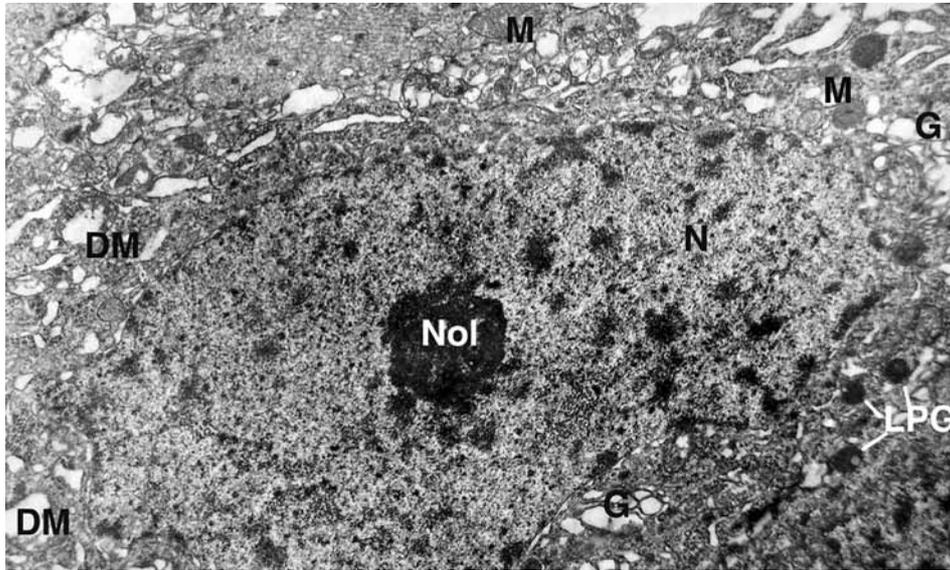The most prominent ultrastructural changes and tissue damage were observed in this experimental group. De-

Fig. 2. EG-10. A perikaryon with slightly deformed shape and heterochromatic nucleus (N) including very active nucleolus (Nol) is seen. The cytoplasm contains dilated Golgi complex (G), damaged mitochondria (DM), electron dense lipofuscin pigment granules (LPG), and widespread Nissl bodies. In neuropil, many vacuoles, very rare synaptic points, and myelinated as well as unmyelinated axon and dendrite sections are seen. × 12500.
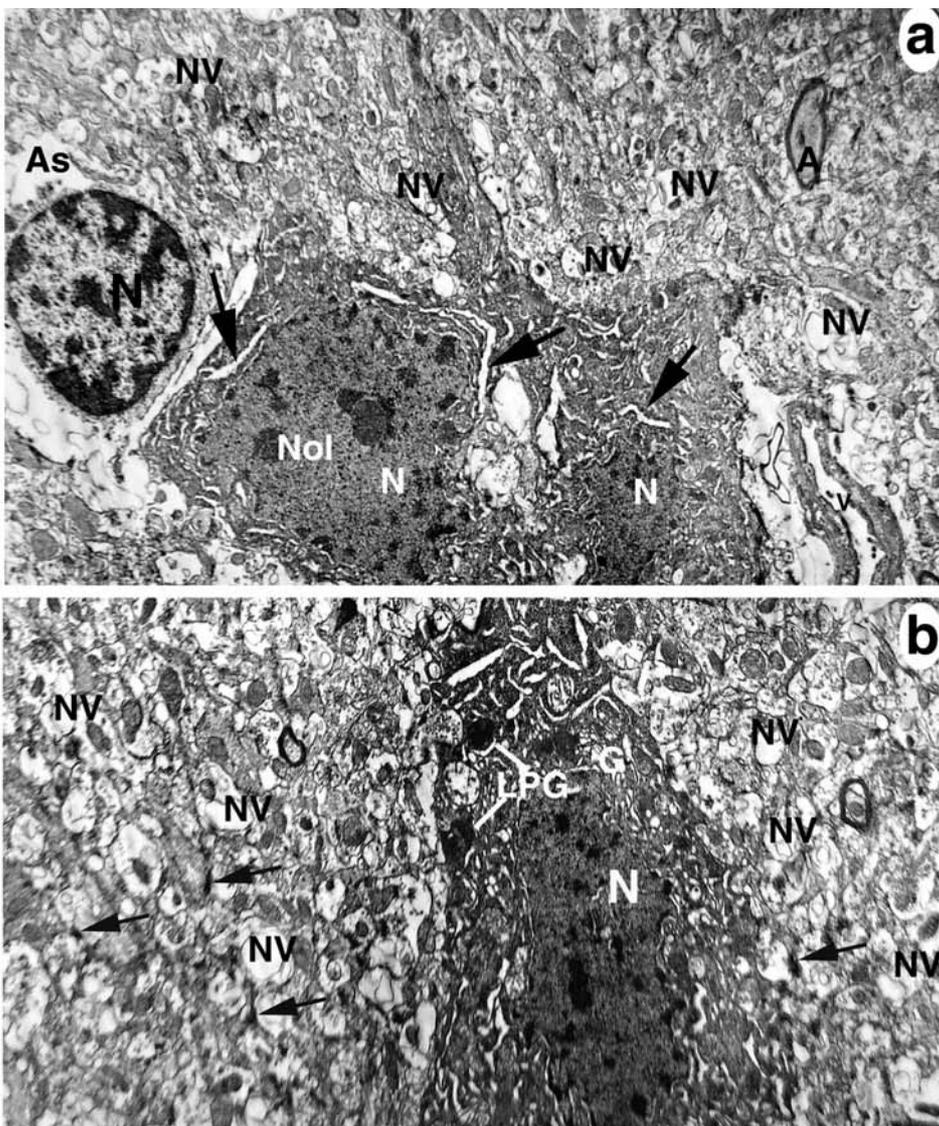

Fig. 3. EG-15. Two pyramidal neurons with axonal hillocks and astrocyte (As) are seen in this micrograph. **(a)** Cellular and neuropil damage is seen due to the effect of high-dose vitamin B_6 treatment; heterochromatic nuclei (N) with nucleoli (Nol) and electron dense cytoplasm with vacuoles, dilated cisternae (arrows) and many damaged cell organelles are seen. An edematous area around the vessel (V) can clearly be observed. **(b)** This electron micrograph shows typical neuronal and neuropil damage after long-term high-dose vitamin B_6 treatment. In neuropil, many neuropil vacuoles (NV) and edematous areas, very rare synaptic junctions (arrows) and myelinated as well as unmyelinated axons (A) are seen. As - astrocyte. a: × 6500; b: × 8500.
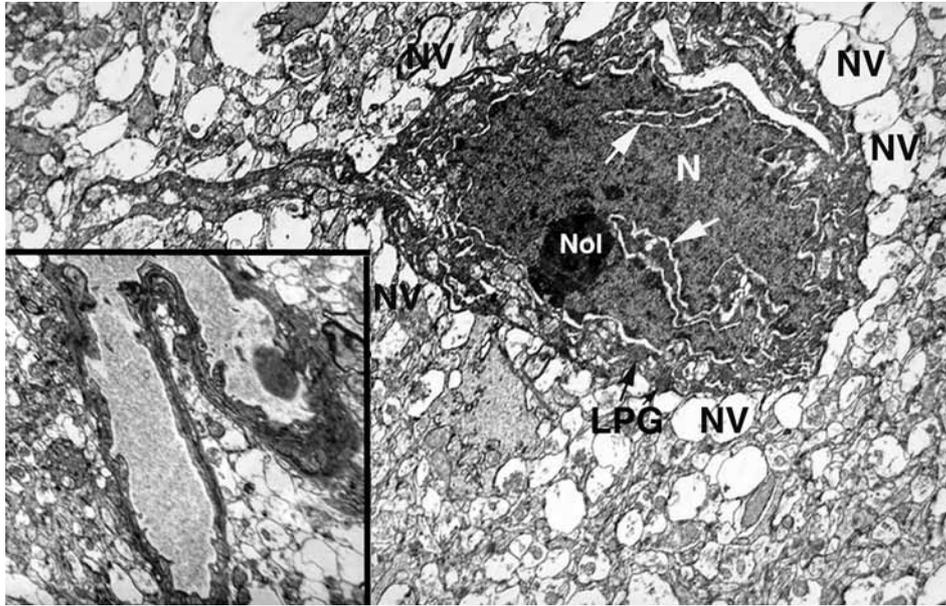
Fig. 4. EG-20. A typical damaged pyramidal neuron with irregular shape and deformed axonal hillock is seen in the cerebral cortex. Nucleus (N) with peripherally located nucleolus (Nol) shows lobulation and fragmentation (arrows), characteristic of nuclear pycnosis. Multimellar and vesicular bodies in electron dense cytoplasm containing damaged cell organelles and electron dense lipofuscin pigment granules (LPG) are seen. Note edematous areas with large neuropil vacuoles (NV) localized around the perikaryon, dispersed throughout the neuropil, and around the vessel (inset). × 6.500; inset × 8 .500.
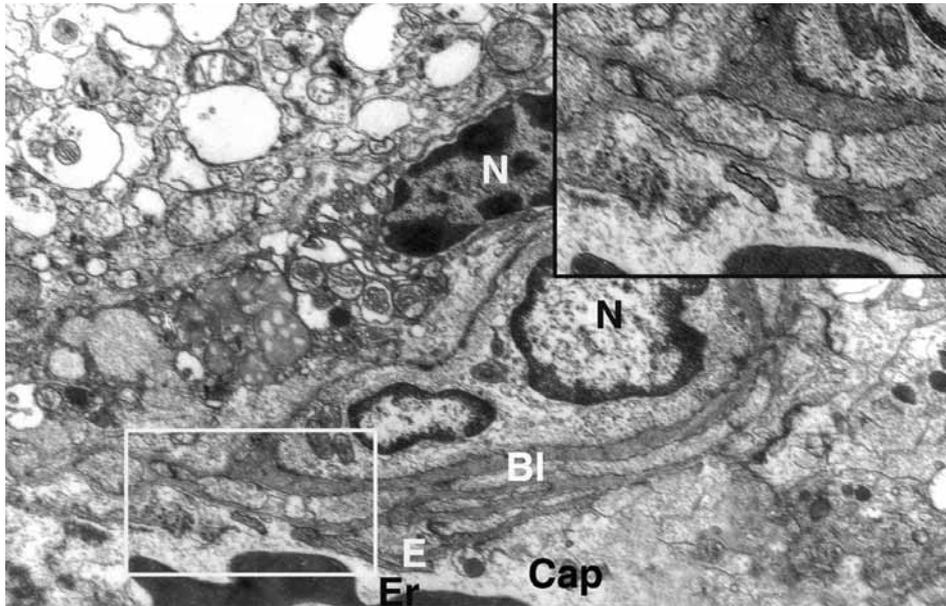

Fig. 5. EG-20. The damaged brain-blood barrier. Fragment of the capillary wall (Cap) with erythrocyte (Er) is shown in the inset. Note damaged capillary endothelium (E) and the underlying basal lamina (Bl). × 16500; inset × 33000.

formed pyramidal neuron structures with pycnotic nuclei showing lobulation and sometimes fragmentation, as well as damaged cell organelles were frequently seen. Increased numbers of vacuoles and vesicles, probably due to damage to mitochondria and other cell organelles in the perikaryons, excessive dilatation of the Golgi complex, regional hyperplasia of granular endoplasmic reticulum, frequently observed lysosomes, and lipofuscin pigment granules, were also significant findings (Fig. 4).

Mildly loosened neuropil with more vacuoles, multilamellar and vesicular bodies were observed. In addition to numerous edematous areas, the blood-brain barrier was also damaged and vessel layers showed shrinkage in all electron micrographs analyzed (Fig. 4). Under higher magnification of the blood-brain barrier, the damage in some areas of capillary endothelium and its basal lamina was clearly seen (Fig. 5). Numerous unmiyelinated axons with vacuolated and degenerated areas were also observed in neuropil (Fig. 6).

Quantitative analysis

Electron micrographs from experimental groups (EGs) and control groups (CGs) were evaluated quantitatively (Table 2). The number of damaged mitochondria and lipofuscin pigment granules in perikaryons of pyramidal neurons, as well as the number of vacuoles in neuropil were increased in parallel to vitamin B$_6$ treatment time. The number of synaptic contacts in neuropil was found
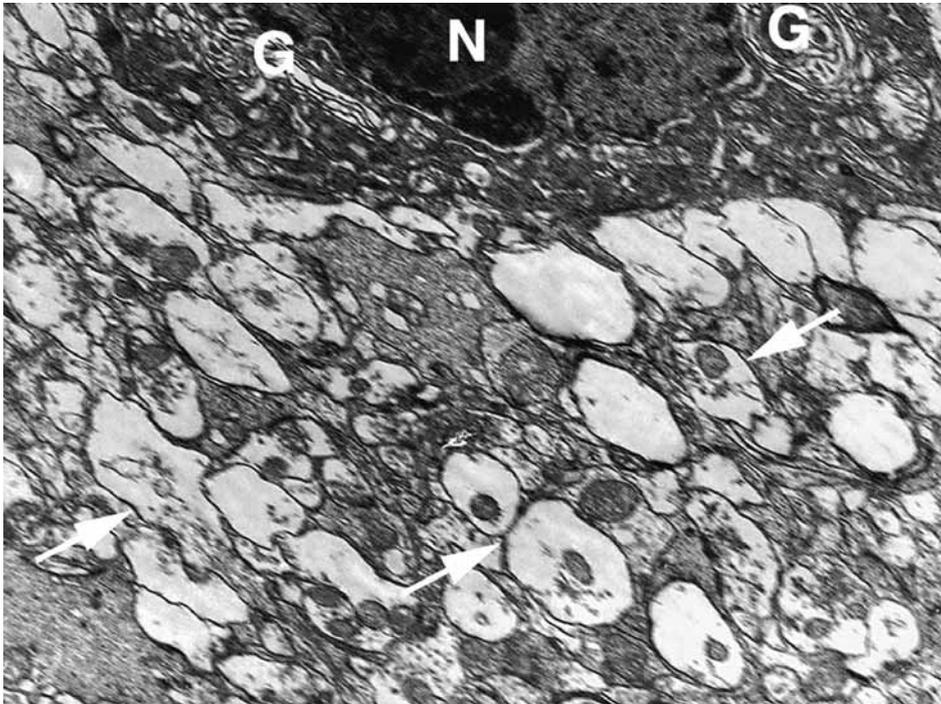
Fig. 6. EG-20. Increased number of unmyelinated axons (arrows) with smooth surfaced vesicles or vacuoles are seen. Note dilatation of Golgi apparatus (G) in the perikaryon and pycnotic nucleus (N). × 12500.

to be decreased when EGs were compared with controls and with each other (P<0.05, EG-10 *vs* EG-20 and EG-15 *vs* EG-20). Differences in these parameters were statistically significant (P<0.05). However, the number of unmyelinated axons in neuropil was similar in all the EGs. All the parameters examined were significantly altered in the EGs compared to the CGs, except for the unmyelinated axons.

Discussion

Brain cortex consists of typical cellular and fibrillar elements and specific extracellular matrix. The structural appearance of perikaryons and processes of the neurons were histologically normal under light microscope. Transmission electron microscopy revealed that some pyramidal cells of the cerebral cortex showed partial to nearly complete synaptic loss. These changes were more typical for the experimental group receiving excess vitamin B_6 intake for a long period (20 days) and, paradoxically, they are reminiscent of those resulting from vitamin B_6 deficiency in rats [27]. According to the results of this study, ultrastructural changes observed in the perikaryons and neuropil of animals treated with excess vitamin B_6 for short period were, however, less pronounced than those observed in vitamin B_6 deficiency [17, 20, 21, 27].

It has been suggested that severe dendritic loss and perikaryonal swelling at the apical and basal poles of the affected pyramidal neurons do not influence the neighboring neurons. In the excessive intake of vitamin B_6 experiments, it has been observed that neurons with long processes and large cytoplasmic volume were especially affected and neuropil areas consisted of degenerated mitochondria, lysosomes and abnormal neurofibrils [37].

Under normal conditions, oral intake of vitamin B_6 is lower than recommended [29]. Particularly in elderly people, it gradually decreases [13]. The experiments on rats have demonstrated that dietary deficiency of vitamin B_6 causes very important morphological changes such as dendrite loss, perikaryonal swelling, vacuolization of dendrites, neuropil degeneration in cortical layers, glial proliferation in the area of neuronal loss [35], and decreased number of Purkinje cells in the cerebellum [6, 21, 26]. Interestingly, we have observed similar changes in the cerebral cortex in the experimental groups receiving excessive vitamin B_6 doses, in a time-dependent manner. On the other hand, Fairfield and Fletcher [15] have suggested that neuropathic cases resulting from a deficiency of vitamin B_6 may be treated with vitamin B_6 excess intake.

According to the results of this study, widespread ultrastructural damage was observed in the pyramidal neuron perikarya and in neuropil dendrites and axons. It is well known that the development of the cerebral cortex is a complex process consisting of cortical maturation including cell proliferation, migration, maturation and establishment of extracellular architecture for functional processes. Increased mitochondrial damage, lipofuscin pigment granules and vacuolization in neuropil, demyelinization of axons, and decreased synaptic density may indicate that the motor cortex neurons were affected by high dose treatment with vitamin B_6 for long

Table 2. Quantitative analysis of selected structures in pyramidal neurons and neuropil of control and vitamin B$_6$-treated rats

Groups	Pyramidal neurons		Neuropil		
	DM (M±SE) per neuron	LPG (M±SE) per neuron	UMA (M±SE) per micrograph	NV (M±SE) per micrograph	S (M±SE) per micrograph
CG	2.96±0.35	2.54±0.28	5.12±0.72	8.25±0.77	86.37±3.07
EG-10	13.12±0.77*	20.35±0.76*	3.62±1.87	42.62±1.61*	58.12±2.06*
EG-15	23.08±0.83*	20.19±0.90*	3.75±0.62	46.37±2.04*	41.12±1.99*
EG-20	30.15±1.02*	20.61±0.77*	4.37±1.22	50.62±1.90*	29.50±1.13**

Eight electron micrographs containing 26 pyramidal nerve cells were evaluated for control group (CG) and for each experimental group (EG). Distribution and mean numbers of damaged mitochondria (DM), lipofuscin pigment granules (LFG) in pyramidal neurons, and numbers of unmyelinated axon (UMA), neuropil vacuoles (NV) and synapses (S) in neuropil of cerebral cortex according to EGs injected with high dose vitamin B$_6$. *P<0.05 compared to CG, **P<0.05 compared with CG, EG-10 and EG-15.

periods. Vitamin B$_6$ plays an important role in both neurogenesis and neuron longevity in the cerebral cortex. Maternal restrictions in vitamin B$_6$ reduce the number of dendrites of stellate neurons in layer II and of pyramidal neurons in layer V of the neocortex [16, 17].

Dendritic processes establish the synapses that are the contact points of the neurons. Thus, it is reasonable to conclude that neural tissue with poor dendritic processes would suffer from malfunction and loss of interconnections. In the present study, decreased number of synaptic junctions was observed after high-dose vitamin B$_6$ treatment for an extended period; this could result from disorientation of dendritic processes and might affect the functional condition of synaptic fields. So, would it be reasonable to expect an improvement in old patients with vitamin B$_6$ deficiency after treating them with high doses of vitamin B$_6$ for a limited period? Our results suggest that the answer to this question is that time of treatment and dose of vitamin B$_6$ should be taken into consideration when vitamin B$_6$ supplementation is given.

Previously, Schaeffer *et al.* [29, 30] used vitamin B$_6$ with high dose daily intake and showed no neurotoxicity depending on behavior action, but reported effects on amino-acid concentration and on binding properties of cortical serotonin receptor in brain tissue.

The ultrastructural findings of this study suggest that long-term administration of high-dose vitamin B$_6$ is likely to result in some biochemical imbalance at the intracellular level, as manifested *e.g.* by mitochondrial damage. Our findings such as partial loosening of the neuropil, formation of smooth surfaced vesicular vacuoles in the unmyelinated axons, or severe dilatation of Golgi apparatus in the perikaryon, may suggest alterations in membrane permeability and imbalanced cytophysiology of the neurons.

In conclusion, excessive vitamin B$_6$ administration with increasing treatment periods is likely to damage neurons and the neuropil structure of the motor cortex. It seems that excess vitamin B$_6$ is a double-edged sword.

Thus, a careful attention should be paid to the time and dose of vitamin B$_6$ recommended for patients under supplementation. We believe that it is necessary to investigate the advantages and disadvantages of high-dose vitamin B$_6$ treatment at the biochemical and pharmacological levels, in order to extend our results.

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