Original Article

Effect of Sodium Fluoride on the Cerebellar Cortex of Adult Albino Rats and the Possible Protective Role of Vitamin B6: A Light and Electron Microscopic Study

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ABSTRACT

Introduction: Sodium fluoride (NaF) toxicity has been a subject of extensive studies over the last years because of their environmental persistence and world widespread distribution. Its toxicity on many organs has received considerable attention, but its neurotoxicity has not been entirely studied.

Aim of the Work: Studying the histological changes occurring in the cerebellar cortex of albino rats following chronic exposure to NaF and the possible protective role of vitamin B6.

Materials and Methods: Twenty adult male albino rats were divided into group I (control), group II (each animal was daily injected I P with 3.6 mg vitamin B6), group III (each animal received 10 mg/kg NaF orally once daily) and group IV (each rat was given vitamin B6 concomitantly with NaF at the same dose, period and route of group II and III). After three months the cerebellum was dissected and prepared for light and electron microscopic study.

Results: The cerebellum of NaF treated animals exhibited severe degenerative changes especially in Purkinje cells. There was multilayer disposition of these cells associated with structural changes in the form of dilated rER and Golgi complex, swollen mitochondria in addition to marginated nuclear chromatin. The surrounding neuropil appeared vacuolated with accumulation of neuroglial cells. Many myelinated nerve fibers displayed disruption in myelination and irregular neurofilaments. Silver stained sections showed accumulation of cytoskeletal elements in Purkinje cells. On the other side, these changes were ameliorated in rats of group IV which received vitamin B6 concomitantly with NaF.

Conclusion: Chronic administration of NaF induced degenerative changes in the cerebellar cortex which could be ameliorated by vitamin B6.

Key Words: Sodium fluoride, vitamin B6, cerebellar cortex, rats.

INTRODUCTION

Fluorine is a chemical electronegative element in the periodic table and a member of the halogen family, which is widely distributed in the environment. It is a natural component of the earth’s crust and occurs in varying concentration in rocks, soil, water and air. It is present in several chemical forms either organic or inorganic, one of the commonest inorganic fluorides is sodium fluoride\(^1\). Like many elements, it is beneficial to human health in trace amounts, but can be toxic in excess\(^2\).

Since the mid of 1940s, fluoride has been added to tap water to reduce dental caries in American communities. Then, it is added to drinking water for the purpose of clarification in many countries. The water based beverages such as beers, colas, carbonated soft drinks, sport drinks and reconstituted juices contain variable amount of fluoride. Sodium fluoride was the first fluoride compound used in fluorination of drinking water and it is still commonly used for that purpose. It is also commonly used as pesticide, rodenticides, fungicide, and a constituent in glass, enamel and dental laboratories\(^3\). It is present in high level in some sea foods and in bone products as gelatin. The dark green vegetables are considered a major source of fluoride, as it accumulates in their leaf which is obtained from the soil and water\(^4\). Cooking in Teflon (fluorinated ethylene) lined cookware also increase the concentration of fluoride in our daily foods prepared inside them\(^5\).

Commercially, it is used to etch glass, ceramics and computer chips, refine metals and petroleum products, make ceramic materials more porous, inhibit fermentation in breweries and wineries, polish aluminum and is used as a refrigerant and as a rust remover. As a result, exposure to fluoride is greater than had been anticipated and because of its multiple sources, it is difficult to determine the daily fluoride consumption\(^6\).
Fluoride is toxic by all routes especially when consumed in excessive amounts. Long-term intake of high levels of fluoride in human causes neurological complications such as paralysis of limbs, vertigo, spasticity in extremities and impaired mental acuity. In experimental animals, chronic fluoride toxicity causes altered neuronal and cerebrovascular integrity.

Vitamin B6 is a water soluble vitamin and is a part of vitamin B complex group. It could be helpful in some neurological condition as peripheral neuropathy and its deficiency has been linked to several neuropsychiatric disorders. It is a collective term comprising pyridoxine, pyridoxol and pyridoxamine. It is involved in several key biological processes, where it acts as a coenzyme for homocysteine re-methylation, and its deficiency is associated with an increase in blood homocysteine levels which is a risk factor for cerebrovascular diseases, Alzheimer’s and dementia.

The effects of fluoride compounds on human health have received worldwide attention because of their environmental persistence, industrial exposure and widespread distribution. Its neurotoxicity is well proven clinically but, the detailed histological changes have not been fully studied and it is not well recognized if these neurological symptoms could be prevented or minimized by protective agents. So, the present work aimed to study the structural changes which might result from sodium fluoride on the cerebellar cortex of adult male albino rats and to evaluate whether vitamin B6 was able to reduce or improve these changes.

MATERIALS AND METHODS

The present study was carried out on twenty adult albino rats, weighing 180-200 gms each. All animals were kept in clean properly ventilated cages and received balanced diet and water. The animals were divided into four groups (five rats each): Group I (control), Group II (vitamin B6 group): Each animal was injected I P with 3.6 mg vitamin B6 daily. The dose was calculated after applying the interspecies dosage conversion scheme, on daily human high therapeutic dose of vitamin B6 as 200 mg/day. Group III (NaF received group): Each animal received 10 mg/kg sodium fluoride orally by gastric tube once daily. Sodium fluoride was obtained from El-Gomhuria Company for Chemicals and Medical Trading, Tanta, Egypt in the form of white powder. Group IV (protective group): Each rat was given vitamin B6 at the same dose and route as in group II concomitantly with the NaF at the same dose and route of group III.

The animals were examined daily for neurological signs by simple observational methods. Three months after the experiment, all rats were anaesthetized and perfused with 4% paraformaldehyde in 0.1M sodium phosphate buffer at pH 7.4 containing 2.5% gluteraldehyde solution. The cerebellum from each animal was dissected out and specimens were taken from the lateral lobe and processed for light and electron microscopic study.

For light microscopic study, the specimens were fixed in 10% neutral-buffered formalin, processed for preparation of paraffin section. Sections were cut and stained with hematoxylin and eosin (H&E) for general examination and Glees and Marsland’s silver stain for cytoskeleton demonstration.

For electron microscopy, very small pieces were processed and embedded in Epon. Semithin sections were cut, stained with toluidine blue and examined by light microscope to choose the selected areas. Ultrathin sections were cut with LKB ultramicrotome using diamond knife on copper grids and stained with uranyl acetate followed by lead citrate for examination with JEOL transmission electron microscope.

RESULTS

General examination:

The water and vitamin B6 received rats appeared normal and did not exhibit any signs of neurological abnormality. On the other hand, three rats from NaF received animals exhibited some neurological signs in the form of reduction in activity, muscle weakness, tremors with movement, loss of equilibrium and walked like to be drunk and at the end of the experiment their hind limbs were paralysed. Animals of group IV which received vitamin B6 concomitantly with NaF showed only reduction of activity up to the end of the study.

Microscopic results:

Control Group:

H & E sections of the cerebellar cortex of this group (I) as well as vitamin B6-treated group (II) showed the well known normal structure. They showed three distinct layers from outside inwards; the molecular layer, the Purkinje cell layer and the granular cell layer. The outer molecular layer was formed of nerve fibers with few scattered stellate and basket cells. The Purkinje cells were arranged in one row between the molecular and granular layers. They showed large pyriform or flask shaped cell bodies, centrally located vesicular nuclei with apparent nucleoli and basophilic Nissel’s granules in their cytoplasm. The granular layer was composed of closely packed numerous small granular cells with dark spherical nuclei (Fig. 1). By Glees and Marsland’s silver stain, the molecular layer showed many nerve fibers including regularly arranged axons of granular cells and dendrites of Purkinje cells. The cytoplasm of Purkinje cells showed homogenous brown stained cytoplasmic
cytoskeletal elements (Fig. 2). By transmission electron microscope, the cerebellar cortex did not demonstrate substantial differences among rats in control group and vitamin B6-treated group and it was similar to the well known normal ultrastructural picture. The Purkinje cells showed large spherical central euchromatic nuclei and cytoplasm containing free ribosomes, rough endoplasmic reticulum, Golgi complexes and mitochondria (Fig. 3). The surrounding neuropil showed some neuroglial cells and many nerve fibers either unmyelinated or myelinated with regular compact myelin sheath (Figs. 4, 5). The axoplasm of these nerve fibers contained regularly arranged microtubules and neurofilaments (Fig. 6).

**Sodium fluoride received group:**

Examination of the H&E stained sections of this group (III) revealed multifocal neuronal affection especially on the Purkinje cell layer. The monolayer arrangement of Purkinje cells was disrupted in many areas, as they disappeared completely in several areas while they revealed multilayer deposition with loss of the normal pyriform shape in others (Figs. 7, 8). Most of Purkinje cells either in multilayer or monolayer areas, were shrunken having irregular outlines with deep homogenous cytoplasm and absence of Nissel’s granules. They were surrounded with vacuolated neuropil (perineurial spaces) with accumulation of neuroglial cells around some of them. As regard to nuclei, few cells showed vesicular nuclei, others showed pyknotic nuclei or complete karyolysis (Figs. 7, 9, 10). The molecular and granular layers were less affected; multiple vacuolated areas associated with increased microglial nuclei are seen in molecular layer while the granular layer showed pyknosis of their nuclei (Figs. 8-10). Glees and Marsland’s silver stained sections showed irregular arranged nerve fibers in the molecular layer while the Purkinje cells showed a massive accumulation of the cytoplasmic cytoskeletal elements that displayed deep argyrophilia (Fig. 11).

The ultrathin sections of the Purkinje cells of this group showed dilatation of rER and Golgi, in addition to swollen mitochondria with destroyed cristae while the nuclei were irregular in outline but exhibited euchromatin (Fig. 12). The neuropil of the cerebellar islands showed loss of structural details and accumulation of many neuroglial cells inbetween the affected axons. The myelinated axons revealed areas of degenerative changes in the form of disruption, splitting and loss of the lamellar compact structure of myelin layers. Many axons appeared swollen with irregular outline and revealed disorganization of their neurofilaments and many swollen mitochondria with destroyed cristae (Figs. 13-15).

**Sodium Fluoride and Vitamin B6 Received Group:**

H&E stained sections of animals of this group (IV) showed monolayer arrangement of Purkinje cells with mild disorganization. Few Purkinje cells still affected inbetween many apparently normal cells with central open face nuclei. Many cells still surrounded with small vacuolated neuropil. The molecular and granular layers were more or less similar to control (Fig. 16). As regards silver stained sections, the cerebellar cortex appeared more or less as those of control sections (Fig. 17).

Ultrastructurally, the animals that received NaF and vit B6 simultaneously showed partial improvement and preservation of the normal structure of cerebellar cortex. Most of the Purkinje cells appeared nearly similar to the control and few of them displayed irregular outline of their nuclei (Fig. 18). Most of nerve fibers were normal with regular compact myelin sheath around normal axons while few scattered fibers were mildly affected (Fig. 19).
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Fig. 3: An electron micrograph of a section in the cerebellar cortex of a control rat showing Purkinje cell containing euchromatic nucleus (N) with fine dispersed chromatin and prominent nucleolus (Nu). The cytoplasm contains many free ribosomes (→), rER, Golgi apparatus (G) and mitochondria (M). X 7500.

Fig. 4: An electron micrograph of a section in the cerebellar cortex of a control rat showing neuropil (perineural spaces) containing neuroglial cells (Ng) around blood capillary (Bc) and transverse section of many myelinated and unmyelinated nerve fibers (→). X 5000.

Fig. 5: An electron micrograph of a transverse section in the cerebellar cortex of a control rat showing many myelinated nerve fibers containing many mitochondria (M). X 7500.

Fig. 6: An electron micrograph of a section in the cerebellar cortex of a control rat showing an axon (→) with regularly arranged microtubules and neurofilaments (F). X 7500.

Fig. 7: A photomicrograph of a section in the cerebellar cortex of a NaF treated rat showing severely affected area with disappearance of many Purkinje cells. Notice, few shrunken irregular and scattered Purkinje cells (P) surrounded with vacuolated neuropil. H&E X 400.

Fig. 8: A photomicrograph of a section in the cerebellar cortex of a NaF treated rat showing many layers of irregular, distorted Purkinje cells (P) inbetween outer molecular (Mo) and inner granular (Gr) layers. H&E X 400.
Fig. 9: Higher magnification of the previous figure showing Purkinje cells with absence of Nissel's granules. The surrounding neuropil is vacuolated and many neuroglial cells (→) accumulate around Purkinje cells. H&E X 1000.

Fig. 10: A photomicrograph of a section in the cerebellar cortex of a NaF treated rat showing a monolayer of Purkinje cells (P) with deep homogenous eosinophilic cytoplasm surrounded with vacuolated neuropil. H&E X 400.

Fig. 11: A photomicrograph of a section in the cerebellar cortex of a NaF treated rat showing many layers of distorted Purkinje cells (P) with massive accumulation of cytoskeletal elements (P). The molecular layer showed irregularly arranged nerve fibers (→). Gies & Marsland's silver stain X 250.

Fig. 12: An electron micrograph of a section in the cerebellar cortex of a NaF treated rat showing cytoplasm of Purkinje cell containing dilated Golgi (G) and vacuolated mitochondria (M). Notice nucleus with normal euchromatin (N). H&E X 75000.

Fig. 13: An electron micrograph of a section in the cerebellar cortex of a NaF treated rat showing many neuroglial cells (Ng) inbetween axons with irregular outline (→) adjacent to a blood capillary (Bc). X 5000.

Fig. 14: An electron micrograph of a section in the molecular layer of cerebellar cortex of NaF treated group showing many nerve fibers with vacuolated mitochondria (M) and focal areas of splitting of myelin sheath (→) in the vacuolated neuropil (V). X 10000.
DISCUSSION

Fluoride is a two-edged sword for human health, because fluoridation of drinking water seems to be effective for its clarification and treatment of dental caries, whereas undesirable adverse effects of fluoride have been reported. Fluoride is completely and quickly absorbed from the gastrointestinal tract and affects many organs. A few studies related to the effect of oral ingestion of NaF for long period on cerebellum have been reported so, the current study was designed to evaluate the effect of oral intake of NaF, which is one of the major routes of fluoride on the structure of the cerebellar cortex with or without vitamin B6.

In this work, sodium fluoride induced some neurological abnormality and selective structural changes in the Purkinje cells while the molecular and granular...
layers were less affected. The neurological signs noticed in this group could be attributed to cerebellar affection with subsequent cerebellar ataxia. The cerebellum is responsible for smooth and accurate movements and any lesions in it manifest many signs of cerebellar ataxia which were noticed in some animals of this group as muscle weakness, tremors with movement, loss of equilibrium, gait disturbance and hind limbs paralysis. Another previous study confirmed that ingested fluoride was retained by the cerebellum, interfering with its physiology and inducing neurotoxicity, cell damage and even cell death.

This study revealed that NaF induced selective structural changes and disorganization in Purkinje cells. Their arrangement was disrupted; some disappeared completely while in other areas they exhibited multilayer accumulation. This might be caused by displacement from some areas to be accumulated in others. Most of them lost the pyriform shape and showed irregular outline. These findings could be correlated with the increased argyrophilia which was shown in silver-stained sections and cytoskeletal disorganization noticed by electron microscope in this study. It was suggested that the neurofilaments accumulation is an essential factor in the development of distal axonal degeneration. Similar findings were observed by Shivarajashankara et al. who found that chronic fluoride intoxication caused marked neurodegenerative changes in rats especially in the early stages of life. In addition, these changes may form the neural basis for impaired learning and memory, abnormal behavior patterns and disturbed overall body physiology.

In this study the appearance of distorted shrunken Purkinje cells seemed to be a result of damage to the system associated with structural and functional biosynthesis of cell proteins. Some authors suggested that the appearance of dark neurons might reflect a certain phase of apoptosis as they displayed markedly condensed cytoplasm and nucleoplasm, while others believed that dark small neurons is usually ischemic due to possible substantial abnormalities in the capillary wall of the cerebellar cortex with subsequent disorders in the structural elements of the blood-brain barrier to neurons.

Degenerative structural changes in axons with splitting and disruption of their myelination in cerebellar cortex of NaF treated rats were recorded as a component of a dying – back process of neuronal injury. The disruption in myelination was attributed to the changes in myelin basic protein secondary to membrane damage and axonal degeneration after exposure to toxic substance. Defect in myelination was also attributed to increased water content in degenerating nerve causing intramyelinic edema with separation of myelin lamellae.

During this work, the neuropil revealed vacuolation and accumulation of glial cells in the NaF treated group. As regards the vacuolation, it might be attributed to the shrinkage of Purkinje cells and withdrawal of their processes secondary to cytoskeletal affection and leaving pericellular spaces. Concerning, the increased neuropil cells (gliosis), this might be a common rapid response to CNS injury and is regulated by neurons through soluble mediators and cell – cell contact. In the healthy and resting condition, these cells function as supportive glial cells. Their activation is a key factor in the defense of the neural parenchyma against inflammation, trauma, ischaemia, brain tumours and neurodegeneration. They transform into potentially cytotoxic cells in response to neuronal or terminal degeneration, or both. Activated microglia are mainly scavenger cells but also perform various other functions in tissue repair, neural regeneration and induction of immune responses. The microglia activation may sustain a chronic inflammation leading to neuronal dysfunction and cell death. This might be mediated by the microglial release of extracellular toxic reactive oxygen and nitrogen species.

The major ultrastructural changes in the form of dilated Golgi, mitochondrial swelling with disturbance in their cristae and nuclear irregularity were seen in NaF treated group. Many investigators mentioned that these disorders were considered to be secondary to direct toxicity on neuronal cells that induced profound disorder of intercellular biochemical events, such as inhibition of oxidative phosphorylation, abnormal production of proteins, and dysfunction in the detoxication.

There are many major pathophysiological mechanisms through which fluoride ingestion might cause intoxication in many organs. Formerly, it was reported that fluoride inhibits Na+/K+-ATPase, leading to extracellular release of potassium with subsequent hyperkalamia. In addition, fluoride ions chelate calcium leading to hypocalaemia and interference with calcium function. Subsequently, it had been known that fluoride induced inflammatory reaction, cell cycle arrest and apoptosis in different systems. Also, it showed diverse actions on a variety of cellular and physiological functions including inhibition of a variety of enzymes as metalloenzymes that were proved to be involved in many essential metabolic processes as glycolysis, oxidative phosphorylation and neuro-transmission.

However, the mechanisms underlying the neurotoxicity of endemic fluorosis still remain unknown. It was found that the administration of fluoride resulted in decreased cerebellar and cerebral protein levels. Fluorine was able to pass through the blood-brain barrier and accumulates in the central nervous system of exposed animals. Chronic fluoride intoxication was accompanied by behavioral disorders, degenerative changes and abnormalities of aerobic metabolism of the neurons. Recently, it was found that fluoride induced significant increase in lactate dehydrogenase release, intracellular
reactive oxygen species (ROS), malondialdehyde levels and the percentage of apoptosis. On the other hand, fluoride induced decrease in the glutathione levels and glutathione peroxidase activities, accompanied by the markedly reduced superoxide dismutase activity. So, fluoride collectively could cause oxidative stress, apoptosis, and decreased mRNA and protein expression levels of neural cell adhesion molecules in rat neurons, contributing to the neuronal dysfunction and synaptic injury.

In the present work, the concomitant administration of vitamin B6 and NaF reduced the signs of neurotoxicity caused by NaF and revealed mild neurotoxic effect on the Purkinje cells of adult albino rats. This was in parallel with the report that vitamin B6 may be able to increase the amount of energy available to the neuron via alternative pathways and it is important for proper neuronal connections and myelination. In addition, vitamin B6 acts as cofactor in production of essential fatty acids and have a role in the synthesis of neurotransmitters including serotonin, dopamine, norepinephrine and gamma-aminobutyric acid; all of which have a role in the proper function of nervous system.

There is growing evidence in the literatures that there is a relationship between low serum B-vitamins, elevated homocysteine, and cognitive impairment. The high-dose of B vitamin supplementation even in individuals with normal levels of B vitamins was effective in reducing the level of homocysteine which is sulfur amino acid involved in essential metabolic pathways. Homocysteine elevation induces neuron loss which might be via impaired DNA repair and induction of apoptotic cell death. There is intriguing evidence that reduction of homocysteine levels can be readily achieved with high doses of folic acid, vitamin B12 and vitamin B6.

From this current work, it could be concluded that NaF induced severe toxic effect on the cerebellar cortex. These changes could be partially reduced by concomitant administration of vitamin B6. Considering the mild degenerative changes still observed in some cells it might be due to low dose of vitamin B6 used in the present work. The dose of vitamin B6 might be needed to be adjusted to cope with the toxicity of NaF or its pretreatment would be earlier before drug intake.

REFERENCES


تأثير فلوريد الصوديوم على قشرة مخيل الجرذان البالغ والدور الوقائي المحتمل لفيتامين ب6 دراسة

بالمجهر الضوئي والالكتروني

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ملخص البحث

إن سمية فلوريد الصوديوم أصبحت محل دراسات عديدة في السنوات القليلة الأخيرة وذلك لوجوده المستمر بالبيئة وانتشاره على مستوي عالمي. وقد حقن تأثيره السمى على أعضاء كثيرة بكثير من الاهتمام ولكن تأثيره السمى على الجهاز العصبي لم يدرس كثيرا. ولذلك فقد كان الهدف من هذا البحث هو دراسة التغيرات الهستولوجية الحادثة في قشرة مخيل الجرذان البالغ بعد تعرضه المزمم لفلوريد الصوديوم وكذلك تقييم الدور الوقائي المحتمل لفيتامين ب6. وقد استخدم 20 جردأ أيضاً بالعظام تم تقييمها إلى: المجموعة الأولى عملية، والمجموعة الثانية تم حقنها بالبروتين ب 10 مجم فيتامين ب6 والمجتمعات الثالثة وقد أعطي كل منها 10 مجم فلوريد الصوديوم /كم بالغم يومياً أما المجموعة الرابعة فقد أعطي كل منها فلوريد الصوديوم متلازمة فيتامين ب6 نفس الجرعة والطريقة والمدة للمجموعة الثانية والثالثة وبعد ثلاثة أشهر شرخت قشرة المخيل وتم تحصيلها لتقصي بالميكروسكوب الضوئي والالكتروني. وقد أظهرت قشرة المخيل للجرذان المعالجة بفلوريد الصوديوم تأثيرات هامة خاصة في خلايا بيغنجي التي ظهرت في طبقات عديدة وكذلك تغيرات تركيبية كالاندماج في الشبكة الانتُدرازية الخشنة و الجهاز جولجي وانتشار في الميتوكندريا بالإضافة إلى تطور في الكروماتين. وقد ظهرت بعض الالفوات في الحيز المحيط بالخلايا مع زيادة في خلايا الدبق العصبي. أما الألياف العصبية المحاطة بالميلين فقد حدث اختلاس في عملية تكون غلاف الميلين وعدم انتظام الألياف العصبية. وقد أظهرت صبغة الفضة تجمع في الناصر المكونة لدعامة الخلية. من هنالك وجه الآخر فإن هذه التغيرات قلت في جرذان المجموعة الرابعة التي عولجت بفيتامين ب6 مع فلوريد الصوديوم. ومن هذه الدراسة نستنتج أن تناول فلوريد الصوديوم المزمم يؤدي إلى تغيرات هامة في قشرة المخيل التي يمكن تقليلها بواسطة فيتامين ب6.