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Original Article

Neuropathies of spinal cord development in rat pups maternally fed with fried potato chips

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Fried potato chips;
Neuropathy;
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Abstract

Objective: Acrylamide is a neurotoxic material and recently elevated levels of acrylamide in varieties of foodstuffs were reported. The present study aimed to illustrate the demyelination of spinal cord of pups maternally fed a diet containing fried potato chips.

Methods: Eighty fertile virgin female Wistar rats were made pregnant after mating with healthy male. Zero dates of gestation were determined and dams were arranged into three groups as control, acrylamide-treated (15 mg/kg body weight, p.o.) and 50% fried potato chips containing diet group. Treatments were carried every other day from 6th day of gestation until 3-week post-partum. The cervical spinal cord was separated and subjected for SDS-PAGE analysis and light and transmission electron microscopy.

Results: Comparing with acrylamide-treatment, protein expression in spinal cord of pups maternally fed with fried potatoes was altered. Necrosis of motor neuronal cells within grey matter, hyperplasia of ependymal lining cells and fragility of white matter was detected. At ultrastructural level, the sensory and motor neuronal cells showed convoluted nuclear envelope and either chromatolysis or compacted chromatin material. Fragmentation of rough endoplasmic reticulum and damage of mitochondria become well evident in pups maternally fed with potato chips. The neuronal axons possessed vacuolation and demyelination associated with apparent damage of mitochondria.

Conclusion: Supplementation of fried potato chips exerted neurotoxicity either directly through their content of acrylamide or via its metabolite glycidamide. Both components were reported to find their way across the placenta during gestation and breast milk during the lactation period, interfering with spinal cord differentiation and adversely affected demyelination.

INTRODUCTION

French fries and potato chips are common parts of children's menus in fast-food restaurants, over the past 30 years; these familiar foods contain high levels of toxic and carcinogenic by-products, mainly acrylamide [1, 2]. Recently, high levels of acrylamide were unexpectedly detected in widely consumed food items, notably french fries, potato chips and bread [3]. Hirvonen *et al* [4] analysed the food consumption data over 2038 adults (25-74 years old) and 1514 children of 1, 3 and 6 years of age, with the data on foods' acrylamide content and found that acrylamide exposure was highest among the 3-year-old children and lowest among 65-74-year-old women. Among adults, the most important source of acrylamide exposure was coffee, followed by casseroles rich in starch, then rye bread. Among children, the most important sources were casseroles rich in starch and then biscuits and, finally, chips and other fried potatoes.

Acrylamide is a major environmental chemical found in tobacco smoke reaching 1 to 2 µg of acrylamide per cigarette [5] as well as widely used for industrial application such as soil conditioning, wastewater treatment and cosmetic, paper and textile industries [6]. Moreover, long-term acrylamide-treatment was found to induce neurotoxicity [7-10], especially axonal damage [11].

Recently, experimental studies revealed that ingestion of diet containing deep-fried potato chips led to fetal growth defects and development of histopathological lesions in hepatic, renal and myocardial tissues [12]. We previously reported that rat pups maternally fed on fried potato chips possessed markedly retarded cerebellar cortex which pointed out the close relation of acrylamide in fried potato chips [13]. The present work aimed to illustrate the developmental defects of neuronal cells and myelination of their axons in rat pups maternally fed on fried potatoes during suckling period.

MATERIALS AND METHODS

Acrylamide treatment

Acrylamide of highest purity 99.9% supplied from Sigma (St. Louis, MO, USA) was used in the present work. The applied dose of 15 mg/kg body weight was dissolved in 0.2 ml saline solution and orally dosed by a stomach tube to pregnant rats from 6th day of gestation until parturition and continued weekly until the pups became 3-week-old.

Fried-potato chips supplementation

Fried potato chips were supplied from the market and mixed with standard diet at a concentration of 50% and used for feeding the pregnants from the 6th day of gestation until parturition as well as 1, 2 and 3 weeks post-partum.

Experimental work

Eighty fertile virgin females and males of Wistar rats weighing 150-180 g were obtained from Hellwan Animal Breeding Farm (Ministry of Health, Cairo, Egypt) and used for experimentation. Rats were housed in individual cages and maintained at 23°C with a 12 h light-dark cycle. Free access of standard diet composed of 30% protein, 20% grinding yellow maize, 15% carbohydrates, 2.5% minerals, butterfat 4%, moisture 7%, fiber 13% and vitamins was supplied. Free excess of water was allowed *ad libitum*. The rats were kept under good ventilation.

Females were made pregnant by keeping them with fertile males overnight and examining in the next morning for the presence of vaginal plugs and sperms in their vaginal smears to give a precise determination of the onset of gestation. The pregnants were arranged into three groups (n = 20) including control, acrylamide-treatment and fried potato chips.

Sodium dodecyl sulfate-polyacrylamides gel electrophoresis (SDS-PAGE)

A set of five pups per mothers of five colonies of both control and experimental groups were sacrificed at the end of treatment, and biopsies of cervical spinal cord were incised and kept frozen at -20ºC for SDS-PAGE according to Laemmli [14]. The gel used for SDS-PAGE is made out of acrylamide and composed of two layers; the top one is the stacking gel and the lower layer called separating gel. SDS-PAGE of the plasma membranes-enriched preparation (PMEP) proteins was performed on 4.8% stacking and 11.5% separating gels. Prior to electrophoresis, PMEMs containing 100 µg of proteins were re-suspended in 0.2 ml sample loading buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 0.1 M DTT, 20% glycerol and a trace of bromophenol blue). After centrifugation at 12,000g for 12 min, the supernatant was loaded into gel wells. The SDS-PAGE was run at 20 mA on the polyacrylamide stacking gel and at 40 mA on the separating gel. After completion of electrophoresis, the separated protein bands were visualized using Coomassie brilliant blue G-250 [15]. A low molecular weight calibration kit (Bio-Rad) was used as the standard molecular weight marker.

Light microscopic investigations

The cervical spinal cord of pups at parturition as well as 1, 2 and 3 weeks of age of both control and study groups were separated and immediately fixed in 10% phosphate buffered formalin. The specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten Paraplast at 58-62°C. Five micrometer histological sections were carried out and stained with Harris hematoxylin & eosin.

Transmission electron microscopic (TEM) investigation

The cervical spinal cord at 2 and 3 weeks of pups were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, (pH 7.4) overnight at 4ºC. The tissue was washed with cacodylate buffer and postfixed with 1% osmium tetroxide for 2 h. Then re-washed again with 0.1 M cacodylate buffer, serially dehydrated in ethanol and propylene oxide and embedded in Eponate-resin (Ted Pella, Redding, CA, USA). Eighty nanometer thin sections were cut with a diamond knife on a ultratome (LKB Instruments, Bromma, Sweden), mounted onto 300 mesh copper grids and stained with saturated uranyl acetate in 50% methanol and then with lead citrate. The grids were viewed in a transmission electron microscope (Jeol Ltd., Tokyo, Japan).

RESULTS

Proteomic (SDS-PAGE) analysis

After fractionation by polyacrylamide gel electrophoresis, there were marked alterations in experimental groups. In 2-week-old pups, experimental groups expressed extra formation of stressed protein band, however both treatments lacked expression of one protein band: at 43kDa, acrylamide treatment revealed missing of two expressed protein bands. In 3-week-old

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pups, fried potato group was less susceptible compared to acrylamide treatment at 200 kDa; a defect was detected only in one expressed protein compared with missing of two proteins in acrylamide group. However, at 43 kDa new expression of a protein band was detected in both experimental groups. Expressions of protein bands were illustrated in Fig.1.

Light microscopic observations

In control 2-week-old pups, the multipolar neurons attained more growth and possessed prominent centrally nuclei and thin basophilic cytoplasm. In the ventral column, the neuronal cells are much larger and differentiated from those of the dorsal column (Fig.2A1-A2). However, in those maternally treated with acrylamide or fed on diet containing fried potato chips, there was a considerable atrophy of ependymal canal associated with widespread pyknotic nuclei in their lining cells. The apical and ventral margin of the ependymal canal showed massive necrosis. The grey matter possessed hyalinization and massive necrosis of their neuronal cells. Many of the multipolar neuronal cells showed eosinophilic appearance. The white matter showed marked fragility and spongy appearance (Fig.2B1-C2).

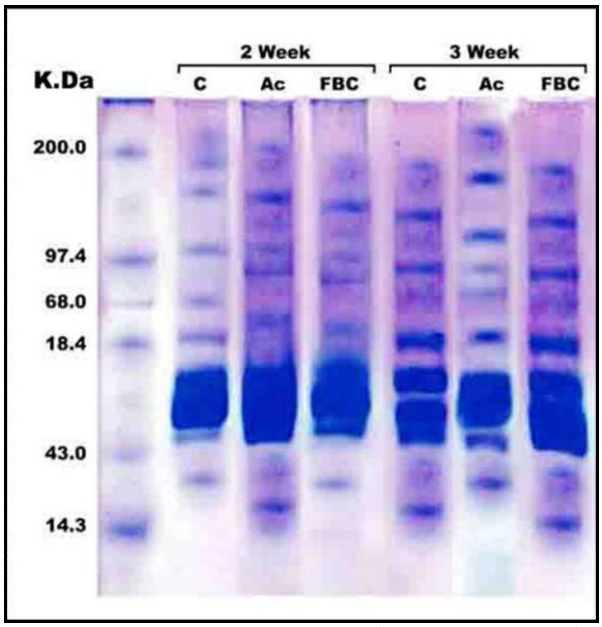

Figure 1. SDS-PAGE protein expression of cervical spinal cord of 2- and 3-week-old control pups and those maternally fed with diet containing fried potatoes or acrylamide. C, control; Ac, acrylamide; FBC, fried potato chips.

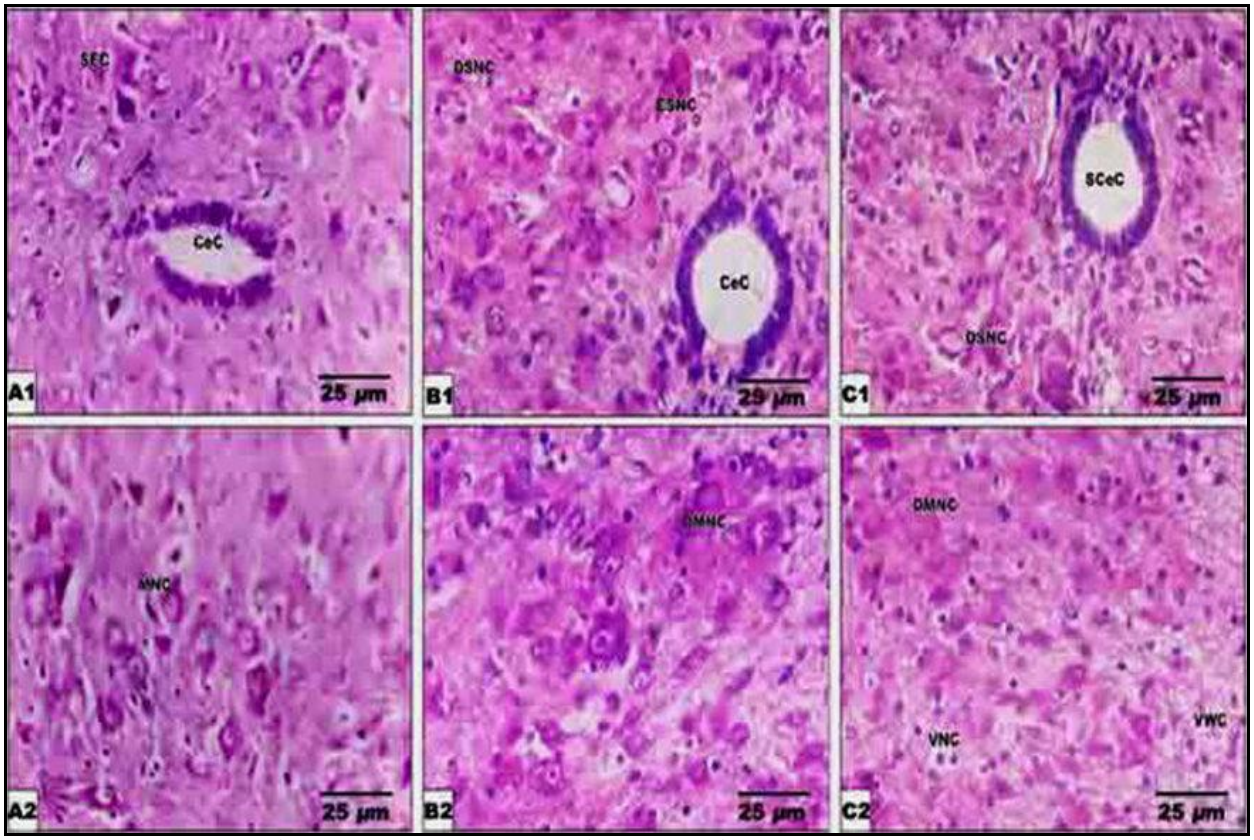

Figure 2. Photomicrographs of transverse histological sections of spinal cord of 2-weeks-old pups. **A1-A2:** controls showing differentiated ependymal canal and multipolar neuronal cells; **B1-B2:** maternally treated with acrylamide showing bilateral apical degeneration of ependymal lining cells; **C1-C2:** maternally fed with fried potatoes showing degeneration of neuronal cells similar to acrylamide treatment (H&E). CeC, central canal; DSNS, degenerated sensory neuron cell; DMNC, degenerated motor neuron cell; MNC, motor neuron cell; SNS, sensory neuron cell.

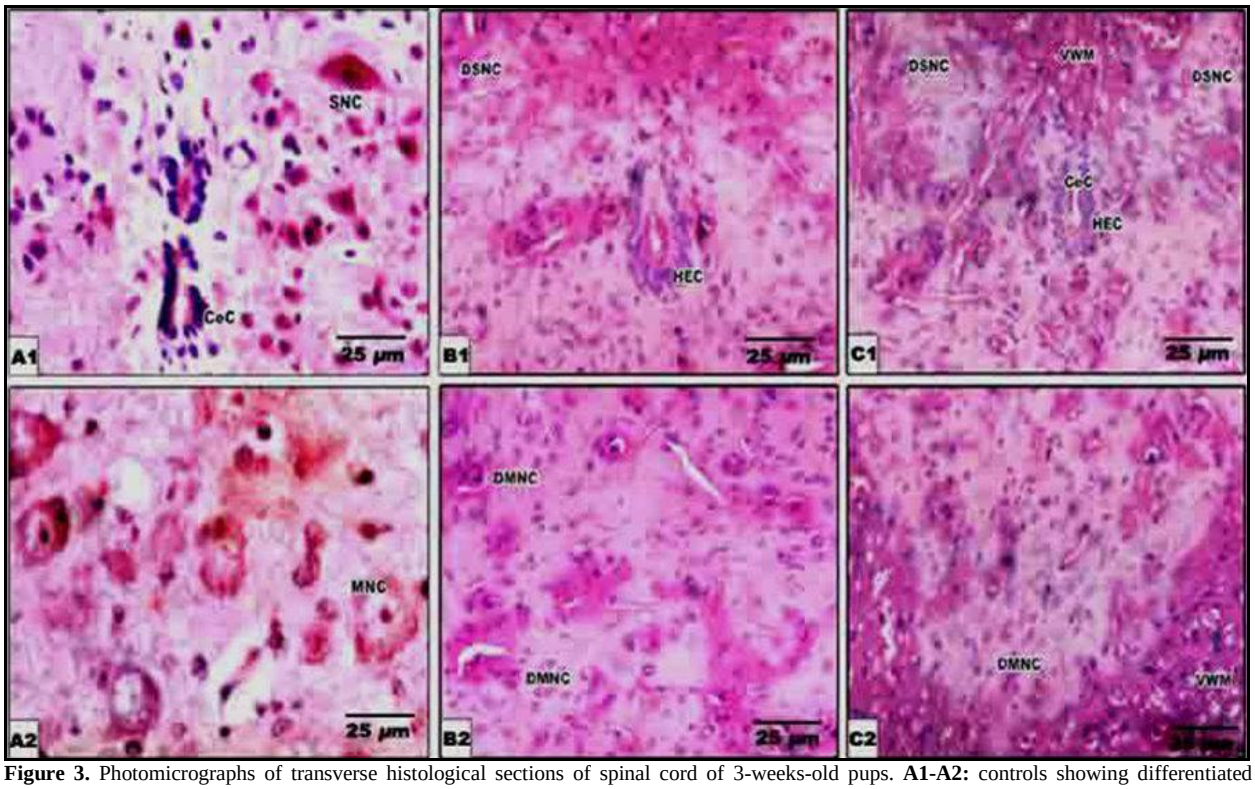

Figure 3. Photomicrographs of transverse histological sections of spinal cord of 3-weeks-old pups. **A1-A2:** controls showing differentiated ependymal canal and multipolar neuronal cells; **B1-B2:** maternally treated with acrylamide showing hyperplastic ependymal lining cells and marked neuronal cell death; **C1-C2**: maternally fed with potato chips showing similar neuronal dysfunction to acrylamide treatment (H&E). CeC, central canal; DSNS, degenerated sensory neuron cell; DMNC, degenerated motor neuron cells HEC, hyperplastic ependymal cell; MNC, motor neuron cell; SNS, sensory neuron cell; VWC, vacuolated white matter.

In control 3-week-old pups, numerous multipolar neuronal cells of varying sizes are clearly identified. The cell structural pattern attained much more maturity. The white matter composed of dense nerve endings (Fig.3A1-A2). In those maternally treated with acrylamide or fed with diet containing fried potatoes, there was a considerable damage of the spinal cord. Most of the histopathological lesions were detected around the central canal and consisted of severely necrotic areas. The gray matter showed apparent chromatolysis of the motor neurons. Vacuolation of the white matter becomes more apparent. There was a close similarity between the pathology of acrylamide and feeding on diet containing fried potatoes (Fig. 3A1-C2).

Transmission electron microscopic observations

In control 2-week-old pups, the motor neuronal cells possess large, round centrally located nuclei and prominent nucleoli. The nuclear envelope is membranous, with peripheral chromatin arrangement. The cytoplasm is rich in rough endoplasmic reticulum, spherical and elongated mitochondria and Golgi complex .The white matter exhibits the presence of myelinated axons. Each axon is formed of several lamellate sheets with markedly enlarged extracellular spaces separating the axon from its myelin sheath (Fig.4A1-A2). In those maternally treated with acrylamide or supplemented fried potato chips, the neuronal cells possessed chromatolysis of their nuclei. The cytoplasmic organelles exhibited vesicuolated rough endoplasmic reticulum and swollen of mitochondria with missing of their internal compartments. The Golgi apparatus was abnormal degenerated. The white matter revealed marked variations in the size of demyelinated axons. Demyelination becomes prominent in many of the axons. Others were invested with varying amounts of myelin in different stages of breakdown. Vacuolation of myelinated axons was detected in many of them. In 2-week-old pups maternally fed with diet containing fried potatoes, the spinal cord exhibited closely similar alteration of both neuronal cells and nerve axons (Fig.4B1-C2).

In control 3-week-old pups, the gray matter reveals prominent distribution of neurons with centrally located nuclei having peripheral marginated heterochromatin on nuclear envelope and abundant euchromatin. The cytoplasm possesses dense arrangement of rough endoplasmic reticulum and free polysomes in between the cristae. A moderate distribution of mitochondria and Golgi apparatus is detected. The white matter possesses abundant distribution of axons closely

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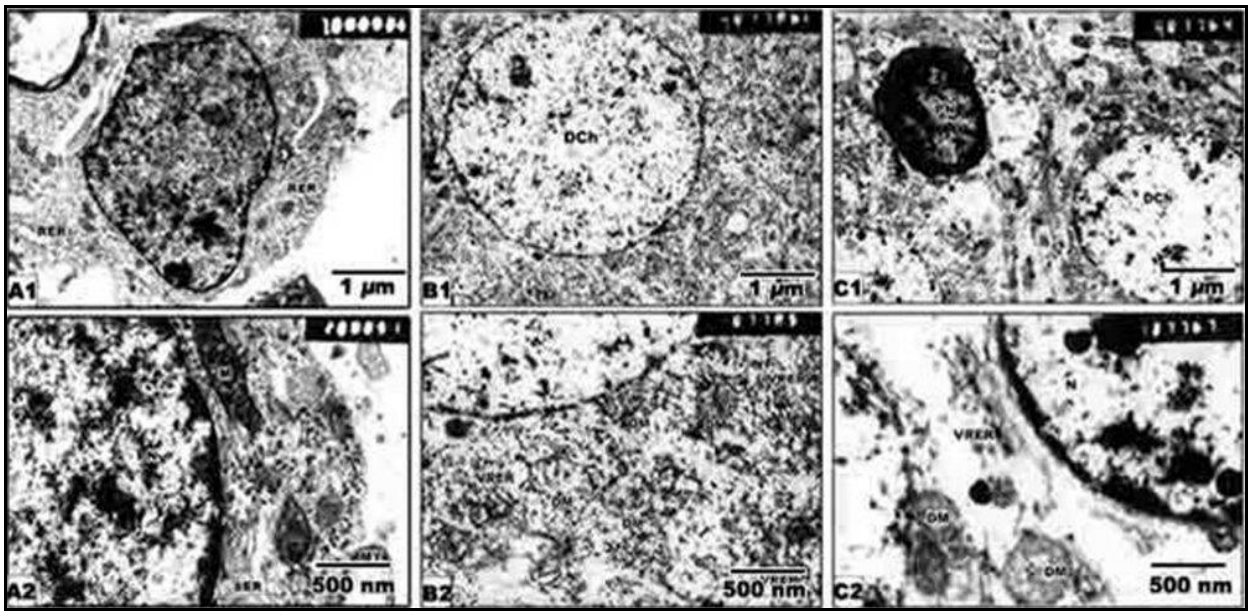

Figure 4. TEM micrographs of spinal cord of 2-weeks-old pups. **A1-A2:** controls showing multipolar neuronal cells with normal nuclei and cytoplasm rich in rough endoplasmic reticulum, mitochondria and ribosomes; **B1-B2:** maternally treated with acrylamide showing karyolysed nuclei and degenerated cytoplasmic organelles; **C1-C2:** maternally fed with fried potatoes showing either karyolysis or pyknosis of nuclei and degeneration of cytoplasmic organelles (lead citrate & uranyl acetate). RER, rough endoplasmic reticulum; VRER, vesiculated rough endoplasmic reticulum; N, nucleus; M, mitochondria; DM, degenerated mitochondria; DCh, degenerated chromatin; PN, pyknotic nuclei.

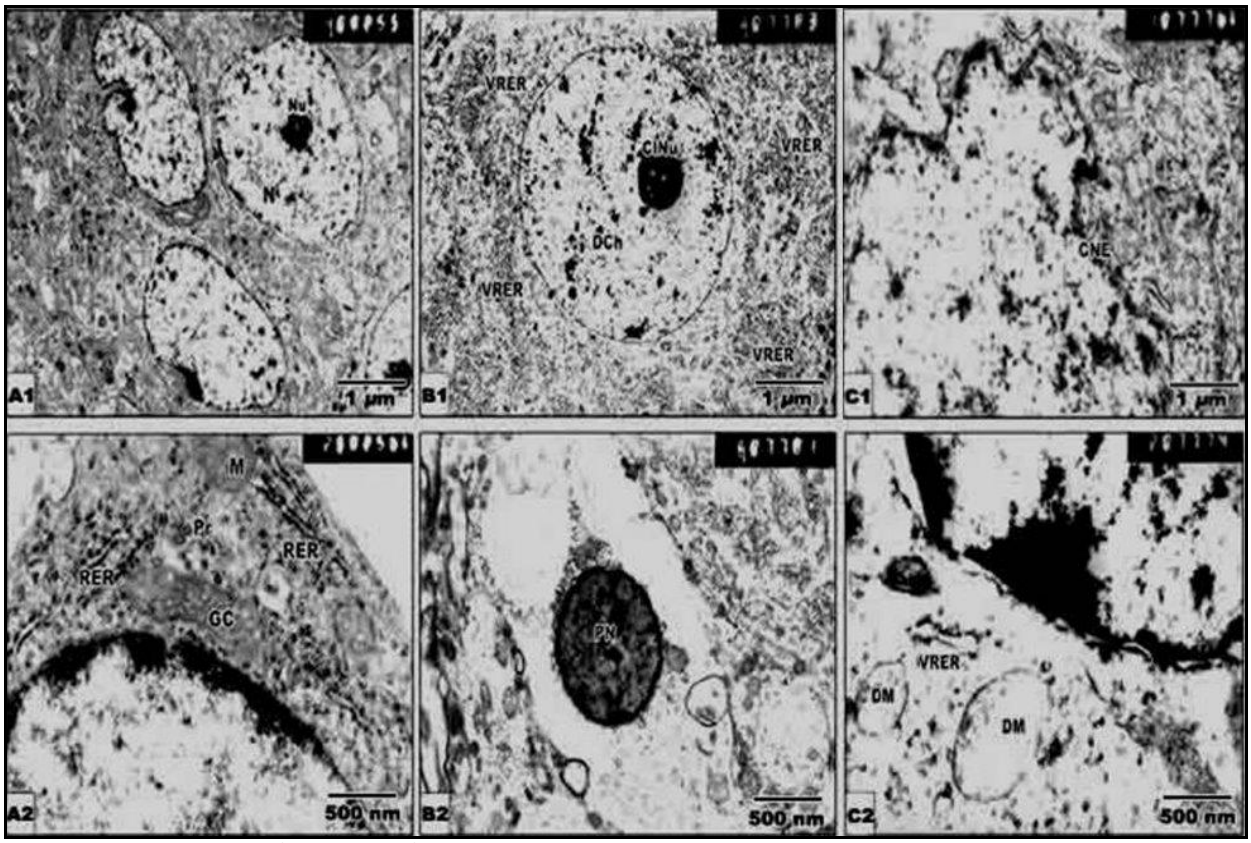

Figure 5. TEM micrographs of spinal cord of 2-weeks-old pups. **A1-A2:** controls showing multipolar neuronal cell with normal nuclei and cytoplasm rich in rough endoplasmic reticulum, mitochondria and ribosomes; **B1-B2**: maternally treated with acrylamide showing either karyolysed or pyknotic nuclei and degenerated cytoplasmic organelles; **C1-C2**: maternally fed with fried potatoes showing karyolysis or pyknosis of nuclei and degeneration of cytoplasmic organelles (lead citrate & uranyl acetate). PN, pyknotic nuclei; SM, swollen mitochondria; VRER; vesiculated rough endoplasmic reticulum.

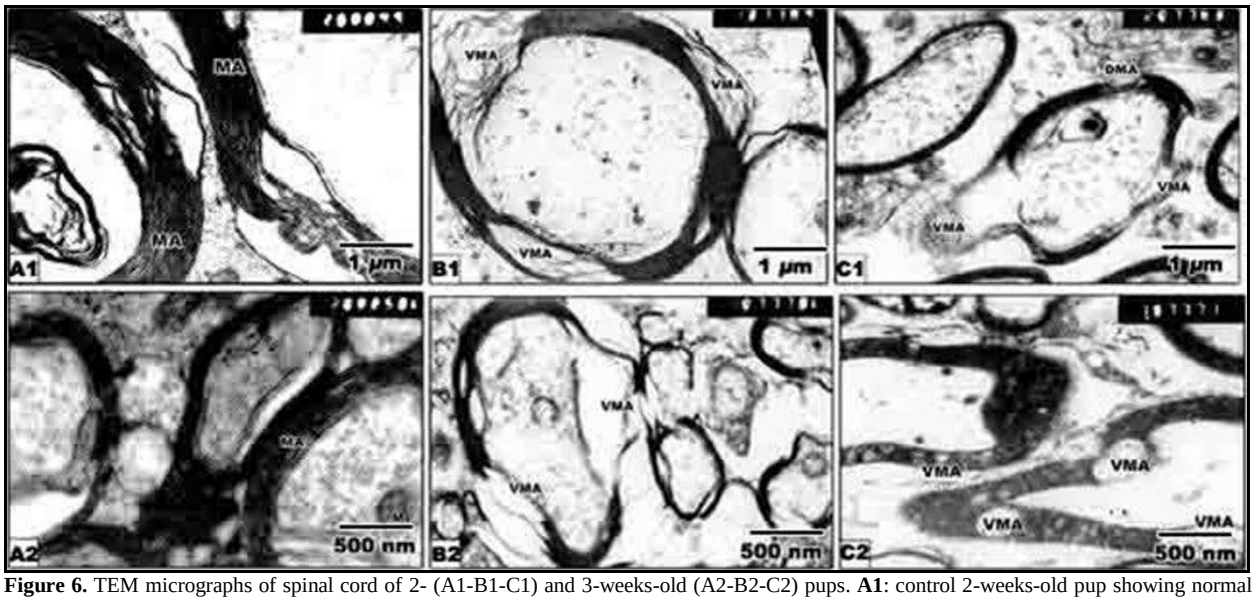

Figure 6. TEM micrographs of spinal cord of 2- (A1-B1-C1) and 3-weeks-old (A2-B2-C2) pups. **A1**: control 2-weeks-old pup showing normal myelinated axons; **A2:** control 3-weeks-old pup showing normal myelinated axons; **B1-B2:** 2- and 3-week-old pup maternally treated with acrylamide showing vacuolar degeneration of myelinated axons; **C1-C2:** 2- and 3-week-old pup maternally fed with fried potato chips showing similar demyelination (lead citrate & uranyl acetate). DMA, degenerated myelinated axon; MA, myelinated axon; VMA, vacuolized myelinated axon.

aligned with each other. Each axon is ensheathed by several layers of electron-dense myelin membranes regularly arranged in a circular manner. The axons, containing characteristic neurofibrils and mitochondria, are surrounded by myelin sheaths of relatively uniform thickness. Few numbers of non-myelinated axons are detected. The cytoplasm of the axons possessed abundant microtubules, mitochondria and vesiculated rough endoplasmic reticulum (Figs.5&6A1-A2).

In those pups maternally treated with acrylamide or fed on diet containing fried potatoes, the neuronal cells possessed nuclei with densely aggregated chromatin material. The cytoplasm exhibited vesicuolated rough endoplasmic reticulum, swollen mitochondria and abnormally degenerated Golgi apparatus. The white matter possessed massive destruction of nerve axons with either totally or partially degenerated myelin. The myelin lamellae showed massive detachment, separation, dissolution and vacuolation. Breakdown may start within or at the inner or outer surface of the sheath and yield in some axons a cleft of the demyelinated axons. The inner cytoplasm compartment becomes degenerated. The axolemma lacked normal regularity (Figs.5&6A1-C2).

DISCUSSION

From the present findings, the spinal cord of pups maternally fed diet containing 50% fried potato chips developed neuropathological alterations in both gray and white matter at 1, 7, 14 and 21 days. There was a marked damage of neuronal cells of gray matter. The number of multipolar sensory and motor neuronal cells was comparatively reduced. The axons showed massive spongiform vacuolation prominent in the white matter. At the ultrastructural level, the neuronal cells showed apparent vesiculation of rough endoplasmic reticulum with derangement of ribosomes, swollen of mitochondria with missing of cristae and degeneration of Golgi complex. The detected findings clarified close similarities of pathological alterations between acrylamide-treated and potato chips fed pups.

Similar findings of acrylamide neurotoxicity were reported in cerebellar purkinje cells of mature rats [16] or in pups maternally received either fried potatoes or acrylamide [13]. The similarities of neuropathological alterations may be attributed to the generation of acrylamide in fried potatoes and which is absorbed during feeding and consequently exerted its toxicological aspects. Tareke *et al* [17] showed that acrylamide was formed by heating certain starch-based foods, such as potatoes, bread and processed cereals, above 120°C. French fries and potato chips exhibit relative high values of acrylamide. The acrylamide level of potato chips was shown to be increased depending on the elevated temperature degree of cooking [18]. The higher susceptibilities of pups may be attributed to the primitive growth and less differentiated neuronal tissues.

Acrylamide can be generated during the heating of specific foodstuffs as a result of a Millard reaction between amino acids and sugars [19, 20]. Potatoes and cereals which had the highest measured levels of acrylamide were found to be rich in asparagine [21].

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Our findings reported vacuolation and demyelination of spinal axons in 2- and 3 weeks-old neonates maternally fed with diet containing fried potato chips compared to acrylamide treatment. A considerable thinning of lamellated myelin was detected. According to Confavreus *et al* [22], axonal degeneration is a key of the progressive disability detected in multiple sclerosis. Acrylamide exposure was found to cause nerve terminal damage in both the central and peripheral nervous system [10, 23].

Proteomic analysis of spinal cord altered protein expression clarified disruption of protein synthesis as a result of acrylamide cytotoxicity. The present findings were confirmed by the work of Lakshmi *et al* [24] who reported increased levels of lipid peroxidation, protein carbonyl content, hydroxyl radical and hydroperoxide in cerebellar cortex of rats received acrylamide-treatment.

Zhang *et al* [25] mentioned that acrylamide form adducts with the nucleophilic sulfhydryl groups on cysteine residues leading to dissociation of the transcription factor, nuclear factor erythroid 2-related factor (Nrf2) which up-regulated gene expression of phase II detoxification enzymes and consequently, impair the protection of neuronal regions.

Axonal damage may be attributed to hypoxia caused by the acrylamide-formed hemoglobin adduct decreasing oxygen transport [26] and consequently leading to disruption of microcirculation, production of toxic metabolites and diminishing mitochondrial energy metabolism [27]. Mitochondria are the major source of free radicals within the cell and are thought to play a key role in many neurodegenerative diseases [28]. Decreased number of mitochondria per axon has been described in animal models of demyelination [28, 29].

In conclusion, maternal supplementation of fried potato chips during gestation and suckling period led to consume the large amount of acrylamide generated during cooking as well as its metabolite glycidamide. Both components pass across the placenta during gestation and breast milk during lactation period interfering with spinal cord differentiation. These led to demyelination and cell death of sensory and motor neuron cells, possibly as a result of liberation of free oxygen radicals from the damaged mitochondria.

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