Acrylamide disrupts The Development of Brachial and Lumber Spinal Cord: Morphological Studies

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Abstract: Acrylamide is a type-2 alkene monomer with established human neurotoxic effects. The primary source of human exposure to acrylamide is occupational; other exposure sources include food, drinking water and smoking. The present study was carried out to investigate the effects of acrylamide on the development of rat newborn spinal motoneurons at prenatal and perinatal maternal acrylamide exposure. Acrylamide orally administered daily to non-anesthetized pregnant females by gastric intubation as a water solution at a dose of 10 mg/kg/day. The newborns were divided into 3 groups. ◆ Normal newborns (Group A). ❖ Group B; the newborns which their mothers received acrylamide from day 7 of gestation till birth (prenatal intoxicated group). ⬩ Group C; the newborns which their mothers received acrylamide from day 7 of gestation till day 28 after birth (perinatally intoxicated group). The current results show that acrylamide leads to disturbances in the developed spinal cord. This action induces different dramatic tissue changes. These abnormalities reflected on the external features of the newborns and newborns body weights. In conclusion, acrylamide and its toxic metabolites induce malformations in the brachial and lumber spinal cord if their mother exposed to them chronically during gestation and lactation periods.

[Allam A, Abdul-Hamid M, Bakry A, El-Ghareeb A. **Acrylamide disrupts The Development of Brachial and Lumber Spinal Cord: Morphological Studies.** *Life Sci J* 2013;10(3):1806-1813] (ISSN:1097-8135). http://www.lifesciencesite.com. 270

Keywords: acrylamide, motorneurons, spinal cord, development, newborns

Introduction

The literature on the neurotoxicity of acrylamide on the adult animals is huge. However, the data on the acrylamide-induced effects on the embryonic and postnatal development of spinal cord is relatively small. Acrylamide is neurotoxic to the experimental animals and humans (Zhang et al., 2011). It has mutagenic and carcinogenic effects (Maier et al., 2012). It has been reported that acrylamide can be detected in starchy food treated by high temperature (120 °C). People could be exposed to acrylamide in factory, laboratory, or even in daily life via diet and drinking water. Recently, the toxicity of acrylamide receives more attention (Ma et al., 2011; Seale et al., 2012).

Acrylamide intoxication produces peripheral neuropathy characterized by weakness and ataxia in both humans and experimental animals (Ko et al., 2002). Early manifestations of acrylamide appear in the hind-limb. The neurological manifestations of acrylamide intoxication among motor nerves have been extensively characterized (Sterman, 1982; Cavanagh and Gysbers, 1983). Pathologically the longest and largest axons in both peripheral and central nervous system are the most susceptible ones and the major changes are in the most distal parts of axons (Spencer and Schaumburge, 1977 a, b). Axonal swelling affecting neuromuscular junctions contributes to the development and progression of weakness after intoxication (LoPachin and Lehning, 1997).

Previous studies have mainly focused on adult animals and only a few observations have explored the issue of age on the susceptibility to acrylamide. During maturation of the nervous system, there is an extensive reorganization of axonal structure and the effects of acrylamide during this process remain elusive (Ko et al., 1999). Also, Ko et al. (1999) reported that the earlier occurrence and faster progression of neurological abnormalities in 3- week old mice is more than in 8-week old mice, while early pathology in motor axons innervating neuromuscular junctions preceding neurological symptoms.

The recent studies in rat have suggested that axon degeneration might not be a primary effect of acrylamide (LoPachin et al., 2000; Lehning et al., 2003). Specifically, degeneration in peripheral nerve (sciatic, tibial, sural nerves) was restricted to a low-dose/long-term acrylamide intoxication paradigm (21 mg/kg/day), i.e. a higher acrylamide dose-rate (50 mg/kg/day) did not produce degeneration (Crofton et al., 1996; Lehning et al., 2002). A recent silver stain study of rat cerebellum revealed that both acrylamide dose-rates (21 & 50 mg/kg/day) produced progressive degeneration of Purkinje cell axons (Lehning et al., 2003; Allam et al., 2011). Acrylamide has been shown to produce a central-peripheral neuropathy in laboratory animals, including rats and monkeys as

well as in humans (LoPachin, 2004). Acrylamide neurotoxicity appears to be dose-and time dependent, with axonal degeneration accruing over time with repeated exposure despite no apparent accumulation of acrylamide at site of toxicity (Crofton et al., 1996). Moreover, neurotoxic effects have been documented in the brain regions of rat which are associated with higher congnitive function (Lehning, 2003).

Regardless of acrylamide dose-rate, many brainstem and spinal cord regions exhibited moderate-to-heavy nerve terminal degeneration. It is likely that this level of afferent damage impacts the overall functional output of affected nuclei. Consequently, damage to specific nuclei in the brainstem and spinal cord might play a significant role in the somatosensory, somatomotor and autonomic dysfunction that characterizes acrylamide neurotoxicity (Spencer and Schaumburg, 1977 a, b; Gold et al., 2000). In spinal cord, the dorsal nucleus of Clarke was significantly affected by acrylamide. The principal synaptic input to this nucleus is primary afferents from dorsal root ganglion neurons in the mid-thoracic, lumbar and sacral regions (Tracey, 1995; Lehning et al., 2003).

Clarke's nucleus is the initial relay nucleus in the dorsal spinocerebellar tract that carries proprioceptive information from the lower body, hind limbs and tail to the cerebellum (Tracey, 1995). These findings in conjunction with evidence for structural and functional damage to peripheral somatic sensory receptors (Spencer and Schaumburg, 1977b). The somatosensory processing in acrylamide-intoxicated animals is impaired significantly by damage to associated nerve terminals in central and peripheral nervous system (Lehning et al., 2003).

The present study was designed to determine the malformation effects of oral (gavage) maternal exposure of albino rats to acrylamide monomer during pregnancy and lactation on the development of rat newborns weights, external features and brachial and lumber regions of spinal cord.

Materials and Methods

Chemicals: Pure Acrylamide (99%) and other chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals used were of analytical grade.

Animal dosing schedule: sixty of albino rats (*Rattus norvegicus)* have been used in the study. Forty five mature virgin females and 15 mature males weighing 140-150g were purchased from the Organization for Vaccine and Biological Preparations, (Helwan laboratory farms, Egypt). Animals were marked, housed 4 per cage and fed standard rodent pellet diet manufactured by the Egyptian Company for Oil and Soap (Cairo, Egypt). Tap water was given *ad libitum*. Daily examination of vaginal smear of each virgin female was carried out to determine the estrous cycle. Estrous females exhibited the presence of comified cells in vaginal smear. Mating was done by overnight housing of 2 pro-esterous females with one male in separate cages. The presence of sperm in vaginal smear determined the D0 of gestation. Acrylamide was dissolved in distilled water and orally administered to non-anesthetized pregnant rats by gastric intubation at a dose of 10 mg/kg/day. The present chronic dose was applied because the over-doses will reduce reproductive activity of mothers and cause paralysis (Tyl et al., 2000).

The newborn baby's mothers were labeled into 3 groups as follows:

Group A: Pregnant rats were given saline (control).

Group B: Pregnant rats were administered acrylamide from D7 of gestation till birth (prenatal intoxication).

Group C: Pregnant rats were administered acrylamide from D7 of gestation till D28 after birth (perinatal intoxication).

Postnatal investigations: The newborns were investigated by the experimenter every day and the following notes were recorded in each group.

1. The weights of 6 newborns from each group daily recorded.
2. The time of fur appearing.
3. The time of ear opening
4. The time of eye opening.

Histological preparation

Segments from brachial and lumber spinal cord (5mm in length) at D7, D14, D21 and D28 were fixed in 20% buffered formalin (pH 7.4) for 24 hour. The tissue was dehydrated in ethyl alcohol followed by two changes of xylene. The tissue was impregnated in paraffin wax and then embedded in paraffin wax. Sections (4-5µm) were cut, de-waxed, hydrated and stained in Mayer's haemalum solution for 3 min. The sections were stained in Eosin for one min, washed in tap water and dehydrated in ethanol as described above. Haematoxylin and Eosin stained sections were prepared according to the method of Mallory (1988). Toludine blue stain for Nissl granules and protein was used according to Carleton et al. (1967). Feulgen method was used for staining DNA (Feulgen and Rossenbeck 1924).

Results

1. General Developmental Observations

Group B newborns suffered from prenatal acrylamide exposure while group C newborn suffered from perinatally acrylamide exposure. Acrylamide

toxicity Signals were observed postnatal on the treated mothers represented by ataxia, splayed hind limb, weakness of hind-limb muscles and finally paralysis causing alteration in maternal behavior, so their newborns suffered from bad lactation and consequently malnutrition especially in group C. At birth, the newborns of all groups were hairless. The time of fur appearing, ear and eye opening was retarded in groups B and C (Table 1). The mean weights of the newborns of all experimental groups varied between D1 and D28 (Table 2).

2. Histological study of brachial spinal cord

In the present rat newborns, the spinal cord was surrounded by meninges. The inner most layer of meninges was the pia matter which adheres closely to the superficial membranes of the cord. The second membrane was the arachnoid. Externally, there was a thick fibrous dura matter separated from the arachnoid by subdural space. The brachial spinal cord was consists of centrally located gray matter and a peripheral white matter. Neurons and glial cells were distributed in the gray matter. The white matter had the axons of neurons and some glial (neuroglia) cells.

At D7, the normal motorneurons were large in size with many processes and had oval nuclei (Fig. 1a). In group B, most motorneurons appeared small in size. Also, pyknosis was noticed (Fig. 1b). In group C, motorneurons chromatolysis was recorded (Fig. 1c). Between D14 & D28, the normal motorneurons increased in size with a well differentiated chromatin and a distinct thick coat of cytoplasm (Figs. 1d, g & j). Treated groups displayed neurocyte chromatolysis and pyknosis (Figs. 1e, f, i & l). Group B showed almost normal structure of motorneurons at D21 & D28 (Figs. 1h & k).

During the growth, Nissl granules were found in the perikaryon and in the proximal parts of the dendrites of the neurons. At D7, the normal motorneurons were well-stained so the intensity of Nissl granules was high (Fig. 2a). In groups B and C motorneurons were faintly-stained (Figs. 2b & c). From D14 to D28, the normal motorneurons were deeply-stained; this is a translation to the high amount of Nissl granules in normal group (Figs. 2d, g & j). Motorneurons were stained moderately in groups B and C (Figs. 2e, f, h, i, k & l).

The DNA content of the brachial motorneuron was demonstrated by using Feulgen stain. It was noticed that the color intensity in the normal cells increased with age progress (Figs. 3a, d, g & j). The nuclei of groups B and C showed markedly decrease in the color intensity specific for DNA, in addition to the presence of central chromatolysis (Figs. 3b, c, e, f, h, i, k & l). Group B showed improvements in DNA intensity at the late ages.

3. Histological study of lumber Spinal Cord

The histological structure of the lumber spinal cord is similar to that of the brachial spinal cord but the difference between both regions depends on the degree of the neuronal maturation. The lumber motorneurons were relatively smaller than the brachial motorneurons at the same age (Fig. 4). At D7, in groups B and C, most lumber motorneurons appeared small and less differentiated compared to the normal. Also, pyknosis and neurocyte chromatolysis were detected (Figs. 4a, b & c). At D14, D21 and D28, the normal lumber motorneurons increased in size while pyknosis and neurocyte chromatolysis were still represented in the treated groups (Figs. 4e, f, i & l). Group B showed improvement in lumber motorneurons structure at D21 & D28 as in the brachial region (Figs. 4h & k).

Between D7 & D28, the normal lumber motorneurons had a relatively high intensity of Nissl granules as in the brachial motorneurons (Figs. 5a, d, g & j). At D7 & D14, motorneurons of groups B and C were faintly-stained, while at D21 & D28, they were moderately-stained (Figs. 5b, c, e, f, h, i, k & l).

Feulgen stain showed that the DNA content of normal lumber motorneurons nuclei similar to the normal brachial motorneurons but the nuclei of lumber motorneurons were small in size (Figs. 6a, d, g & j). In treated groups, it was observed that the color intensity was markedly decreased compared to the brachial region, especially in group C (Figs. 6b, c, e, f, h, i, k & l).

Discussion

The present study was designed to study the effect of acrylamide on the appearance of some external features, body weights and the development of lumber and brachial regions of spinal cord in rat newborns at different condition of maternal acrylamide exposure. The effects of the present low dose of acrylamide were recorded in several sections in spinal cord at different ages.

Acrylamide and its metabolism products as glycidamide pass readily through placenta due to its solubility in water (Sorgel et al., 2002) and distributed in many fetuses tissues during gestation (Marlowe et al., 1986; Sumner et al., 2001). Acrylamide passes through mother's milk to here newborns during lactation (Sorgel et al., 2002). Acrylamide leads to bad lactation which resulted from bad maternal behaviors and consequently leads to postnatal malnutrition (Frieda and William 1999; Shaheed et al., 2006). Therefore, the newborns of groups B and C suffered from exposure to acrylamide and malnutrition.

In group B, the newborns suffered from prenatal acrylamide exposure. In addition, postnatal

malnutrition regarded to maternal acrylamide exposure during gestation period which leads to alteration of maternal behaviors (Shaheed et al., 2006). In group C, the newborns suffered from acrylamide exposure during gestation and lactation period, so they exposed to malnutrition.

The fur of the present normal newborns appeared at D9. Its appearance delayed in the treated groups. Gold et al. (2000) reported that acrylamide causes growth retardation. This retardation resulted from growth and protein deficiencies due to the malnutrition during the development (Allam et al., 2010). In the present normal newborns, ear opening detected at D12-13. Smart et al. (1971) detected similar results in rat newborns. In treated groups, ear opening delayed (at D15). This retardation showed that acrylamide exposure impairs organogenesis as mentioned by Marlowe et al., (1986). These results are in agreement with the results reported by Garey et al. (2005). The eye opening occurred at D14-15 in the present group A as observed by Bolles and Woods (1964) while it was detected in groups B and C at D16-17. The retardation in treated groups is in-agreement with Sumner et al. (2001) who mentioned that acrylamide causes developmental alterations.

The newborns of the treated dams suffered from body weight loss. The main reason of prenatal weight reduction resulted from intrauterine acrylamide exposures that lead to growth deficiency of the developing fetus (Tyl et al., 2000). Newborns body weight was the most sensitive indicator of developmental toxicity (Wise et al., 1995). The present effects of acrylamide on embryos were intrauterine because fetuses have not the enzymes required to break down the substances once they have entered the blood supply (Adlard and Dobbing 1971). In treated groups, the acrylamide affects on the function of mammary glands because it leads to prolactin reduction in animals thus it impairs lactation (Uphouse et al., 1982). So, there were nutritional deprivations and consequently the newborns body weight loss was noticed. Frieda and William (1999) stated that postnatal weight reduction in treated newborns because they suffered from alterations in maternal behaviors caused by acrylamide as well as a decrease in lactation index.

The detected spinal cord in normal and treated rat newborns is surrounded with meninges and consists of centrally located gray matter where neurons and glial cells are distributed and peripheral white matter. In Mongolian gerbil, the motorneurons of the ventral horn of spinal cord are already well-differentiated in the day of birth, but the neurons of the dorsal hom are smaller, less differentiated and more densely packed (Cabana et al., 1993). These findings agree with Tanaka et al. (1997) and Dalia (2002) in rat newborns.

In the present normal rat newborns, the brachial and lumber motomeurons were well differentiated at D7. Their number and size increased with age progress. The brachial motor neurons appeared more differentiated than the lumber neurons. Dalia (2002) reported the brachial motomeurons are large in size and number compared with the lumber motomeurons. The neurotransmitters, like serotonin and noradrenaline are supposed to play a crucial role in the differentiation and proliferation of the spinal cells (Rajaofetra et al., 1989).

In the present treated groups, the motomeurons were small and less differentiated compared with the normal ones. Between D7 & D28, the normal motomeurons increased in size, while pyknosis and neurocyte chromatolysis were observed in the treated groups due to acrylamide exposure. Group B showed some improvement in the structure of motorneurons at D21 & D28 due to the absence of acrylamide during lactation. The present results improved that acrylamide ingestion by pregnant dams will cause cells loss during the development as reported by Shaheed et al. (2006). Acrylamide exposure reduced motorneurons functions (Ko et al., 1999). Moreover, acrylamide consumption impairs motor coordination, motor control and behavioral activities these are regulated by functional integration of neuronal activity in various regions of the brain and the spinal cord. It reduced motorneurons ability to generate action potentials (Wise et al., 1995; Lehning, 2003). Also, it affects on the pattern of neuronal axons and fibers as well as synapse (LoPachin et al., 2004). Therefore, acrylamide induced dysfunction of neuronal electrical activity in the mammalian spinal cord.

The intensity of Nissl granules in the neurons refers to the high metabolic activity of these neurons (Stevens and Lowe, 1997). In group A, the intensity of Nissl granules in spinal cord neurons was high at investigated stages, which indicate the high rate of metabolism in newborns neurons of this group. The present study showed that the intensity of Nissl granules in the neurons were more compact and have thickened cytoplasm coat. These evidences are in agreement with Stevens and Lowe (1997) who found the necessary protein so-called Nissl granules are present in the cell body and dendrites.

In group C, the investigated motorneurons appeared faint in all ages due to the low amount of intracellular Nissl granules. This result indicates the low rate of metabolism in the neurons this group which may affects on cells functions and inturn affects on the activation of the newborns. In group B, the cells appeared pale at D7 & D14 due to the low amount of

Nissl granules and proteins but stained moderate at D21 & D28 which reflected the slow improvement due to acrylamide withdrawals. The Nissl granules intensity in treated groups was low due to the inability of the fetus to synthesize required proteins, where Nissl granules in neurons are essential protein (Stevens and Lowe, 1997). Acrylamide directly impairs protein synthesis in fetal tissues through its effect on RNA and DNA as well as total and subcellular protein contents (Klaunig and Kamendulis, 2005). There are other metabolic effects of acrylamide on the cell function include formation of free radical, alteration of cytoskeleton elements, cells membrane necrosis and mitochondrial destruction (Sridevi et al., 1998; Yousef and El-Demerdash, 2006). Also, acrylamide indirectly affects on the protein synthesis because it resulted in nutrients deficiency which are essential in protein synthesis due to pre- and postnatal acrylamide exposure impair the functions of placenta and mammary glands (Sorgel et al., 2002 and Shaheed et al., 2006). The above factors collectively reduce the metabolic activities of cell and protein synthesis due to inadequate nutrients so the intensity of Nissl granules was low in the treated group. This leads to reduction in the activity of the treated newborns.

DNA in the investigated motorneurons was stained red to pink using Feulgen stain. It was observed that color intensity in the normal cells increased with age progress reflected the increasing in DNA content of the cells with age development till D28 due to the change in cells size. The motorneurons nuclei of groups B and C showed a markedly decrease in the intensity of the color specific for DNA demonstration, indicating a marked loss of DNA. This color reduction decreased with age development in group B but increased in group C. Central chromatolysis was observed in most ages in treated groups due to DNA damage induced by acrylamide (Sega et al., 1989; Tyl et al., 2000). The present observed chromatolysis in the investigated motorneurons of the developed rat newborns due to acrylamide and its metabolic derivative glycidamide which have high potential to form DNA adducts through their reaction with DNA (Sega et al., 1990; Klaunig and Kamendulis, 2005). In Chinese hamster lung cell lines, acrylamide induced alteration in cell division and chromosomal aberrations (Warr et al., 1990).

In conclusion, acrylamide affects on brachial and lumber regions of spinal cord in the developed rat newborns if their mothers exposed to acrylamide during gestation and lactation periods. These effects appeared as histopathological changes.

Acknowledgement

This project was supported by King Saud University, Deanship of Scientific Research, College of Science Research Center.

Figures

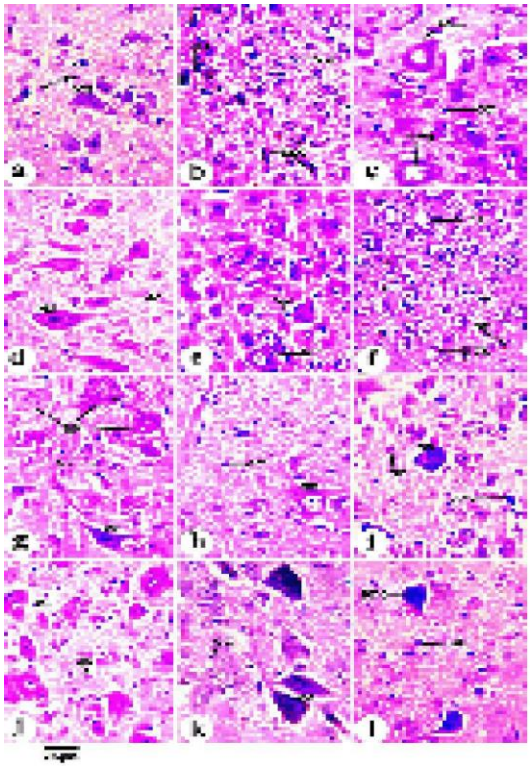

Fig. 1. Transverse sections in the brachial spinal region showing the glial cells (GC), motorneurons (MN), neurocyte chromatolysis (NCH) and pyknosis (PKC). (**a**) normal group at D7. (**b**) group B at D7. (**c**) group C at D7. (**d**) normal group at D14. (**e**) group B at D14. (**f**) group C at D14. (**g**) normal group at D21. (**h**) group B at D21. (**i**) group C at D21. (**j**) normal group at D28. (**k**) group B at D28. (**l**) group C at D28. Scale bar = 25µm. (**H. & E.**)

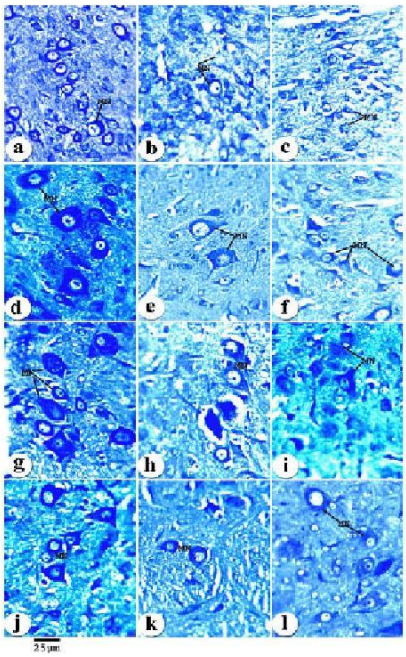

Fig. 2. Transverse sections in the brachial spinal region showing the distribution of Nissl granules in motorneurons (MN). (**a**) normal group at D7. (**b**) group B at D7. (**c**) group C at D7. (**d**) normal group at D14. (**e**) group B at D14. (**f**) group C at D14. (**g**) normal group at D21. (**h**) group B at D21. (**i**) group C at D21. (**j**) normal group at D28. (**k**) group B at D28. (**l**) group C at D28. Scale bar = 25µm. (**Toludine-blue stain**)

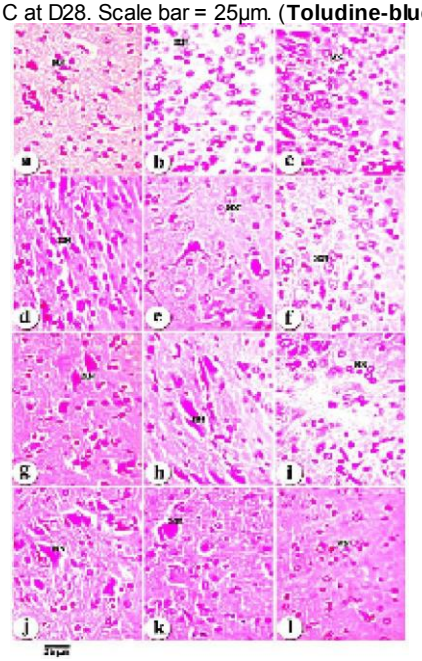

Fig. 3. Transverse sections in the brachial spinal region showing DNA in the nuclei of motorneurons (MN). (**a**) normal group at D7. (**b**) group B at D7. (**c**) group C at D7. (**d**) normal group at D14. (**e**) group B at D14. (**f**) group C at D14. (**g**) normal group at D21. (**h**) group B at D21. (**i**) group C at D21. (**j**) normal group at D28. (**k**) group B at D28. (**l**) group C at D28. Scale bar = 25µm. (**Feulgen staining technique**)

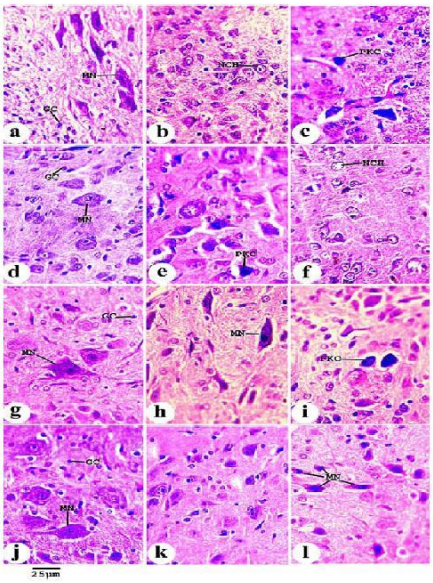

Fig. 4. Transverse sections in the lumber spinal region showing the glial cells (GC), motorneurons (MN), neurocyte chromatolysis (NCH) and pyknosis (PKC). (**a**) normal group at D7. (**b**) group B at D7. (**c**) group C at D7. (**d**) normal group at D14. (**e**) group B at D14. (**f**) group C at D14. (**g**) normal group at D21. (**h**) group B at D21. (**i**) group C at D21. (**j**) normal group at D28. (**k**) group B at D28. (**l**) group C at D28. Scale bar = 25µm. (**H. & E.**)

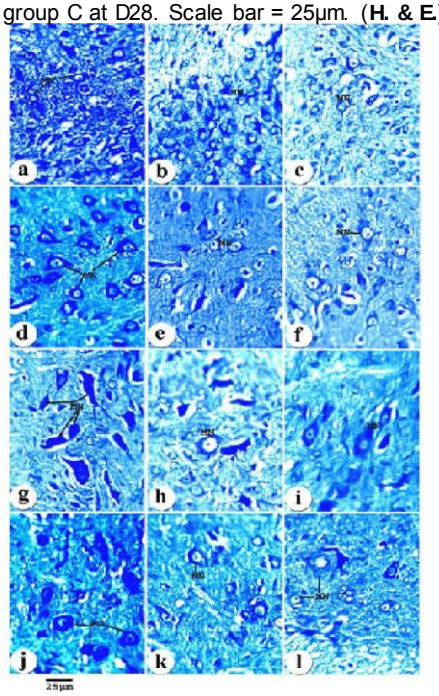

Fig. 5. Transverse sections in the lumber spinal region showing the distribution of Nissl granules in motorneurons (MN). (**a**) normal group at D7. (**b**) group B at D7. (**c**) group C at D7. (**d**) normal group at D14. (**e**) group B at D14. (**f**) group C at D14. (**g**) normal group at D21. (**h**) group B at D21. (**i**) group C at D21. (**j**) normal group at D28. (**k**) group B at D28. (**l**) group C at D28. Scale bar = 25µm. (**Toludine-blue stain**)

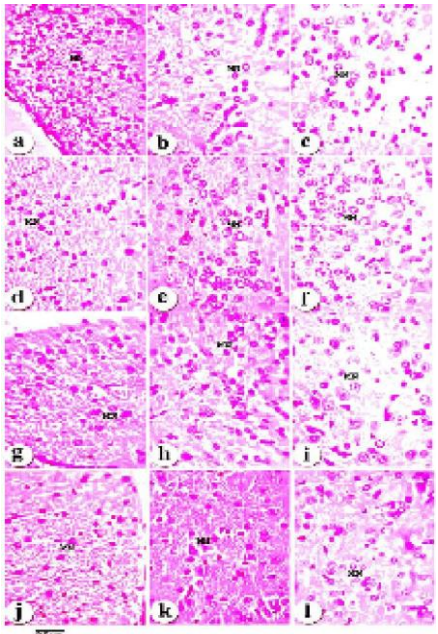

Fig. 6. Transverse sections in the lumber spinal region showing DNA in the nuclei of motorneurons (MN). (**a**) normal group at D7. (**b**) group B at D7. (**c**) group C at D7. (**d**) normal group at D14. (**e**) group B at D14. (**f**) group C at D14. (**g**) normal group at D21. (**h**) group B at D21. (**i**) group C at D21. (**j**) normal group at D28. (**k**) group B at D28. (**l**) group C at D28. Scale bar = 25µm. (**Feulgen staining technique**)

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8/23/2013