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Acrylamide Administration Induces Neuromuscular Junction Degeneration

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Acrylamide, a widely used vinyl monomer, is known to induce central-peripheral axonopathy. In the present study, investigate the effect of acrylamide (ACR) on the neuromuscular junctions of the Swiss albino mice. Animals were given acrylamide (10 and 20mg Kg^{-1}) interapertioneally 6 days weekly for 4 weeks. Intoxication with ACR at the high dose results in the prominent neurological signs and a clinical illness characterized by weakness and ataxia of the limbs. Pathological evidences of neurotoxicity were indicated morphologically by degeneration of many motor endplates from the flexor digitorum muscle in the gold chloride preparations. Accumulation of neurofilaments, diminished neurovesicles and vacuolization of mitochondria in motor endplates were observed ultrastructurally. The present data supports the hypothesis that the mechanism of the toxic action of ACR involves its interaction with neurofilaments and other molecules within the axon.

Key words: Acrylamide, albino mice, neuromuscular junctions

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Introduction

Although acrylamide monomer is now known as a potent neurotoxicant capable of producing central-peripheral distal axonopathy (Kemplay and Cavanagh, 1984; De Grandchamp and Lowndes, 1990; Madrid *et al.*, 1993; Ko *et al.*, 1999 & 2000; Stone *et al.*, 2001), it is widely used in the production of non-toxic polymers, which enjoys important applications in the industry. The neurotoxicity of the chemicals appears to be related to cumulative dose (Smith *et al.*, 1986). The mechanism by which acrylamide produces this type of nerve lesion is unknown. Some authors suggested that ACR interferes with the metabolic machinery of the nerve cell body, which gradually fails in its function to provide material for axon (Cavanagh, 1964). It is also believed that ACR inactivates the axonal transport system by which substances, assembled in the neuronal cell body, are transported along the axon (Pleasure *et al.*, 1969). Acrylamide may enter the nerve fibre at the terminals and can interact with neurofilaments and other molecules within an axon (Schoental and Cavanagh, 1977).

Skeletal neuromuscular junctions (NMJs) have been extensively used for studying the process of chemical transmission at synapses. The general morphology of motor endings has been established by the light microscopical studies, moreover, our knowledge of NMJs, supplying the structural basis to interpret the process of synaptic vesicle turnover and transmitter mechanisms have been extended by an electron microscopical studies (Peters *et al.*, 1976; Heuser and Reese, 1977).

The ultrastructural features of NMJs morphogenesis have been reported in the mouse (Carry *et al.*, 1983) and the rat (Kelly and Zacks, 1969). Some of the studies attempting to quantify ACR-induced deficits, in the neuromuscular functioning of rodents, were carried out by Gipon *et al.* (1977), Kemplay and Cavanagh (1984) and Madrid *et al.*, (1993) . There has been a some what surprising lack of success in demonstrating the specificity of the neuromuscular deficit produced by ACR. DeGrandchamp and Lowndes (1990), reported that prolonged ACR administration produces motor nerve terminal branch degeneration and impairs axonal outgrowth following nerve crush.

It becomes apparent that although the neurotoxicity of acrylamide has been well documented, very little data is available regarding its effect on NMJs. Owing to this fact, this research work is a trial to ascertain and evaluate the influence and toxicity of ACR monomer on the NMJs of the superficial flexor digitorum muscle of the Swiss albino mouse.

Materials and Methods

Experimental animals: 100 adult male Swiss albino mice (*Mus musculus*) having weight 30-35g each, were used. Animals were allowed to free access of food and water throughout the experimental period (4 weeks). Animals were assigned randomly to 1 control and 2 experimental groups and kept in large glass cages in an environmentally controlled room (22-24°C). The first group (control animals) received 1.c.c distilled water. Mice of the second group were injected intra peritoneally (i.p.) with aqueous ACR solution, at a dose of 10mg Kg^{-1}, 6 days/week. Animals of the third group received i.p. injection of 20mg Kg^{-1}, 6 days/week. These doses of ACR were reported to produce neuropathies which, are mild to moderate (Lowdnes *et al.*, 1978). Acrylamide chemical was purchased from BDH Chemicals Ltd, U.K.

Twenty-four hours after the last injection, randomly selected animals from different groups were anaesthetized with diazepam. For demonstrating the motor endplates, pieces of the superficial flexor digitorum muscle were surgically removed and prepared according to the method as described previously by Löwit (1875). For electron microscopic studies, animals from the different groups were regionally perfused through the ventral aorta with 2% glutaraldehyde in 0.1M Cacodylate buffer (pH: 7.4). Pieces of the muscle about 1mm long were kept in the same perfusate for a further 12 hours. This was followed by immersion in 1% buffered osmium tetra oxide (OSO_4), the tissue pieces were then dehydrated in serial alcohol (C_2H_5OH), transferred to propylene oxide and were embedded in a 1:1 mixture of epon-araldite. Thin sections were cut on an LKB ultramicrotome, double-stained with uranyl acetate and lead citrate and were then examined with a JEOL transmission electron microscope.

Results

Behavioral and Neurological observations: Swiss albino mice intoxicated with 10 mg ACR Kg^{-1}, 6 days a week up to 4 weeks, suffered no serious illness and the percent of mortality of these animals did not shows any significant difference from controls. However, animals which received 20 mg ACR Kg^{-1}, 6 days a week exhibited the following signs of behavioral and motor abnormality: ataxia, weakness, decreased appetite, progressive loss of limb grasp and impaired ability to balance. The results also showed that this dose increased the percent of mortality by 5% (Table 1).

Macroscopic and Microscopic observations: Anatomical examination of the various organs in all the experimental groups showed only one case of tumor in the form of small nodules appearing on the surface of the liver and spleen in only one mouse out of 30 that received the high dose of ACR. No histopathological identification to these tumors were carried out in this study. With the aid of dissecting microscope no focal surface injuries could be recognized in any limb muscle including the superficial flexor digitorum and their related peripheral nerves. To appreciate the details seen in electron micrographs of NMJs in the muscle used, it was necessary first to give a brief summary of its general morphology revealed by optical microscopy.

Control muscle teased preparations impregnated with gold chloride showed that most nerve fibers were normal (Figs. 1 and 2). In these preparations motor nerve trunks, that were situated perpendicular to the muscle fibre, ramified repeatedly in the vicinity of NMJs. Each branch abruptly rebranches in the shape of one elongated tree to give an array of myelinated terminal branches, which spread along fibre surface. Moreover, it was found that each terminal branch loses its myelin on approaching the muscle fibre and before it branches to terminal twigs. Many profiles of nerve endings appeared on the surface of muscle fibers, shortly after tapering the nerve endings become closely associated with the muscle fibre. Between 8 and 35 endplate terminals diverged from a single large diameter motor nerve branch. Moreover, our preparations showed that endplates appeared generally as flowers either disc shaped about 14.4 μm length, or oval shaped with an average length of 22.6 μm and average width of 8μm. Only one plate terminal associated with Kühne's granules was found (Fig. 3).

Table 1: Number of animals and survival rate following doses Of acrylamide administrated during the duration of the experiment

Experimental animals	No. of animals in each group	Total cumulative dose $mouse^{-1}$	Survival rate		% of Mortality	Daily dose $mouse^{-1}$
			No. of dead mice	No. of surviving mice		
Control	40	-	10	30	25.0	-
Low dose (10mg Kg^{-1}) ACR group	30	6 mg ACR (I.P)	8	22	26.7	0.25 mg ACR day^{-1}
High dose (20mg Kg^{-1}) ACR group	30	12 mg ACR (I.P)	9	21	30.0	0.5 mg ACR day^{-1}

Fig. 1: A large nerve trunk runs perpendicular to the muscle and diverges into smaller nerve terminals (arrows) each supplying muscle fibre, X 350. Gold chloride stain.

Fig. 2: A myelinated nerve fiber with motor endplate. Notice that the preterminal part of the axon lacks myelin (arrow). Notice the accessory endings (ae), X 1200. Gold chloride stain

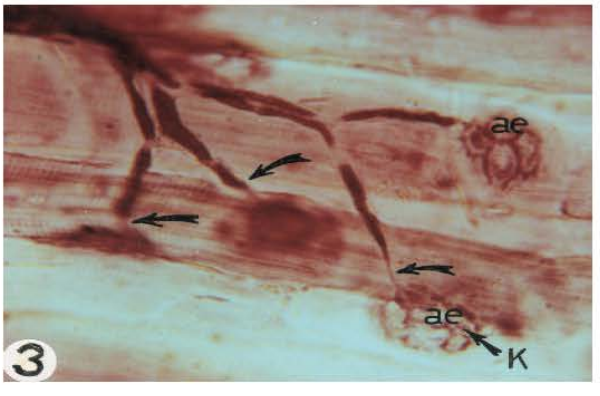

Fig. 3: Light micrograph shows four myelinated nerve terminals with normal accessory endings (ae) with associated Kühne's granules (K), X 1200. Gold chloride stain.

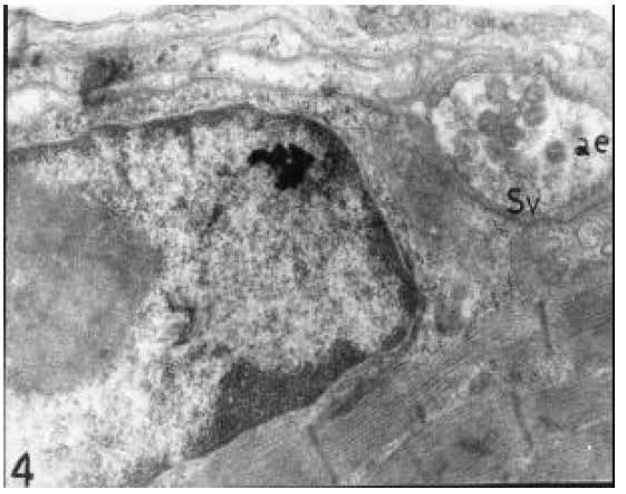

Fig. 4: Electron micrograph of control muscle, showing a normal accessory ending (ae) with numerous synaptic vesicles (Sv) and mitochondria X 30000.

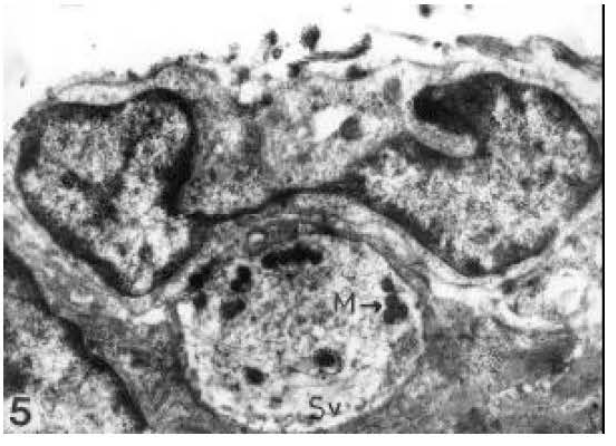

Fig. 5: Electron micrograph of the elements of the axoplasm are distributed in an orderly pattern, Sv: Synaptic vesicles; M: mitochondria X 30000.

Fig. 6: Light micrograph of high dose of ACR-treared mice, showing very few motor endplates are present in this region (arrows) X 350. Gold chloride stain.

Fig. 7: Lysis of an endplate where most of the accessory endings have been lost (arrows), X 1200. Gold chloride stain.

Fig. 8: Abnormal endplates with degenerated accessory endings (arrows), K: Kühne's granules, X 1200. Gold chloride stain.

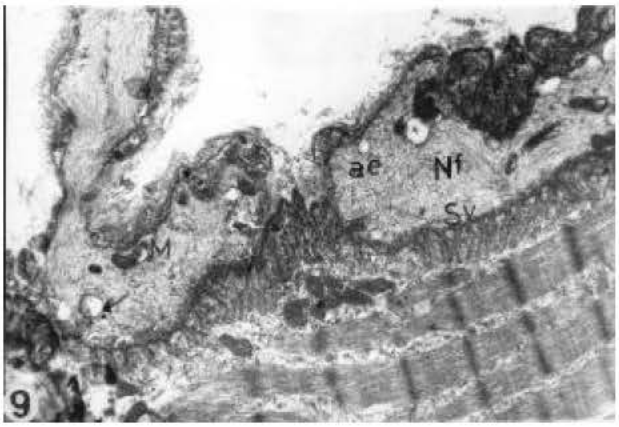

Fig. 9: Electron micrograph of high dose of ACR treated mice, showing two accessory endings (ae) in contact with part of the muscle. Note the accumulation of neurofilaments (NF) and the vacuolation of the axoplasm (arrows), M: damaged mitochondria. Note also the scarcity of synaptic vesicles (Sv) X 30000.

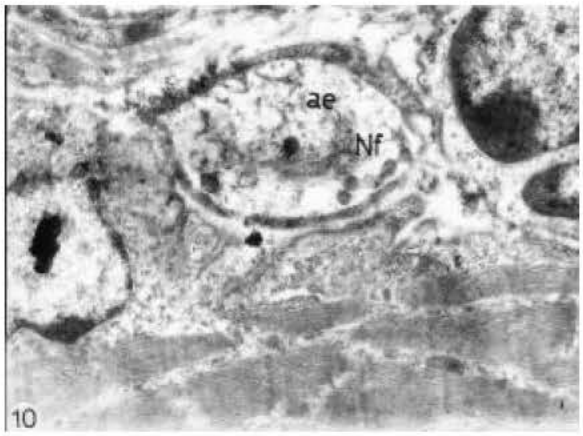

Fig. 10: An altered accessory ending (ae) with an accumulation of neurofilaments (NF) X 30000.

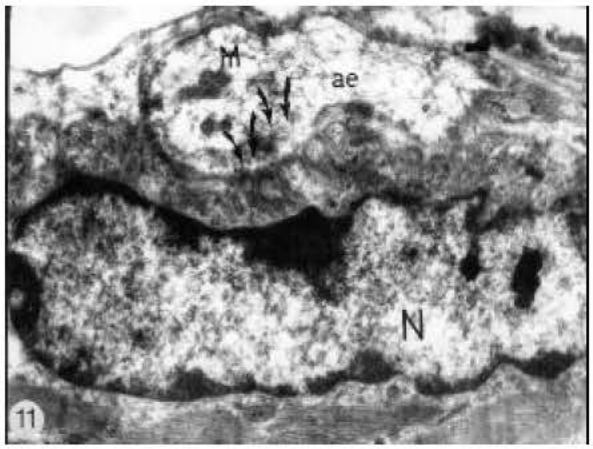

Fig. 11: An altered accessory ending (ae) of a motor endplate, with electron dense mitochondria (M). Arrows point to synaptic vesicles X 30000.

Ultrastructurally, the accessory endings of the terminal branches appeared to parallel to the long axis of the muscle fibers and lay in shallow depressions in the fibre surface. In addition, it was found that the superficial gutters of the muscle fibers were depressed at regular intervals into deeper local "trenches" the so-called junctional folds which run at right angles to the fibre axis. These terminal endings appeared normal in our preparations (Figs. 4 and 5). A cumulative dose of 240 mg mouse^{-1} failed to cause overt clinical signs and morphological alteration in motor endplates. However, animals that received 480mg mouse^{-1} (cumulative dose) were severely affected and revealed widespread nerve terminal degeneration (Figs. 6, 7 and 8).

In some mice the degeneration was restricted to the accessory endings of the terminals, while at other sites there was extensive degeneration of both of the axis cylinder and myelin sheath. Moreover, data analysis indicated that the mean disc endplate diameter was decreased (by about 30%) to 10.1μm instead of being 14.4μm in control. In the case of oval endplates the mean length was found to be 14.6μm as compared to 22.6μm in control. The mean width was found to be 5.9μm composed to 8μm in case of control (i.e. less than control by about 50%). Lysis or complete loss of accessory endings (Figs. 6 and 7)

characterized many specimens, so that Kühne's granules were not detected (Figs. 7 and 8). Other specimens showed increased numbers of these granules. Electron microscopic examination also confirmed the above results and showed that the pathological changes in terminal branches were characterized by hypertrophy of the terminal arborization, and neurofilament accumulation in the axoplasm (Fig. 9), while in other preparations axon terminals appeared to be vacuolated (Fig. 10). Mitochondria were generally altered showing decreased diameter, dense matrix and altered cristae. One of the striking features observed in our preparations was that synaptic vesicles appeared markedly reduced in number and also were often compacted against the axolemma (Figs. 9 and 10). In some specimens the motor endings appeared highly altered. They lost their intimate contact with the sarcolemma. Moreover, accumulations of fibrillar structures were accompanied by disrupted axoplasm (Fig. 11). Complete loss of synaptic elements was observed in these terminals. Some axon terminals appeared normal even in treated mice.

Discussion

Until now the focus of concern for human health hazards from acrylamide has been upon its neurotoxicity. The effect of ACR on NMJs reported here, may represent a more sensitive toxic endpoint. The cumulative effect of prolonged, low-level exposure to ACR monomer is the insidious development of a progressive peripheral neuropathy and may be supplemented by symptoms indicative of CNS damage. In addition, sensory symptoms begin in the hands and feet and may be accompanied by loss of reflexes (Spencer and Schaumburg, 1975).

On the average, it was found that a total intake of approximately 240mg Kg^{-1} was insufficient to produce significant outward signs in mice. A dose of 204 mg Kg^{-1} ACR consumed at an average daily dose of 2.8mg Kg^{-1} failed to result in overt clinical sign of neuropathy or classical axonal lesion under light microscopy (Smith *et al.*, 1986). However, ACR when administered to mice at a high dose level (480mg Kg^{-1} consumed at an average daily dose of 20mg Kg^{-1}), increased the percentage of mortality by 5%, confirming the observation reported by Zenick *et al.* (1985) and produces certain external symptoms reflecting neurological complications as well as dose-dependent changes in the general behavior of animals due to the side effects of the chemical. Similar observations were reported previously in human and experimental animals treated with comparable doses of ACR (Spencer and Schaumburg, 1975; Sickles and Goldstein, 1985 ; Ko *et al.*, 1999; Stone *et al.*, 2001).

We believe that hind limb dysfunction in mice and the consequent inability to get food is one important reason for increased mortality. Similarly, Cabe and Cowell (1981) reported that a reasonable inference is that neuromuscular involvement reduced the ability to get food and water and late deaths may be attributed to starvation or dehydration rather than acrylamide toxicity. The morphology of peripheral nerves and related endplates of Swiss albino mice observed in our preparations was similar to that reported previously (Desaki and Uehara, 1980).

Alteration in the number of Kühne's granules was observed after high dose of acrylamide treatment. This may be responsible for limb muscle dysfunction observed during the experiment. Animals exhibiting finely coordinated muscular activity had NMJs that contained a large number of Kühne's granules which makes possible a relatively greater innervation of the muscle fibre (Gray, 1970).

From our observations, it is apparent that light microscopic findings correlate well with the ultrastructural observations and that both revealed that ACR poisoning produced obvious pathogenic changes that usually involved peripheral nerve alterations,widespread nerve terminal degeneration and disorganization as well as decreases in endplate dimensions in animals treated with 480 mg ACR Kg^{-1}. Similar findings were reported previously by DeGrandchamp and Lowndes, 1990; Stone *et al.*, 2001.

The time at which the earliest lesions have been detected in ACR neuropathy has been reported to be from 4 to 28 days after the first dose, depending on the experimental paradigm and staining technique employed (Tsujihata *et al.*, 1974; Jennekens *et al.*, 1979; Cavanagh, 1982; De Grandchamp *et al.*,1990). In this study well developed pathological changes were detected, after 30 days of treatment. Animals were not examined microscopically before 30 days of treatment so possible early pathological changes were not the object of these experiments. We also found that the motor endplates of ACR-treated groups were swollen along their entire length. It is believed that swelling in motor terminals indicates marked changes in the peripheral nerves of rats (Tanii *et al.*, 1988). Alterations of peripheral nerve myelin sheaths were also observed after high dose ACR administration. These findings are in agreement with the findings reported by Fullerton and Barnes (1966), who observed that axonal degeneration of axis cylinders and myelin sheaths in peripheral nerves affecting predominantly the distal parts.

Accessory endings of terminal nerve fibers supplying the flexor digitorum muscle revealed an ultrastructural appearance comparable to the fine structure of the neuromuscular junctions observed in frogs (Birks *et al.*, 1960). We also agree with the DeGrandchamp and Lowndes (1990), who claimed that within the terminal axoplasm the different subcellular structures are distributed in a consistent and orderly pattern. Two main areas were observed in the axoplasm, a 'mitochondrial region' adjacent to the schwann cell covering and a'vesicular region' near the muscle fibre. However, an obvious decrease in synaptic vesicle content, in degenerating terminals was observed. Diminished synaptic vesicle content has been previously reported by DeGrandchamp *et al.* (1990). These morphological abnormalities could result in diminished endplate potential frequency and reduced release of neurotransmitter. This is in agreement with Tsujihata *et al.* (1974), who correlated the scarcity of synaptic vesicles to the functional performance of synaptic terminals. They reported that functional and morphological changes in motor axon terminals may be indicative of some degree of functional denervatation.

The mechanism of action of ACR in the production of its neurotoxic effect is not understood. Morphological evidence suggested that ACR may affects cytoskeletal proteins, specifically the regulation of neurfilament turnover (DeGrandchamp and Lowndes, 1990). Our findings agree with Schaumburg *et al.* (1974) and DeGrandchamp *et al.* (1990) who observed morphological damage, confined to the distal proteins of the axons that was characterized by accumulated neurofilaments and paranodal swellings, after ACR treatment. In our ACR treated groups, significant obvious accumulation of neurofilaments was often detected at the terminals. This leads us to the suggestion that ACR may interfere with neurofilament degradation in nerve terminal and may exert an inhibitory effect on the calcium activated neutral protease (CANP) which is responsible for the degradation of neurofilaments. This suggestions agrees with the cytoskeletal model of the neuron proposed by Lasek and Hoffman (1976) in which the neurofilaments are continuously conveyed to the terminal where they are degraded by a calcium activated neutral protease (CANP). Since a column of neurofilaments more than 1 mm long is normally degraded each day at the nerve terminal (Lasek and Hoffman, 1976), neurfilament accumulation would be expected to develop rapidly after CANP inhibition. It is of interest that Jones and Cavanagh (1984) observed loss of neurofilaments in the sensory ganglionic cells following ACR intoxication.

The evidences discussed above indicate that, administration of high doses of ACR produces motor nerve terminal and endplate degeneration . We hope that our finding will be of use in future investigations of ACR toxicity.

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