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Adverse Effects of Acrylamide on the Developing Retina of Albino Rats

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ABSTRACT

The adverse effects of Acrylamide (ACR) upon the retina of two developmental stages of the albino rats aged 7 and 14 days from parturition were investigated. Three fold integrated approaches were adopted, namely histological, ultrastructural and proteomic analysis. Histological examination of the retina of the experimental offspring revealed many histopathological changes. These changes included marked loss of the building neuronal cells, massive alterations in retinal cell layers and degeneration, vacuolization and cell loss in the ganglion cell layer. At the ultrastructural level, the retina of experimental offspring exhibited many alterations in the studied development stages. These alterations included accumulation of heterochromatin at the nuclear envelope of the pigment epithelial cells, appearance of electron-dense lysosomes, deteriorated and detached microvilli. The cytoplasm was vacuolated with dispersed electron-dense particles and folding basal lamina. The chorio-capillaries disturbed and blood capillaries were swollen, degenerated retinal cell layers including pyknosis, convolution and karyolysis of the nuclei were observed. The smooth endoplasmic reticulum was vesiculated and mitochondria atrophied. The rods and cones exhibited stacked lamellar disintegration and degenerative features including the presence of heterogeneous electron-dense residual degenerated bodies. Proteomic analysis of retina of the two experimental developmental stages showed variations in the expressed proteins as a result of intoxication which illustrated the adverse toxic effects of ACR treatment.

KEY WORDS: Acrylamide; Retina; Histology; Ultrastructure; albino rats, Teratology.

INTRODUCTION

Acrylamide (ACR) is a water soluble vinyl monomer widely used to synthesize polymers for several industrial applications such as soil conditioning, wastewater treatment, cosmetic, paper and textile industries (Friedman 2003). ACR can be generated during the heating of specific foodstuffs as a result of Millard reaction between amino acids and sugars (Mottram *et al.*2002 and Totani *et al* . 2007). High levels of ACR were unexpectedly detected in widely consumed food items such as fried bread and potato chips (Zhang and Zhang 2007) and in tobacco smoke (Akarsu *et al.*2004). ACR was reported to be a neurotoxicant (LoPachin, 2004) and has been reported to induce varieties of symptoms in both the central and peripheral nervous systems together with the autonomic nervous system. These symptoms include irritation of the skin and mucous membranes, with peeling of the skin of the hands and feet, muscular weakness, paresthesia, numbness in hands, feet, arms and legs, and unsteadiness with difficulties in walking and standing (WHO, 1985). Exposure to ACR was found to produce a distal axonopathy in both humans and experimental animals (Lopachin, 2000 and Lopachin *et al*,. 2003). Other early studies demonstrated that ACR induces cumulative neurotoxicity linked to nerve terminal damage in the CNS and PNS where distal axon swelling and subsequent degeneration were considered to be the hallmark morphological features of this toxic axonopathy (Miller and Spencer 1985, LoPachin and Lehning 1994 and Lehning *et al.* 1998). At the molecular level, it has been postulated by Carlson and Weaver 1985 that covalent binding of ACR to CNS proteins may play an important role in ACR toxicity, resulting in inhibition of a number of enzymes and essential compounds. More recent studies demonstrated that this presynaptic toxicity appears to be mediated by the formation of sulfhydryl adducts on the cysteine residues of many proteins (Barber and LoPachin 2004 and LoPachin *et al.* 2007).

There is evidence that ACR may affect the visual system (Merigan 1989 and Godin *et al.* 2000). Godin *et al.,* (2000) detected abnormal papillary light reflexes in a cow accidentally exposed to ACR and N-methyloacrylamide. Ophthamoscopic examination showed progressive retinal degeneration and degenerative changes in the optic nerves head. Light and electronic microscopic examination revealed pathologic changes in the retina and optic nerves consistent with chronic stages of ACR-toxicity. Axonal swelling and later degeneration in the more central optic tract, lateral geniculate nucleus and superior colliculus were described in ACR -intoxicated animals (Schaumburg and Spencer, 1979).

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From the available literature so far, it is concluded that little works were done on the ophthalmic-toxicity of ACR. Moreover, the developmental aspects of intoxication during postnatal life are not illustrated well. This stimulates us to study the adverse effects of ACR on the developing retina of postnatal young rats maternally treated with ACR.

MATERIALS AND METHODS

Animals and Housing

All the experiments were done in compliance with our institutional guideline for the care and use of Laboratory animals. Thirty fertile virgin females and ten fertile males of Wistar albino rats weighing 100 ±5 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 in a room with good ventilation at 23 ºC. The housing room was maintained on a 12:12 h light: dark cycle. Standard diet composed of 20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5% vitaminzed starch (Egyptian Company of Oils and Soap Kafr-Elzayat Egypt) was supplied. Water was provided *ad-libitum*. Females were made pregnant by keeping them at a ratio of 1 male: 3 females with healthy fertile males for 12 hour between 8 P.M till 8 A.M. During the next morning, the prospective pregnants were examined for the presence of vaginal plugs. Vaginal smears were carried out to give a precise determination of the onset of gestation.

The pregnant rats were arranged into two groups, each was composed of 15 individuals as follows:

1- Control pregnant rats and consequently their delivered newborns until reaching 7 and 14 days old from parturition.

2- ACR -treated pregnant and mother rats: Each individual received daily oral doses of 15 mg/kg starting at the 6th day of gestation till 7 or 14 days post-partum.

Acrylamide Treatment

ACR of highest purity 99.9% (Aldrich chemical company) was used in the present study. The applied dose of 15 mg/kg body weight (Lopachin et al. 2003) was dissolved in 0.2 ml saline solution and orally dosed by gastric tube.

Light microscopic examination

The retina of postnatal youngs aged 7 and 14 days from parturition of both control and experimental ACR mothers were separated, and immediately fixed in 10% neutral formalin. The specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylol and mounted in molten paraplast 58-62 ºC. 5 μ m serial histological sections were cut, stained with Harris hematoxylin, counter stained with eosin and investigated under bright field Leitz microscope.

Transmission electron microscopic (TEM) examination:

The retina at 7 and 14 –days of both control and experimental groups were separated and immediately fixed in 2.5% glutaraldhyde and 2% paraformaldhyde in 0.1M cacodylate buffer (pH 7.4). After rinsing in 0.1M cacodylate buffer, samples were post fixed in a buffered solution of 1% osmium tetraoxide at 4ºC for 1.5 h. This was followed by dehydration in ascending grades of ethyl alcohol and embedded in epoxy-resin (Reynolds, 1963). Ultrathin sections were cut with a glass knife on a LKB microtome and mounted on formvar-coated grids, stained with uranyl acetate and lead citrate and finally examined at Joel transmission electron microscope.

Sodium Dodecyl polyacrylamides gel electrophoresis (SDS-PAGE):

A set of four individuals of both control and experimental groups were sacrificed at the end of treatment and biopsies of retina were taken and processed for SDS-PAGE according to Laemmli (1970). Electrophoresis was carried out with constant volt at 200v. The separated proteins on polyacrylamides gel were stained with coomassie blue R-250 (Andrews 1986).

RESULTS

I. Histological examination

a. Retina of 7 days old offspring

Light microscopic examination of retina of 7 day old offspring of control mothers showed that it is composed of four definite cell layers namely, nuclear, inner plexiform, ganglion layer and pigment epithelial cell layer. The outer plexiform layer appeared, separating the inner from the outer nuclear layer. Internally adjacent to the vitreous humor, the nerve fiber layer attained highly organization and possessed newly formed blood capillaries (Fig.1A). Massive alterations in cell layers are evident in retina of the experimental group. The nuclear cell layers showed dramatic degenerative lesions including pyknosis, vacuolar degeneration and karyolysis in different areas and ill-differentiated nuclear layer. The photoreceptor layer showed abundant distribution of vacuoles. The outer plexiform cell layer attained a considerable thinning in the majorities of specimens and missing in some others. Few numbers of ganglion cells were detected and form a thin layer at the periphery of the inner plexiform cell layer comparing with multicultural arrangement in control group. The nerve fiber layers became vacuolated with massive degenerative lesions. (Fig.1B).

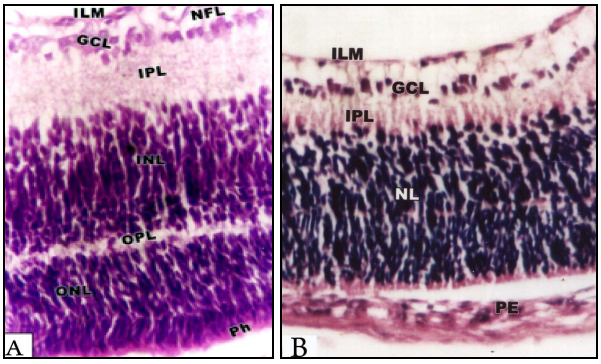

Fig.1 A: section of control retina of 7 days old rat showing, normal differentiation of retinal cell layers including inner limiting membrane (ILM), nerve fiber layer (NFL), gangilionic cell layer (GCL), thick inner plexiform layer (IPL), inner nuclear layer (INL), thin outer plexiform layer (OPL), Outer nuclear layer (ONL), Photoreceptor cell layer (Ph) and pigmented epithelium (PE).
B: section of retina of 7 days old rat of experimental mother showing massive atrophy of inner plexiform layer (IPL), ill differentiated nuclear layer (NL) associated with massive decrease of nuclear cells. (H&E X250).

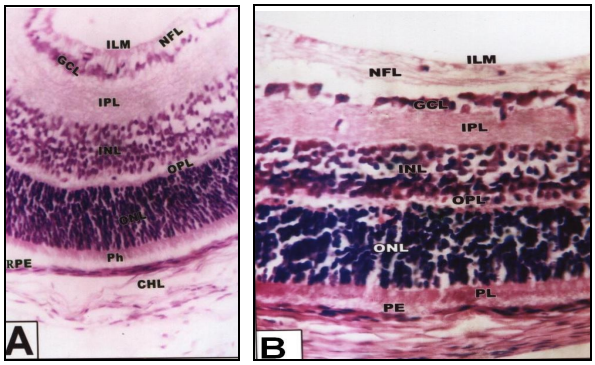

Fig.2.(A): section of control retina of 14 days old rat showing normal differentiation pattern of retinal cell layers including inner limiting membrane (ILM), nerve fiber layer (NFL), gangilionic cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor cell layer (Ph) and pigmented epithelium (PE).
B: eye region of 14 days old experimental mother showing vacuolated nerve fiber layer (NFL) and massive reduction of both inner (INL) and outer (ONL) nuclear cell layers. (H&E X 250).

b. Retina of 14 days old offspring

The retina of 14 days old offspring of control mothers composed of eight cell layers and two limiting membranes arranged from the choroid to the vitreal side as follows: cubical pigmented epithelium, rod and cone cell layers, outer nuclear cell layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer (Fig.2A). After treatment with ACR, the retina appeared comparatively reduced owing to the massive degeneration, vacuolization and cell loss in the ganglion cell layer. The outer plexiform layer was distorted in all investigated specimens. Both outer and inner nuclear cell layer showed numerical increase of pyknotic nuclei (Fig. 2B).

II. Transmission electron microscopic (TEM) results

a. Retina of 7 days old offspring

TEM examination of retina of 7 days old offspring of control mothers revealed that the pigment epithelium consists of a single layer of cubical cells with branched microvilli. They contain large oval-shaped nuclei with a remarkable abundant euchromatin. The basal surface of the pigment epithelial cell rests on a prominent basement membrane adjacent to Burch's membrane. Burch's membrane appears as a precursor of electron-dense granules semi-like lysosome was detected in the apical cytoplasmic margin of the pigment cells. Mitochondria attained marked differentiation as the ribosomes and smooth endoplasmic reticulum. Inner segment of photoreceptor made their first appearance and the outer segment was still undifferentiated. Numerous blood capillaries were detected within the nerve fiber layer and consisted of endothelial and mural cells, separated from one another by extra cellular spaces which were often enclosed by basement membrane lamellae. The capillary walls are surrounded by an outer basement membrane, which is completely unsheathed in a glial tunnel composed of the processes of Miller cells. (Fig. 3 A &B)

Retina of ACR treated offspring showed many alterations. The epithelial cells possessed vacuolar degenerative changes associated with nuclear chromatin condensation. Degenerative lesions of nuclear cell layers including pyknosis, vacuolar degeneration and karyolysis were evident. The photoreceptor layer showed abundant distribution of vacuoles. The outer plexiform cell layer attained a considerable thinning in the majorities of specimens and missing in some others. Few numbers of ganglion cells were detected and form a thin cell layer at the periphery of the inner plexiform cell layer comparing with multicultural arrangement in control. The nerve fiber layers become vacuolated with massive degenerative lesions (Fig.4 A, B &C).

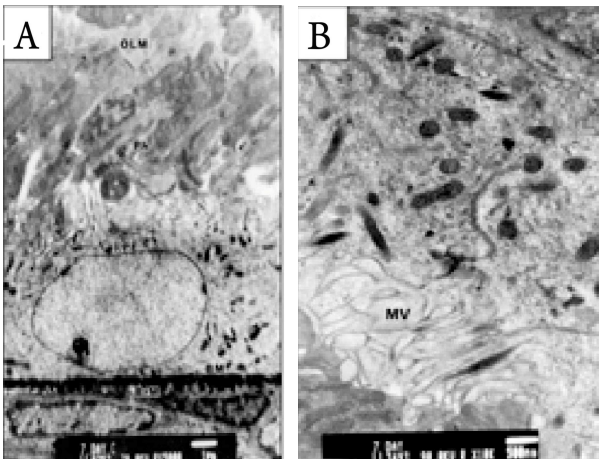

Fig.3 Transmission electron micrographs of control retina of 7 days old offspring rat A: showing retinal pigmented epithelial cells with underlying basement membrane. The cytoplasm is rich in mitochondria, ribosomes and smooth endoplasmic reticulum. The apical part characterizes by radial arranged microvilli adjacent to macrophagosome and newly formed inner segment of photoreceptors (X 7500)
B. showing branched microvilli (MV) of pigmented epithelium, phagosome and newly formed inner segment of photoreceptors (X13000).

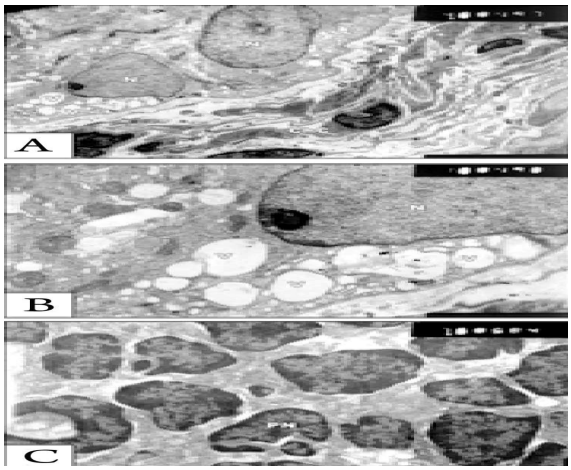

Fig.4. Transmission electron micrographs of retina of 7 day old experimental mother. A&B showing malformed pigment cell. Their cytoplasm enclosed by numerous vacuoles (V) and phagosomes. Inner segment appears degenerated in many of them.
C. showing massive degeneration of nuclear cells building nuclear layer. The nuclear cells appeared with convoluted and pyknotic nuclei (PN) and electron-dense chromatin material. (A&B X 13000-C X 7500).

b. Retina of 14 days old offspring

TEM examination of retina of 14 days old offspring of control mothers revealed that the pigment epithelium of retina attained a considerable development compared with the previous stage. The basement membrane of the pigment epithelium is firmly attached to the Burch's membrane with more differentiated choriocapillaris lined by flattened endothelial cells. While it's inner aspect has a rather fragile attachment to the rods and cones of the retina by means of cilia-like microvilli. The nuclei become spindle shaped with thin peripheral arrangement of chromatin and widespread of euchromatin. The cytoplasm is rich with cytoplasmic organelles. The photoreceptor of outer segments is interdigitated with microvilli of the pigment epithelium. The rod proper is composed of a slender inner segment filled with mitochondria, ribosomes and rosette-shaped glycogen particles. The cone proper has an inner (ellipsoid) segment with numerous mitochondria. The nuclei of photoreceptors are characterized by their unique rosette shaped dense heterochromatin. The inner plexiform layer formed of a layer of synapses of the cells bipolar layer with the next ganglion cell layers. The ganglion cells are disposed, together with some few neuralgia cells. Their nuclei contain large euchromatin with prominent nucleoli and the cytoplasm contains rough endoplasmic reticulum grouped in masses. Other organelles can be distinguished such as mitochondria, Golgi apparatus and lysosome. Dendrite cell processes make synaptic junctions with bipolar neurons and amacrine cells in the inner plexiform layer. The layer of nerve fibers consists of the axons of ganglion cells running parallel to the inner surface of the retina. (Fig. 5A & B).

Examination of retina of 14 days old offspring of ACR- treated mothers showed that the nuclear material of the pigmented epithelium is formed of electron-dense heterochromatin material. The mitochondria were less in number and showed a considerable atrophy. Vacuoles of different sizes were dispersed in cytoplasm. Numerous electron-dense lysosomes were detected in the apical part of the pigment cells. The smooth endoplasmic reticulum became vesiculated. The pigment cell basement membrane showed apparent thickening. The Burch's membrane appeared electron-dense with marked deposition of collagen fibrils with severe loss of choriocapillaris. The blood capillaries appeared swollen. Microvilli of the pigment epithelial cells detached from the apical tips of outer segments and appeared deteriorated. Other specimens exhibited pyknotic pigment nuclei associated with disruption of their cytoplasmic organelles. The outer segments of rods and cones exhibited stacked lamellar disintegration in their apical part adjacent to the pigment cells. Some of the inner compartments of the outer segment were vesiculated. Inner segments of rods and cones showed some degenerative features including the presence of atrophied mitochondria and presence of heterogeneous electron-dense residual degenerate bodies. Pyknotic nuclei were visible within the outer and inner nuclear layers. Where the whole chromatin condensed and the nucleus became darkly stained. The layer of bipolar (inner nuclear layer) expressed massive deterioration, where there were cell loss and the remaining cell nuclei showed signs of karyolysis, while the cytoplasm was completely lyses and no definite organelles could be detected (Fig.6 A&B,Fig.7A,B&C).

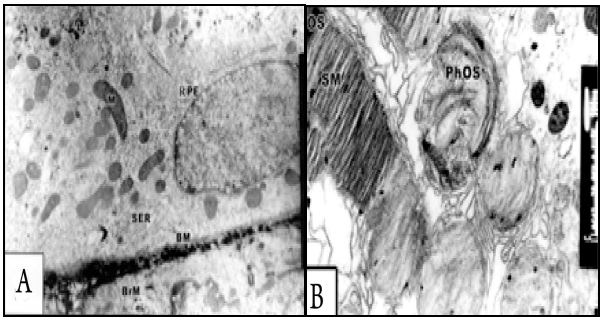

Fig.5. Transmission electron micrographs of control retina of 14 days old rat
A: showing pigmented epithelial cells having cytoplasm rich in mitochondria (M), smooth endoplasmic reticulum (SER), free ribosomes. The base show well development basement (BM) and brush membrane (BrM).
B: showing outer segment (OS) of photoreceptors with regular arrangement of stacked membranes (A&B,X 7500).

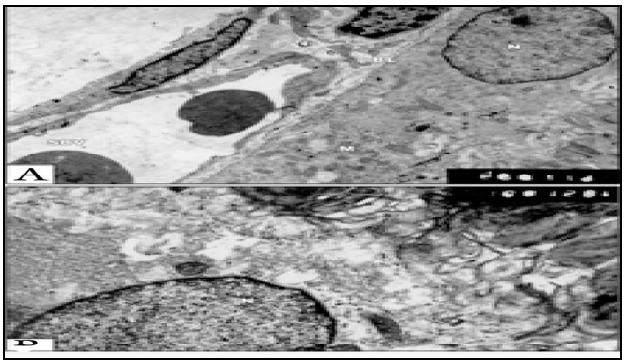

Fig.6. Transmission electron micrographs of experimental retina of 14 days old rats.
A: showing damaged pigment cell. Their cytoplasm enclosed by numerous vacuoles and phagosomes. The underlying chorio-capillaries are swollen.
B. showing pigmented epithelium with vacuolated cytoplasm and degeneration of outer segment of photoreceptors (A&B X 13000).

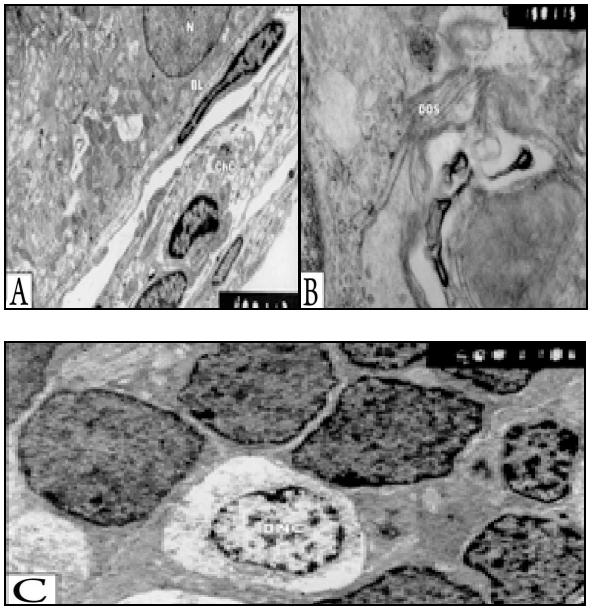

Fig.7. Transmission electron micrographs of experimental retina of 14 days old rats. Showing A: pigmented epithelium with vacuolated cytoplasm vesiculated and fragmented rough endoplasmic reticulum. The chorio-capillaries (ChC) appear swollen.
B: showing degenerated outer segment of photoreceptors (DOS).
C: showing degenerated outer nuclear cells (DNC). (AX 13000, B X 15000 & CX 10000).

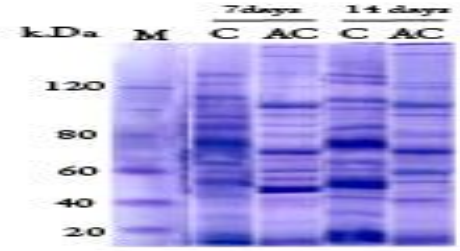

Fig.8.SDS-PAGE of retina of 7 and 14 -days old rats offspring of both control and those maternally treated with acrylamide (M: Marker, C: control, AC: acrylamide).

III. Proteomic (SDS-PAGE) analysis of retina

Total protein bands are illustrated in tables (1&2) and Figure 8. After fractionation by polyacrylamide gel electrophoresis marked differences in pattern were observed between experimental and control groups. Proteomic analysis of experimental retina showed variations in expressed proteins. 7 and 14 days old rats maternally treated with ACR, showed lack expression of protein or expression of new stressed proteins as a result of intoxication which illustrate the adverse toxic effects of ACR.

DISCUSSION

Vision is a complex sensory process permitting eye-bearing organisms to detect the features of any object in their environment such as size, color, orientation, motion, and distance. The first steps in this process take place in the retina. The present findings revealed that the eye is sensitive to the cytotoxicity of ACR-treatment during both fetal and breast-feeding periods. The adverse effects caused an ascending drastic alteration of retina of 7 and 14 days offspring. Because of the high cell-replication rates during foetus development, trans-placental exposure of neonates to ACR might raise concerns (Schettgen *et al.* 2004). It has been suggested that ACR manifested toxicity principally toward growth and development (Garey *et al.*2005, Wang *et al.* 2010).

The obtained results are in agreement with many investigators who studied the neurotoxicity of ACR. Edwards *et al.* (1991) reported that the manifestations of ACR intoxication was closely similar in human and experimental animals and were in the form of swollen axons and or decrease in numbers of large axons diameter. Soergel et al. (2002) found that from 10 to 50% of dietary ACR in pregnant women was transferred via blood through the placenta to the fetus. Breast milk was found to contain up to 18.8/mg/L of ACR. Because water soluble ACR can pass both placental and blood brain barriers, the authors suggested that to protect fetuses' pregnant women should not consume high- ACR food. Takahashi *et al.* (2008) obtained similar results after studying the effect of ACR on rat offspring. *In vivo* and *in vitro* chronic treatment of rats with ACR was found to produce a substrate-dependent, toxicologically specific inhibition of brain mitochondrial respiration. This inhibition of mitochondrial energy production might play a role in the neurotoxic mechanisms of action for ACR (Medrano and LoPachin 1989). Following administration of ACR to rats, Tandrup (2002) found that the somatofugal wave of atrophy moving distally in the axon of primary sensory neurons and led to loss of myelinated nerve fibers.

ACR exposure was found to be linked to nerve terminal damage in the central nervous system and peripheral nervous system (Lehning *et al.* 2003 and LoPachin *et al.* 2003). At the molecular level, this presynaptic toxicity appears to be mediated by the formation of sulfhydryl adducts on the cysteine residues of many proteins (Barber and LoPachin 2004; LoPachin *et al.* 2004, and 2007). Quantitative analyse of whole-brain synaptosomes isolated from ACR-intoxicated rats revealed an accumulation of the cysteine adduct, S-(2-carboxyethyl)-cysteine (CEC) that was closely correlated to the development of neurotoxicity (Barber and LoPachin 2004). It was demonstrated that ACR can produce neurological toxicity in the absence of axonopathy; i.e. whereas equivalent neurotoxicity can be induced by intoxication over a wide range of daily dose-rates, axon degeneration in PNS and CNS occurred only during long-term exposure to lower ACR dose-rates (LoPachin *et al.* 2002; Lehning *et al.* 2003). The dissociation between neurological dysfunction and the presumed underlying morphological lesion suggests that axonopathy might not be importantly involved in the pathophysiological process leading to ACR neurotoxicity. Nerve terminals have been suggested as an alternative site of ACR action based on early structural and functional changes as in PNS and CNS (LoPachin *et al.* 2003). Morphological studies (Lehning *et al.* 2003) using a contemporary amino-cupric silver staining method to detect degenerating neurons and their processes (i.e. dendrites, axons, nerve terminals) indicated that intoxication at a higher ACR dose-rate produced selective, widespread degeneration of nerve terminals in rat brain and spinal cord regions. Intoxication at a lower dose rate was associated with initial nerve terminal degeneration followed later by pre-terminal axon degeneration.

ACR neurotoxicity may be attributed to its higher affinity to form adducts with glutathione, proteins, and DNA directly or after metabolized to its epoxide, glycidamide (2, 3-epoxy-1-propanamide). Glycidamide was found to produce severe lesions. The brain stem exhibited axonal degeneration with chromatolytic necrosis in midbrain medial and lateral reticular nuclei (Abou-Donia *et al.* 1993). In addition, the ability of ACR to form haemoglobin adduct led to dysfunction of oxygen transport causing hypoxia (Schettgen *et al.* 2004). Hypoxia-like metabolic injury led to vascular disturbance and defective microcirculation, local production of toxic metabolites such as nitric oxide (NO), which interfere with mitochondrial energy metabolism (Lassmann 2003).

Zhu *et al.*, (2008*)* reported that ACR-induced neurotoxicity may be associated with the enhancement of lipid peroxidation and reduction of the antioxidative capacity. Allam *et al.* (2011) reported that prenatal and perinatal ACR disrupts the biochemical machinery, cause oxidative stress and induce structural changes in the developing rat cerebellum. It is concluded that ACR affected developing retina in rats and this may be due to oxidative stress induced by ACR or its metabolite.

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