Original Article

A Histological and Immunohistochemically Study on the Possible Protective Action of Lithium Chloride on the Acrylamide Induced Toxicity During the Postnatal Development of Midbrain Red Nucleus in Male Albino Rats

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ABSTRACT

Introduction: Acrylamide is considered as industrial product that is worldwide used . It is identified in humans as a neurotoxic agent.

Aim of the Work: Elucidate the influence of acrylamide on postnatal development of the midbrain red nucleus following maternal administration during pregnancy and lactating periods and the possible protective role of lithium chloride.

Materials and Methods: Thirty pregnant rats were divided into three groups ; Group I: Received distilled water orally daily from the 7th day of pregnancy until day 28 after delivery. Group II & III: Acrylamide (10 mg/kg/day) was given orally to the rats from the 7th day of pregnancy until day 28 after delivery. Group III: Daily intraperitoneal injections of lithium chloride (85 mg/kg) was given concomitantly with acrylamide. The red nuclei specimens of the male pups at the following ages ; neonate ,7days ,21 days and 3 months of the three groups were taken and processed for further histological and Immunohistochemically techniques.

Results: The red nuclei of control rats at different age groups appeared as bilateral ovoid circumscribed collections of neurons on either side of the midline . It had two parts ; magnocellular and parvocellular . The acrylamide treated rats showed disturbed architecture of the red nuclei with darkly stained neurons . Immunohistochemically staining for GFAP showed increase in the number and size of GFAP-positive cells in the treated rats . Morphometric study showed that the mean number of the neurons in the treated group exhibited significant difference when compared with control group . Concomitant treatment with lithium chloride showed less obvious signs of degeneration in comparison with the acrylamide treated group.

Conclusion: Lithium chloride could be effective as a neuroprotective agent against acrylamide induced degenerative changes during the postnatal development of the midbrain red nucleus.

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Key Words: Acrylamide, lithium chloride, rat, red nucleus.

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INTRODUCTION

The red nucleus is one of extrapyramidal nuclei located in the tegmentum of the midbrain. It is divided histologically into two parts; magnocellular and the parvocellular parts[1]. A great attention has been directed to the red nucleus as it plays major role in locomotion and motor control among vertebrates[2].

Acrylamide(ACR) (CH2 CH CONH2) is considered as industrial chemical product that is worldwide used in the manufacture of synthetic organic chemicals[3]. It is used as flocculants to classify drinking and to treat industrial and municipal effluents. In addition acrylamide enter in the production of organic chemicals, dyes , cosmetics and paper products[4]. As a result of its wide industrial use , it can be therefore released to the environment and causes water pollution[5].

ACR is categorized as an industrial neurotoxic that presents in carbohydrate-rich foods which are cooked at high temperatures such as potato chips, cookies and crackers[6]. Therefore it is identified in humans as a neurotoxic agent and it is a public health issue that has been attracted a great attention[7]. It has been known that ACR mainly affects cysteine residues and the nerve terminal found on the functionally presynaptic proteins, this in turn results in in inhibition of the release of the neurotransmitter and obvious degeneration[8].

Lithium chloride(LC) is recognized as a mood-stabilizer that is used as a therapy for the bipolar affective disorder[9]. In addition it has an obvious effects in the recovery state from both unipolar and bipolar depression[10]. In the recent years ,various evidence denotes to the obvious neuroprotective effect of the LC[11]. In addition, it was reported that therapeutic doses of LC markedly increases both the differentiation and the proliferation of neural progenitor in *vitro*[12].

The worldwide use of ACR in many industrial fields, furthermore its presence in the cooked carbohydrate-rich

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foods which are widely consumed makes it an interesting matter to examine the harmful effects of ACR on the postnatal development of the midbrain red nucleus and the possible neuroprotection of LC . So the aim of this study is to elucidate the influence of ACR on the postnatal development of the midbrain red nucleus following maternal administration during pregnancy and lactating periods and the possible protective role of LC

MATERIALS AND METHODS

Chemicals

The chemicals which were used in that work were of high analytical grade. Acrylamide was present in the form of white powder (99% purity) and was purchased from Sigma Chemical Company (St Louis, MO, USA). Lithium chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals used in this study were of the highest purity commercially available.

As regard glial fibrillary acidic protein (GFAP); the primary antibody, it was purchased from (Thermo scientific company, USA).

Experimental Animals

Thirty adult virgin females and fifteen adult male albino rats of Sprague-Dawley strain, weighing about 180-200 gm were used in this study. These animals were obtained from the animal house of Assiut University and are kept in cages with particular care and hygiene at a temperature of (20 ± 2ºC) with a natural 12-h light/dark cycle. The cages contained fine wood bedding which was changed three times every week. Food and water were given to the rats ad libitium . The male rats were separated from the female rats for three weeks in order to be sure that the female ones were not pregnant. Daily vaginal smears were taken from virgin females and examined for the determination of the estrous cycle. Cornified cells were detected in the estrous females. Mating was allowed in the ratio of 2 females to one male[13]. The techniques of this experiment were conducted according the international rules of the care and the use of laboratory animals[14].

Experimental protocol

The pregnant female rats were divided randomly into three groups; each group contained ten pregnant female rats:

Group I (Control group): Distilled water was given to this group orally daily by gastric tube from the 7th day of pregnancy until day 28 after delivery.

Group II (Acrylamide treated group): Acrylamide in a dose of 10 mg/kg/day was given orally to the pregnant rats from the 7th day of pregnancy until day 28 after delivery by means of gastric tube[15,16]. Acrylamide was available as white powder and was dissolved in distilled water.

Group III (Acrylamide / Lithium chloride -treated group): As regard this group, acrylamide in a dose of 10 mg/kg/day was given orally by the way of gastric tube from the 7th day of pregnancy until day 28 after delivery[16] concomitantly with daily intraperitoneal injections of 1.0 mL of 85 mg/kg lithium chloride[17].

At the end of experiment, six male pups at the following ages; neonate, 7days, 21 days and 3 months of the three groups were anaesthetized by inhalation of ether. Then, their chest wall was opened, the rats were intracardially perfused through the left ventricle with saline followed by 4% paraformaldehyde solution. The descending aorta of the animals was ligated and the right atrium was opened once the perfusion began. This process was continued until the blood flow from the right atrium became clear . The rats that were processed for study with the electron microscope were perfused with glutaraldehyde after saline. The brains of the animals were removed. from the skulls[18].

Histological study

To perform the light microscopic study, the brains of the rats of the three groups were fixed in formalin (10%) for two days. Then the brains were dehydrated by the means of ascending concentrations of alcohol, cleared by using xylene and finally embedded in paraffin. Serial sections in a coronal direction (5µm in thickness) were processed to be studied by Einarson's Gallocyanine stain for the demonstration of the cytoarchitecture of the neuronal nuclei and nucleoli[19].

The red nuclei of the animals' brain of the three months age group was dissected by a coronal cut just anterior to the cerebral peduncle and another cut just behind it. 1X1 mm specimens were fixed in phosphate buffered gluteraldhyde for about 24 hours . Then the specimens were post fixed in 1% osmium tetroxide for one hour. Semi-thin sections of 1µm thickness were obtained to be stained with toluidine blue. Ultrathin sections , about 50-60 nm thickness of the selected area were prepared by the use of ultra-microtome and contrasted with lead citrate and uranyl acetate[20]. The ultrathin sections were photographed with transmission electron microscope (Joel- JEM- 100 CXII; Joel, Tokyo, Japan) in the Electron Microscopic Unit of Assiut University.

Immunohistochemically study

The previously prepared paraffin sections were cut serially at 5um thickness, then mounted on coated slides to be stained with modified avidin-biotin peroxidase method used for the glial fibrillary acidic protein (GFAP) to detect the astrocytes . Deparaffinization and hydration of the sections were done . Then the sections were further treated by using 0.01 mol/l citrate buffer (PH 6.0) for about 10 minutes to unmask antigen. They were incubated in 0.3% H2O2 for about half an hour to prohibit the activity of endogenous peroxidase before the blocking with 5% horse serum for 1-2h at the room temperature for the inhibition of the non-specific immunoreaction. Incubation of the slides with the primary antibody(1:100 monoclonal mouse anti GFAP) for 18-20h, except for negative control; then the slides were washed and incubated with

biotinylated secondary antibodies and then with avidin - biotin complex. At the end, sections were prepared with 0.05% diaminobenzidine slides , were processed to be counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted. The glial fibrillary acidic protein positive cells appeared to be brown. For the detection of the negative control, the experiments were done by the incubation of the slides without the primary antibody; so no immune reaction was occurred[21].

Morphometric and Statistical studies

Morphometric study was done by using gallocyanine-stained sections to count the number of the neurons in the M part of the red nucleus in the three months of different groups . An image analysis system (Leica Q500 MC, Leica Microsystems Germany) was used. Ten non overlapping areas that belong to five rats for each group were chosen at magnification (× 400). The measured data were declared in the form of mean values ± standard deviation (SD) and then analyzed by the aid of the software statistical package of social science 'version 16' SPSS (Chicago, Illinois, USA). Mann-Whitney test was done in order to compare the means results of the different groups. As regard the level of significance, *P- Value* ≤ 0.05 was considered to be significant[22].

RESULTS

Histological results

Light microscopic results

Newly born albino rat

Sections stained with gallocyanine stain revealed that the red nuclei of the control group appeared as bilateral ovoid circumscribed collections of neurons on either side of the midline . They were deep in the tegmentum of the midbrain , dorsomedial to the substantia nigra . The red nucleus was divided into two parts ; the magnocellular (M) and the parvocellular (P) parts. The two parts were well differentiated with the presence of neurons of varying sizes in each part. The P part was present in the rostral part of the red nucleus (Figure 1A) .The M part was detected in the caudal pole of the nucleus and had relatively large sized neurons and few medium and small sized cells (Figures 1A,4A) . The large cells were spherical in shape with well-defined nuclei and prominent nucleoli . Neuronal processes were ill defined at this age (Figure 4A).

As regard the acrylamide treated group , light microscopic examination revealed that the red nuclei were hardly differentiated from the surrounding neural tissue as compared to the control group of the same age . The two parts of the red nucleus ; M and P parts were ill differentiated and presented with numerous degenerated neurons (Figure 2A) . The cellular architecture of the M part of the red nucleus showed numerous degenerated neurons with darkly stained nuclei .In addition many patchy area of vacuolation were obvious (Figure 5A).

Some sort of protection was obviously noticed in the rats received lithium chloride in concomitant with acrylamide. Gallocyanine stained sections revealed the position of the red nuclei deep in the tegmentum of the midbrain, dorsomedial to the substantia nigra. The red nucleus was distinctly differentiated into two parts; the M part in the caudal pole of the nucleus and the P part in the rostral pole of the nucleus (Figure 3A). The M part was appeared with some normal neurons; large sized neurons and few medium and small sized neurons ,in addition to few degenerated ones . The large neurons were declared with their characteristic spherical shape, well-defined nuclei and prominent nucleoli (Figure 6A) .

Seven days old albino rats

Gallocyanine stain stained sections of the control rat showed the two parts of the red nucleus ; M and P parts . At this age slight increase in the size of the red nucleus as compared with the previous age (Figure 1B). Large , medium and small sized cells were identified within the M part .These cells had nuclei with prominent nucleoli. Cell processes were seen .These cells are rich in Nissl's granules in comparison to the previous age (Figure 4B).

Acrylamide treated group showed disrupted architecture of the red nuclei with ill differentiated M and P parts . Noticeable darkly stained neurons of the M part of the red nucleus were detected (Figure 2B) . The cellular architecture of the M part of the red nucleus was severely disrupted with the presence of numerous shrunken neurons with distorted lysed nuclei. In comparison to the control group of the same age group , the nerve cells seemed to be poor with Nissl's granules . Cells with darkly stained pyknotic nuclei were shown .Patchy areas of intercellular vacuolization were noticed (Figure 5B).

In the acrylamide / lithium chloride -treated rats, the normal histological character of the red nucleus was conserved. The M and P parts of the red nucleus were detected. Slight increase in the size of the red nucleus as compared with the previous age (Figure 3B) .Within the M part, large neurons with well-defined nuclei and prominent nucleoli could be observed in addition to medium and small sized cells. These observed neurons were present with abundant amount of Nissl's granules. Some neurons with distorted nuclei were still detected within the cellular architecture of the M part (Figure 6B).

Twenty one days old albino rats

Sections stained with gallocyanine stain demonstrated further increase in the size of the red nucleus . The M part had larger neurons as compared with the previous age (Figure 1C) . The cells; large , medium and small sized , had large amount of the cytoplasm with abundant amount of the Nissl's granules. Notice that presence of large vesicular nuclei with prominent nuclei in the large cell type. Cell processes and neuroglia cells were also detected

(Figure 4C).

Acrylamide treated group declared apparent shrunken of the red nuclei in comparison with the control group of the same age group . The M part of the red nucleus had densely stained neurons that were surrounded with vacuolated areas (Figure 2C) . The cellular architecture of the M part was represented with shrunken neurons with rarified nuclei . Some neurons appeared as ghosts with absence of their nuclei . Scanty amount of Nissl's granules was noticed in comparison to the corresponding control group (Figure 5C).

In the animal group treated with acrylamide and lithium chloride , the red nucleus architecture declared noticeable improvement. It seemed to be more or less comparable to the control group of the same age. The M and P parts were shown (Figure 3C). The large neurons of the M part had more or less normal appearance; large vesicular nuclei with prominent nuclei with abundant amount of Nissl's granules. Medium and small sized cells could be detected. Cell processes were also detected. Notice the presence of few shrunken neurons with hardly identified nuclei (Figure 6C).

Three months old albino rats

Sections stained with gallocyanine stain of the control group showed the size of the red nucleus increased in comparison with the previous age. The two parts of the red nucleus; M and P were well differentiated (Figure 1D) . Large well-differentiated nerve cells could be seen in the M part mostly multipolar , some of them were pyramidal and fusiform. The cytoplasm of these cells were darkly stained as a result of increase the amount of Nissl's granules .Their nuclei appeared with homogenous distribution of the chromatin with the presence of prominent nucleoli .Nerve cell processes and neuroglia cells with well-defined nuclei were observed . The medium -sized and small cells at this age had darkly stained cytoplasm . At this age the neurons were widely separated as compared with the previous age (Figure 4D).

Examination of the rats treated with acrylamide declared severely disruption of the architecture of the red nuclei that were obviously shrunken . The M and P parts were poorly differentiated with the presence of extensive vacuolation and darkly stained nuclei (Figure 2D).The M part of the red nucleus had numerous distorted neurons. Some neurons were irregular in shape and had densely stained pyknotic nuclei. Neurons that were surrounded with empty spaces could be detected. Furthermore numerous vacuolated areas were obvious (Figure 5D).

Concomitant treatment with Lithium chloride showed less obvious signs of degeneration in comparison with the acrylamide treated group. The red nucleus was distinctly differentiated into M and P parts (Figure 3D). Multipolar, pyramidal and fusiform neurons were present in the M part. These neurons had well-defined nuclei with prominent nucleoli and darkly stained cytoplasm with abundant Nissl's granules. Few areas was presented with degenerated neurons (Figure 6D).

Sections stained with toluidine blue of the control adult rats showed the presence of normal histological architecture of the M part of the red nucleus. Nerve cells, myelinated nerve fibers and neuroglial cells scattered in between nerve cells could be observed within the M part. The large cell type was characterized with the presence of well-defined round heterochromatic nuclei with peripheral condensation of the chromatin and prominent nucleoli. Numerous Nissl's granules in the cytoplasm were noticed. Indented nucleus with prominent nucleolus was present in the medium-sized cell type (Figure 7A). The treated rats showed interrupted histological structure of the M part of the red nucleus .The large cell type had darkly stained nuclei and vacuolated cytoplasm . Numerous neuroglial cells were seen scattered in between the nerve cells . Nerve fibers with disrupted thick myelin sheath were noticed . The medium-sized cells appeared with rarified nuclei and absent nucleoli (Figure 7B).

Sections stained with toluidine blue of the adult rats that were received acrylamide in concomitant with Lithium chloride declared normal histological appearance to a great extent . The M part of the red nucleus presented nerve cells that were more or less similar to the control . In addition to myelinated nerve fibers and neuroglial cells. The large cells had round heterochromatic nuclei with peripheral chromatin condensation and prominent nucleoli. The medium-sized cell type were presented with indented nuclei with prominent nucleoli. Notice the presence of few disrupted cells with degenerative changes ; rarified nuclei and vacuolated cytoplasm (Figure 7C).

Ultrastructural results

Electron microscopic examination of magnocellular part of the red nucleus of three months old rats revealed the presence of large and medium-sized cells. The large cells had heterochromatic rounded nuclei with peripheral chromatin condensation. Their cytoplasm was abundant with rough endoplasmic reticulum ,mitochondria and numerous free ribosomes. As regard the medium-sized cells, they were presented with heterochromatic nuclei with indented nuclear envelope. The cytoplasm of these cells was characterized with numerous free ribosomes, rough endoplasmic reticulum and mitochondria. Numerous axosomatic synapses were detected as several synaptic contacts with the soma of the nerve cells . The synaptic terminals presented numerous synaptic vesicles . Myelinated nerve fibers appeared as lamellar structure of myelin which wrapped regularly around the axons could be identified (Figures 8 A1,A2).

Obvious neurodegenerative deficits were noticed by the ultrastructure examination of the three months treated rats; the large cells appeared with shrunken nuclei with irregular nuclear membrane. The cytoplasm was rarified with ill-defined cell organelles and marked vacuolated areas. As regard the medium-sized cells , their nuclei

showed peripheral chromatin condensation with marked invagination of the nuclear envelope. They had degenerated vacuolated cytoplasm. Some mitochondria were swollen and had disrupted cristae. The synaptic terminals of the axosomatic synapses showed absence of the synaptic vesicles . The nerve fibers declared disrupted thick myelin sheath (Figures 8 B1,B2).

Electron microscopic examination of magnocellular part of the red nucleus of three months old rats treated with acrylamide in concomitant with Lithium chloride declared histological appearance that more or less similar to the control. The large cells showed heterochromatic rounded nuclei. Their cytoplasm has rough endoplasmic reticulum ,mitochondria and numerous free ribosomes. Some dilated rough endoplasmic reticulum could be noticed. The medium-sized cells could be noticed with the presence of heterochromatic nuclei with indented nuclear envelope. Their cytoplasm had rough endoplasmic reticulum, ribosomes and mitochondria. The synaptic terminals of the axosomatic synapses presented with synaptic vesicles. Myelinated nerve fibers appeared more or less similar to the control (Figures 8 C1,C2).

Immunohistochemically results

Immunohistochemically staining for GFAP in the three months old rats displayed few GFAP-positive cells with short thin processes in the control group (Figure 9A). Apparent increase in the number and size of GFAP-positive cells in the treated group as compared with the control group. The detected GFAP positive cells appeared to be large with long, thick and branched processes (Figure 9B). Acrylamide / Lithium chloride -treated group revealed positive expression of GFAP that was nearly similar to that noticed in the control group (Figure 9C).

Morphometric results

In the three months control rats , the mean number of the neurons in the M part of the red nucleus was 75.50 ± 1.37. A significant difference was observed when compared the mean number of the neurons of the control group with the mean number of the neurons of the corresponding treated group . The mean number of the neurons in the treated group was 34.00 ± 1.00 . As regard the Acrylamide / Lithium chloride -treated group , the mean number of the neurons showed insignificant difference when compared with the corresponding control group (Table 1and Histogram 1).

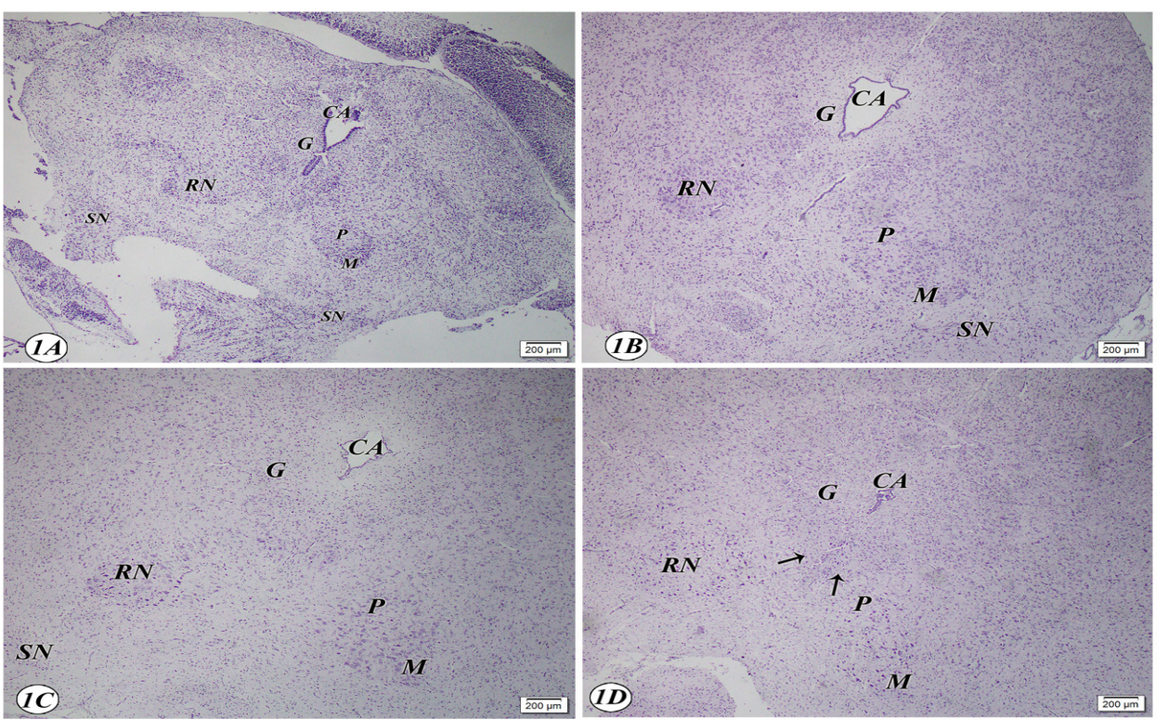


Fig. 1: A photomicrograph of a coronal section in the midbrain showing the position of the red nuclei of the control groups . (1A) Newly born : The red nuclei (RN) appear on either side of the midline as bilateral ovoid circumscribed collections of neurons. They are situated deep in the tegmentum of the midbrain , dorsomedial to the substantia nigra (SN) . The red nucleus has two parts; the magnocellular (M) and the parvocellular (P) parts. Cerebral aqueduct (CA) and periaqueductal gray (G) are shown. Notice the presence of the (P) in the rostral part of the red nucleus .The (M) part is detected in the caudal pole of the nucleus. (1B) Seven days: At this age slight increase in the size of the red nuclei (RN) in comparison with the previous age . Notice the two parts of the red nucleus (RN) ;(M) and (P) parts . Cerebral aqueduct (CA),periaqueductal gray (G) and (SN) are shown. (1C) Twenty one days: The (M) and the (P) parts are apparent in the red nucleus (RN) . Cerebral aqueduct (CA),periaqueductal gray (G) and (SN) are obvious . (1D)Three months : Showing the two parts of the red nucleus (RN);(M) and (P) . As compared with the previous age groups the size of the red nucleus is apparently increases . Cerebral aqueduct (CA) and periaqueductal gray (G) are obvious .Arrows point to the oculomotor nuclei . Gallocyanine stain, X40.

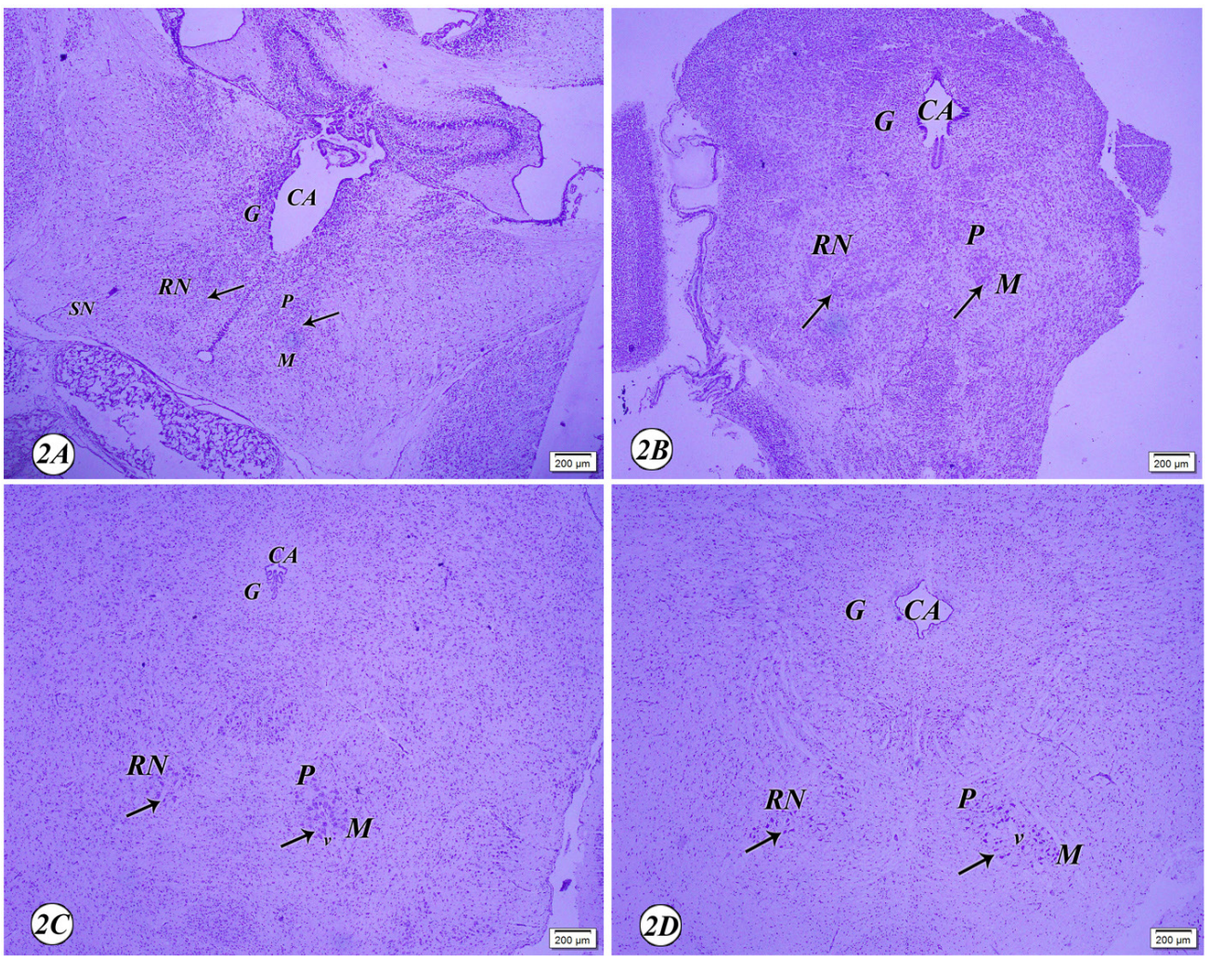


Fig. 2: A photomicrograph of a coronal section in the midbrain showing the red nuclei of the acrylamide treated groups .(2A) Newly born: The red nuclei (RN) are hardly differentiated from the surrounding neural tissue as compared to the control group of the same age . The two parts of the red nucleus ; (M) and (P) parts are ill differentiated and presented with numerous degenerated neurons (arrows) . Cerebral aqueduct (CA),periaqueductal gray (G) and (SN) are shown (2B) Seven days: Disrupted architecture of the red nuclei(RN) with ill differentiated (M)and (P) parts are observed . Noticeable darkly stained neurons (arrows) of the M part of the red nucleus were detected .Cerebral aqueduct (CA) and periaqueductal gray (G) are shown . (2C): Twenty one days: Apparent shrunken of the red nuclei (RN) in comparison with the control group of the same age is noticed . The (M) part of the red nucleus has densely stained neurons (arrows) that were surrounded with vacuolated areas(v) .(P) , Cerebral aqueduct (CA) and periaqueductal gray (G) are obvious . (2D) Three months : Severe disruption of the architecture of the red nuclei(RN) that are obviously shrunken . The (M) and (P) parts are poorly differentiated with the presence of extensive vacuolation (v) and darkly stained nuclei (arrows) . Cerebral aqueduct (CA) and periaqueductal gray (G) are shown. Gallocyanine stain, X40.

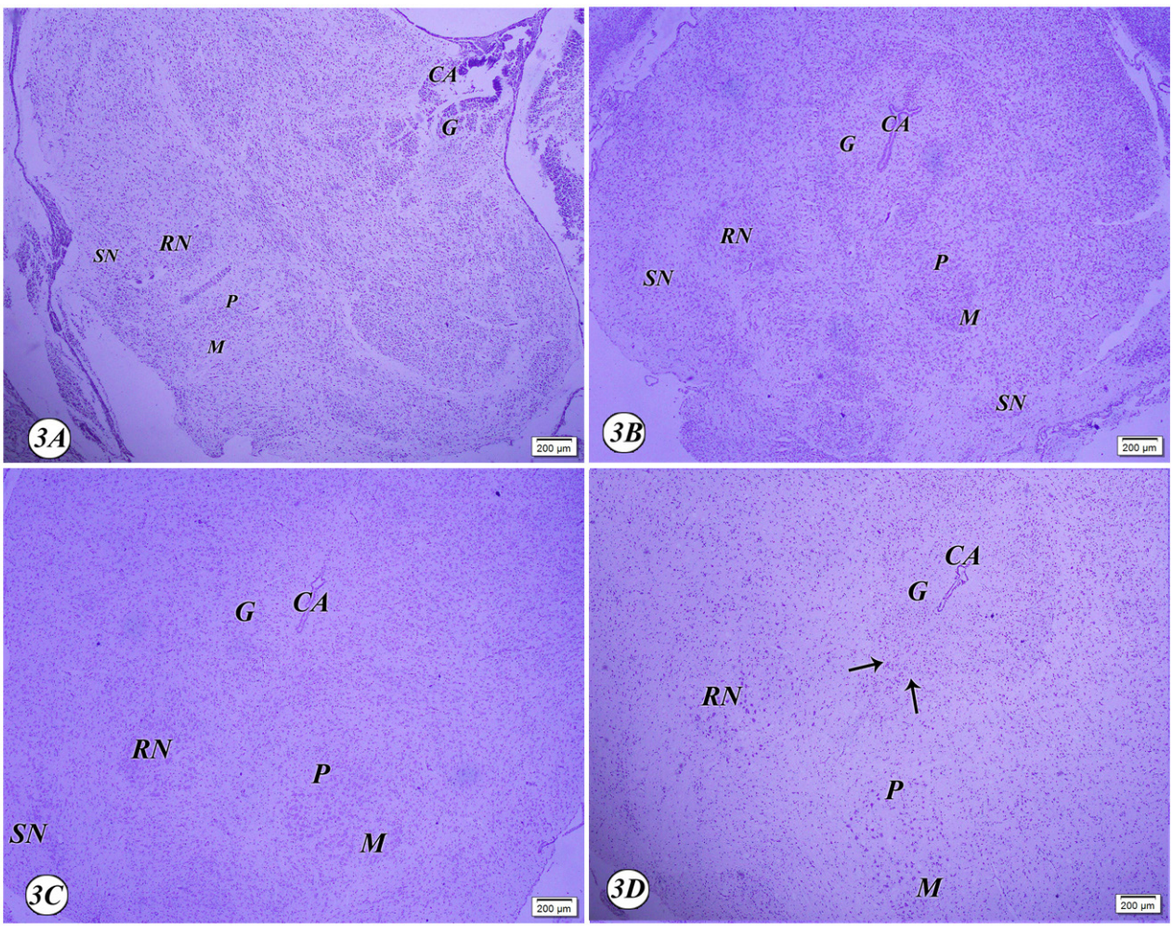


Fig. 3: A photomicrograph of a coronal section in the midbrain showing the red nuclei of the Acrylamide / Lithium chloride -treated group .(3A) Newly born : The red nuclei (RN) are deep in the tegmentum of the midbrain, dorsomedial to the substantia nigra(SN). The red nucleus is differentiated into two parts ;the (M) part in the caudal pole of the nucleus and the (P) part in the rostral part of the nucleus . Notice the (CA) and (G). (3B) Seven days: Conservation of normal histological character of the red nucleus(RN) with slight increase in size as compared to the previous age . The (M) and (P) parts of the red nucleus are detected. (CA), (G) and (SN) are shown. (3C) Twenty one days: The red nucleus (RN) architecture declares noticeable improvement and it seems to be more or less comparable to the control group of the same age . The (M) and (P) parts are detected . (CA), (G) and (SN) are obvious . (3D) Three months: The red nucleus (RN) is distinctly differentiated into (M) and (P) parts . (CA) and (G) are obvious . Arrows point to the oculomotor nuclei. Gallocyanine stain, X40.

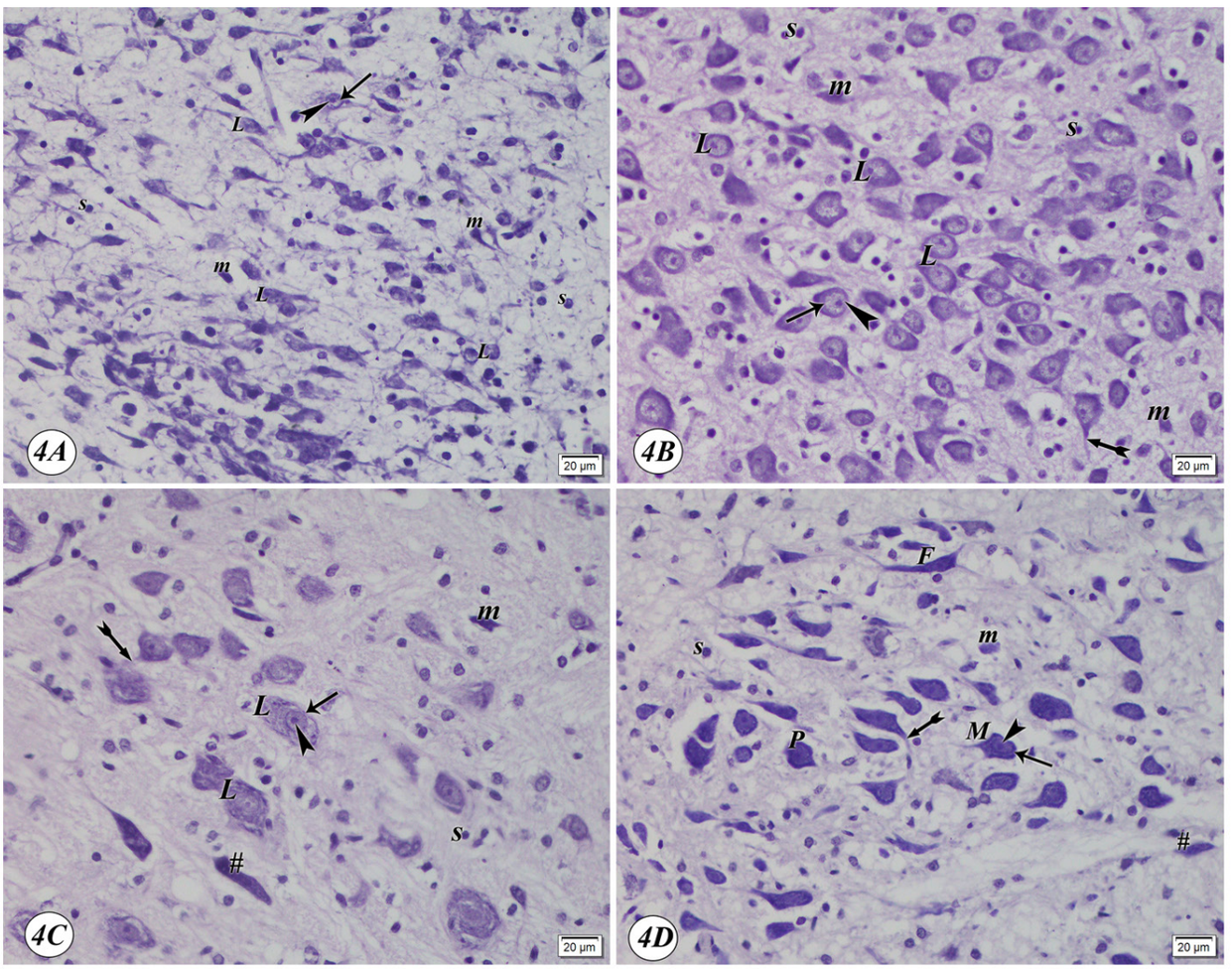


Fig. 4: A photomicrograph of a coronal section in the midbrain showing M part of the red nuclei of the control groups .(4A) Newly born: The M part has relatively large sized neurons(L) and few medium(m) and small (s) sized cells . The large cells are spherical in shape with well-defined nuclei (arrow) and prominent nucleoli(arrow head) . At this age the neuronal processes are ill defined . (4B) Seven days: Large (L), medium (m) and small sized (s)cells are identified .These cells have nuclei(arrow) with prominent nucleoli (arrow head). Cell processes (tailed arrow) are seen .These cells are rich in Nissl's granules in comparison to the previous age . (4C) Twenty one days : The M part has larger neurons as compared with the previous age . Large (L) , medium (m) and small sized(s) cells with large amount of the cytoplasm with abundant amount Nissl's granules. Notice that presence of large vesicular nuclei (arrow) with prominent nuclei (arrow head) in the large cell type . Cell processes (tailed arrow) and neuroglia cells(#) are also detected . (4D) Three months: Large well-differentiated neurons are seen in the M part mostly multipolar (M) , some of them are pyramidal(P) and fusiform(F). The amount of Nissl's granules is increased .Their nuclei (arrow) appear with homogenous distribution of the chromatin with the presence of prominent nucleoli(arrow head) .Nerve cell processes(tailed arrow) and neuroglia cells(#) with well-defined nuclei can be observed . The medium -sized (m) and small cells (s) at this age had darkly stained cytoplasm . The neurons are widely separated as compared with the previous age. Gallocyanine stain, X400.

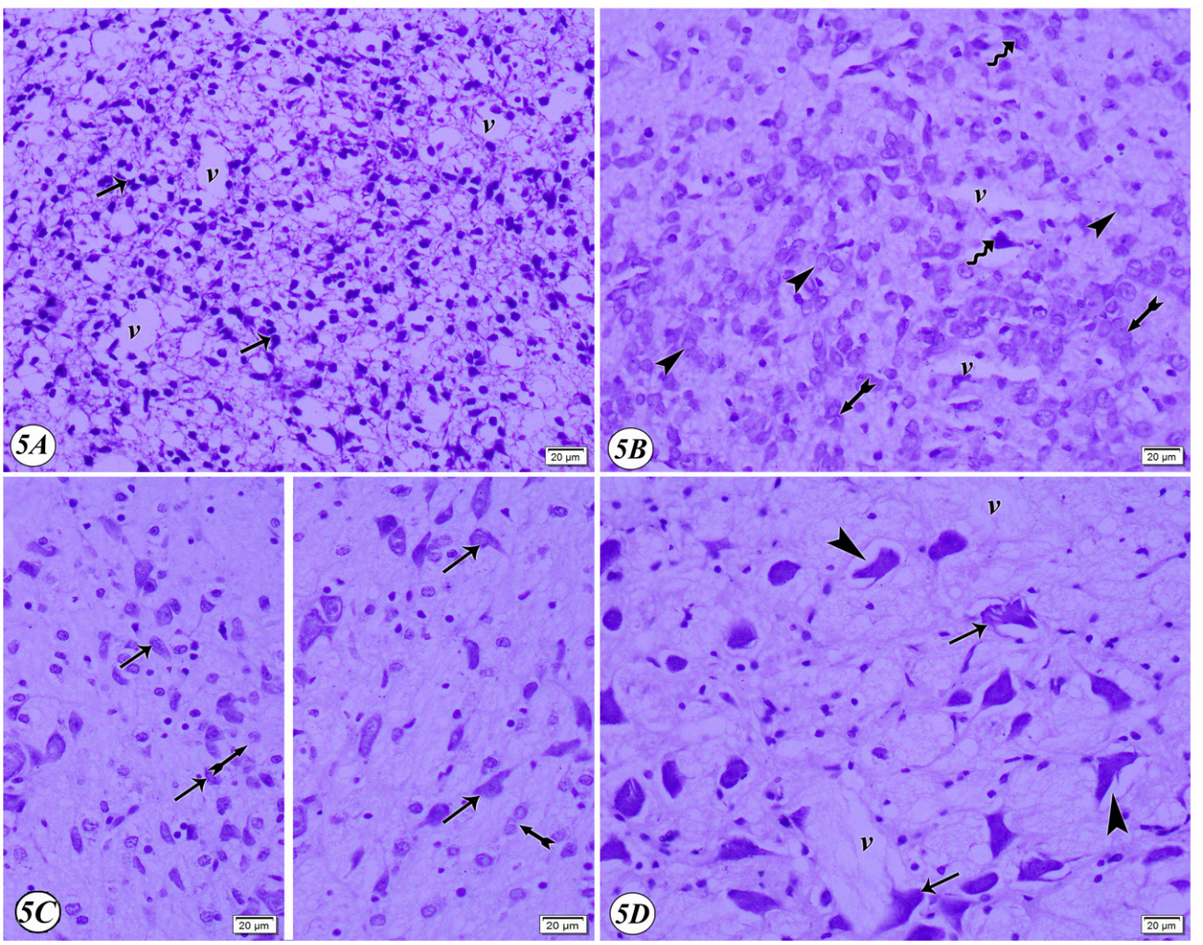


Fig. 5: A photomicrograph of a coronal section in the midbrain showing M part of the red nuclei of the acrylamide treated groups . (5A) Newly born: The cellular architecture of the M part of the red nucleus show numerous degenerated neurons with darkly stained nuclei (arrows) .In addition many patchy area of vacuolation (v) are obvious . (5B) Seven days: The cellular architecture of the M part of the red nucleus is severely disrupted with the presence of numerous shrunken neurons with distorted lysed nuclei (arrow heads). In comparison to the control group of the same age group , the nerve cells seem to be poor with Nissl's granules (tailed arrows) .Cells with darkly stained pyknotic nuclei are shown (wavy arrows). Patchy areas of intercellular vacuolization(v) are noticed . (5C) Twenty one days: The cellular architecture of the M part has shrunken neurons with rarified nuclei (arrows). Some neurons appear as ghosts with absence of their nuclei(tailed arrows) . Scanty amount of Nissl's granules is noticed in comparison to the corresponding control group. (5D) Three months: The M part of the red nucleus had numerous distorted neurons. Some neurons are irregular in shape and have densely stained pyknotic nuclei (arrows). Notice the presence of some neurons that were surrounded with empty spaces(arrow heads). Furthermore numerous vacuolated areas were obvious (v). Gallocyanine stain, X400.

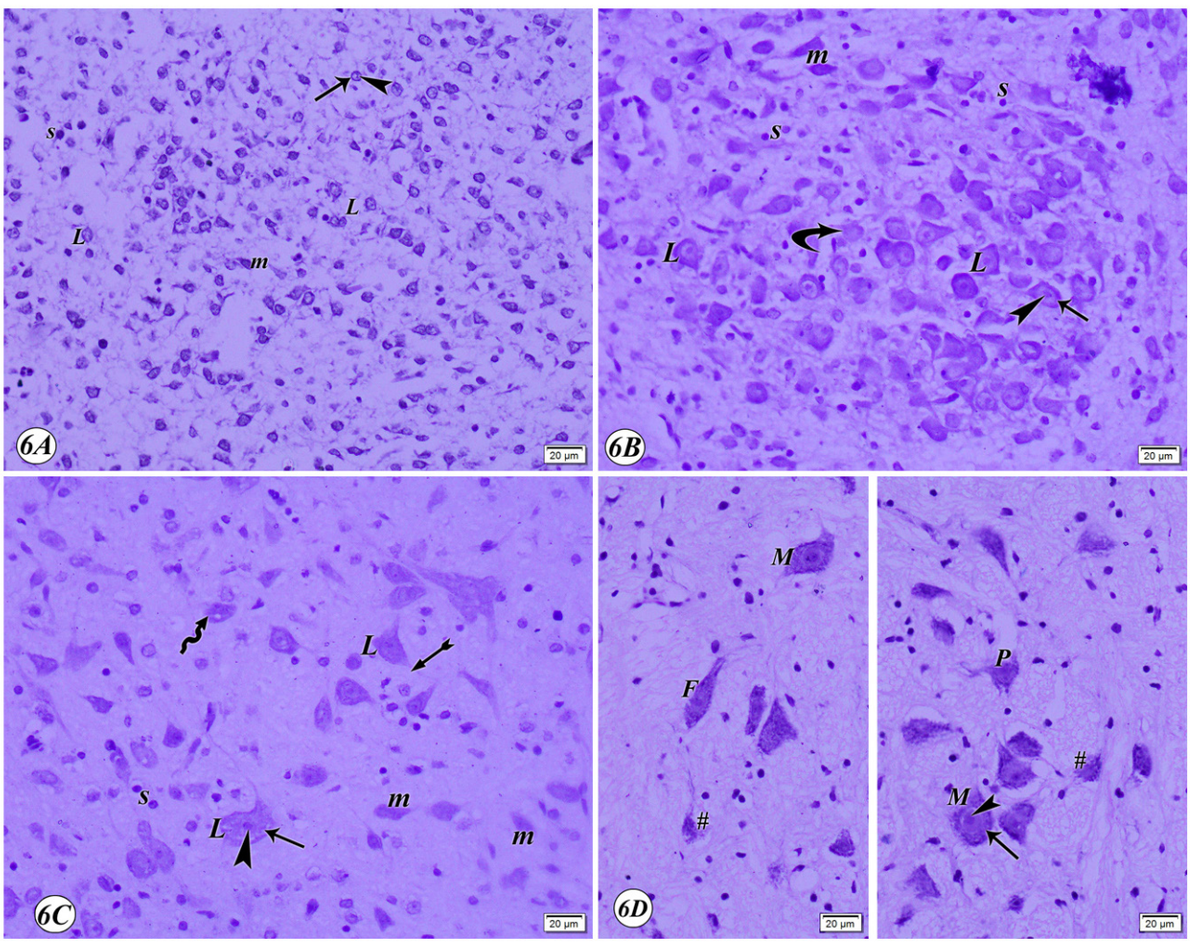


Fig. 6: A photomicrograph of a coronal section in the midbrain showing the M part of the red nuclei of the Acrylamide / Lithium chloride -treated group. (6A) Newly born : Large sized neurons(L) and few medium (m) and small(s) sized neurons are present in the M part. The large neurons were declared with their characteristic spherical shape , well-defined nuclei(arrow) and prominent nucleoli (arrow head). (6B) Seven days: Within the M part, large neurons (L) with well-defined nuclei (arrow) and prominent nucleoli (arrow head) could be observed in addition to medium (m) and small(s) sized cells. These observed neurons are present with abundant amount of Nissl's granules. Notice the presence of some neurons with distorted nuclei (curved arrow) .(6C) Twenty one days: The large neurons(L) of the M part have more or less normal appearance; large vesicular nuclei (arrow) with prominent nuclei (arrow head) with abundant amount of Nissl's granules. Medium (m) and small (s) sized cells are noticed . Cell processes are also detected (tailed arrow) .Wavy arrow points to shrunken neuron with hardly identified nucleus. (6D) Three months: Multipolar (M), pyramidal (P) and fusiform(F) neurons are present in the M part. These neurons had well-defined nuclei (arrow) with prominent nucleoli (arrow head) and darkly stained cytoplasm with abundant Nissl's granules. Some degenerated neurons (#) are noticed. Gallocyanine stain, X400.

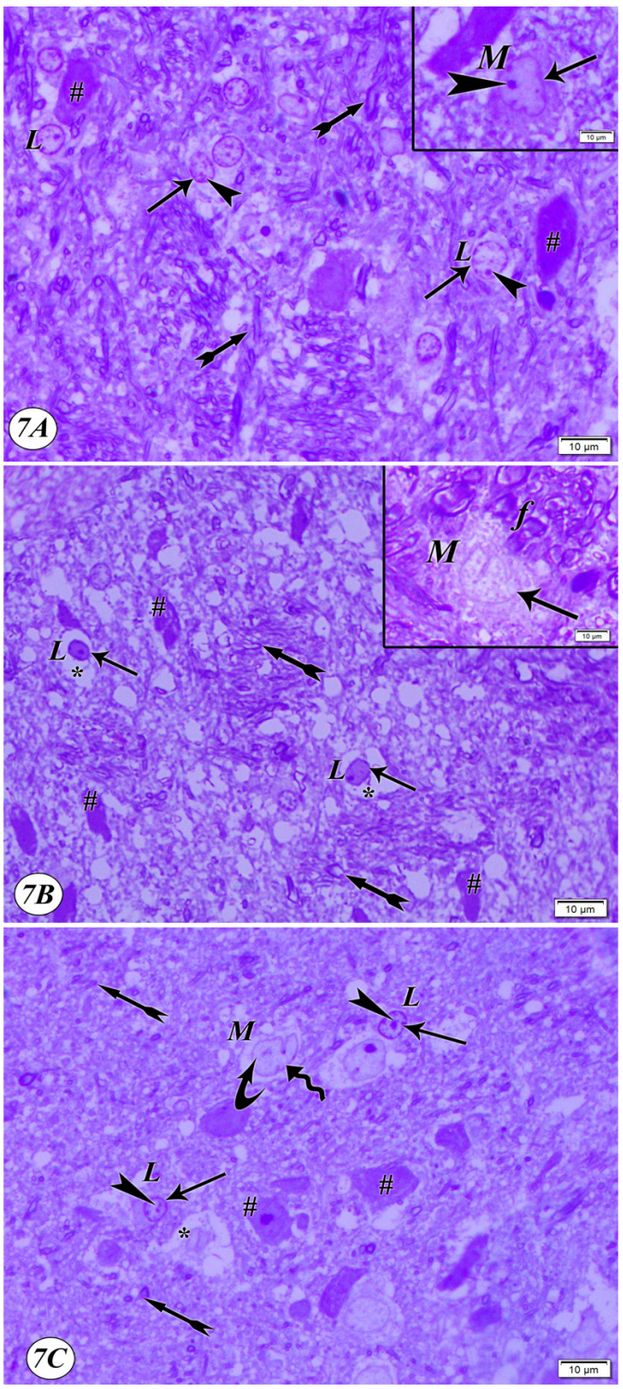


Fig. 7: Semithin sections in the midbrain showing M part of the red nuclei of the three months old rats. (7A) Control group: Showing nerve cells, myelinated nerve fibers (tailed arrows) and neuroglial cells(#) scattered in between nerve cells. The large cells (L) are characterized with the presence of well-defined round heterochromatic nuclei (arrows) with peripheral condensation of the chromatin and prominent nucleoli (arrow heads) . Numerous Nissl's granules in the cytoplasm . Inset : Shows indented nuclei (arrow) with prominent nucleoli (arrow head) in the medium-sized cell type(M). (7B) Acrylamide treated group: Showing interrupted histological structure of the M part of the red nucleus .The large cell type (L) has darkly stained nuclei (arrows) and vacuolated cytoplasm (asterisks) . Numerous neuroglial cells(#) are seen scattered in between the degenerated nerve cells . Nerve fibers with disrupted thick myelin sheath are noticed(tailed arrows) .Inset : Shows the medium-sized cell (M) with rarified nucleus and absent nucleolus (arrow). Notice the thick myelin sheath (f) (7C) Acrylamide / Lithium chloride -treated group: Normal histological appearance to a great extent are obvious. The M part of the red nucleus presented nerve cells that are more or less similar to the control . In addition to myelinated nerve (tailed arrows) fibers and neuroglial cells(#) . The large cells(L) have round heterochromatic nuclei (arrows)with peripheral chromatin condensation and prominent nucleoli(arrow heads). The medium-sized cell type (M) are presented with indented nuclei (wavy arrow) with prominent nucleoli (curved arrow). Notice the presence of few disrupted cells with degenerative changes ; rarified nuclei and vacuolated cytoplasm (asterisk) . Toluidine blue,X1000

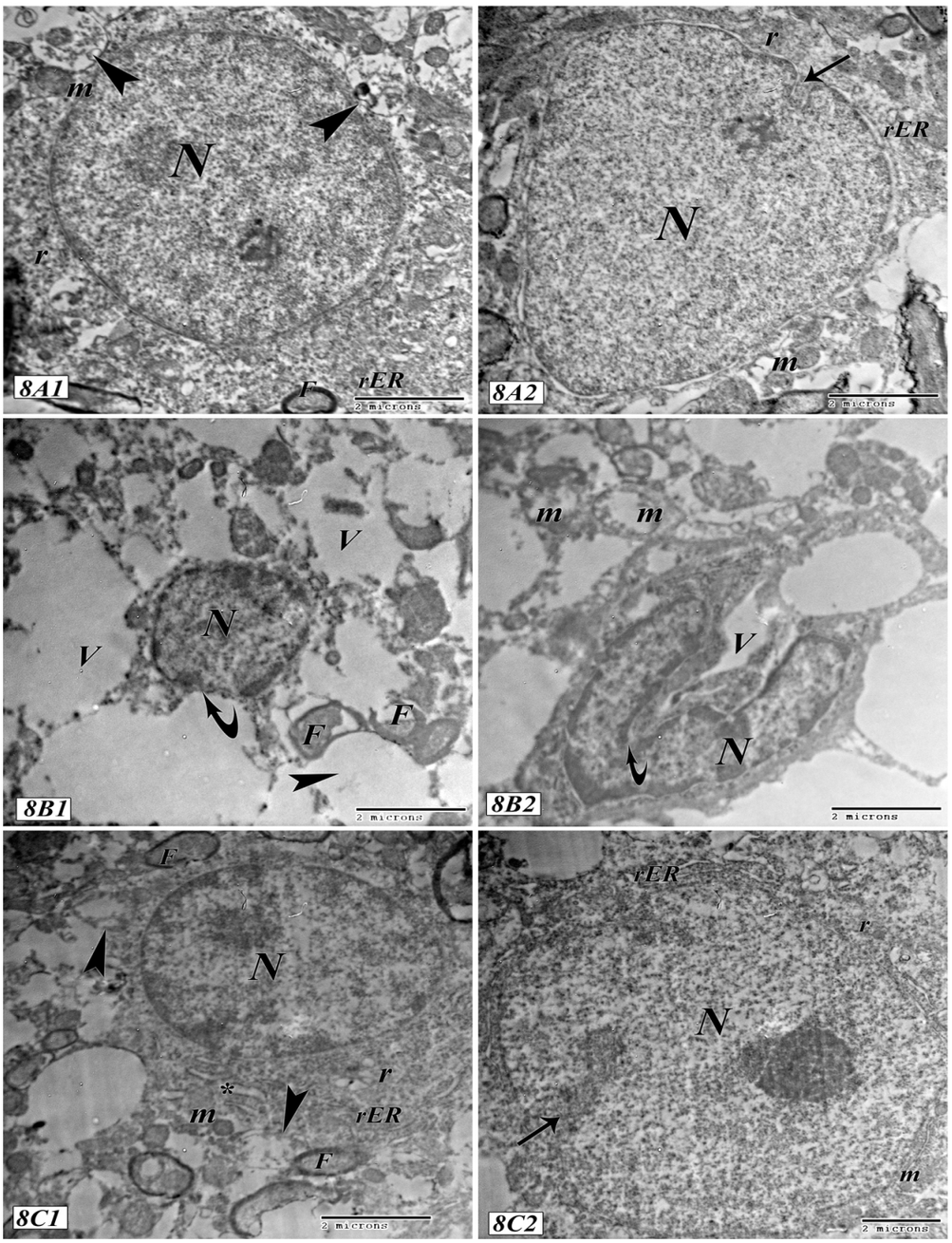


Fig. 8: TEM of the M part of the red nuclei of the three months old rats .(8A1) Control group: Showing the large cells with heterochromatic rounded nuclei (N) with peripheral chromatin condensation .Their cytoplasm has rough endoplasmic reticulum(rER) ,mitochondria (m) and numerous free ribosomes (r) . Arrow heads point to the axosomatic synapses with the soma of the nerve cell. The synaptic terminals have numerous synaptic vesicles .Myelinated nerve fibers (F) appear as lamellar structure of myelin which wrapped regularly around the axons are noticed . (X10000) (8A2) Control group: Showing the medium-sized cells , they have heterochromatic nuclei (N) with indented nuclear envelope (arrow) . The cytoplasm has (r) , (rER) and (m) . (X10000) (8B1) Acrylamide treated group: The large cells appear with shrunken nuclei(N) with irregular nuclear membrane (curved arrow) . The cytoplasm is rarified with ill-defined cell organelles and marked vacuolated areas(V) . The synaptic terminals of the axosomatic synapses show absence of the synaptic vesicles (arrow head) . The nerve fibers (F) declare disrupted thick myelin sheath. (X10000) (8B2) Acrylamide treated group: Shows the medium-sized cells , their nuclei(N) have peripheral chromatin condensation with marked invagination of the nuclear envelope (curved arrow). They have degenerated vacuolated cytoplasm(V). Some mitochondria (m) are swollen and have disrupted cristae(X10000) . (8C1) Acrylamide / Lithium chloride -treated group: The large cells show heterochromatic rounded nuclei(N) . Their cytoplasm has (rER) , (m) and (r). Some dilated rough endoplasmic reticulum can be noticed (asterisk). The synaptic terminals of the axosomatic synapses present with synaptic vesicles (arrow heads). Myelinated nerve fibers (F) appear more or less similar to the control . (X10000) (8C2) Acrylamide / Lithium chloride -treated group: Shows the medium-sized cells with heterochromatic nuclei(N) with indented nuclear envelope (arrow) . Their cytoplasm had (rER), (r) and (m) .(X7200)

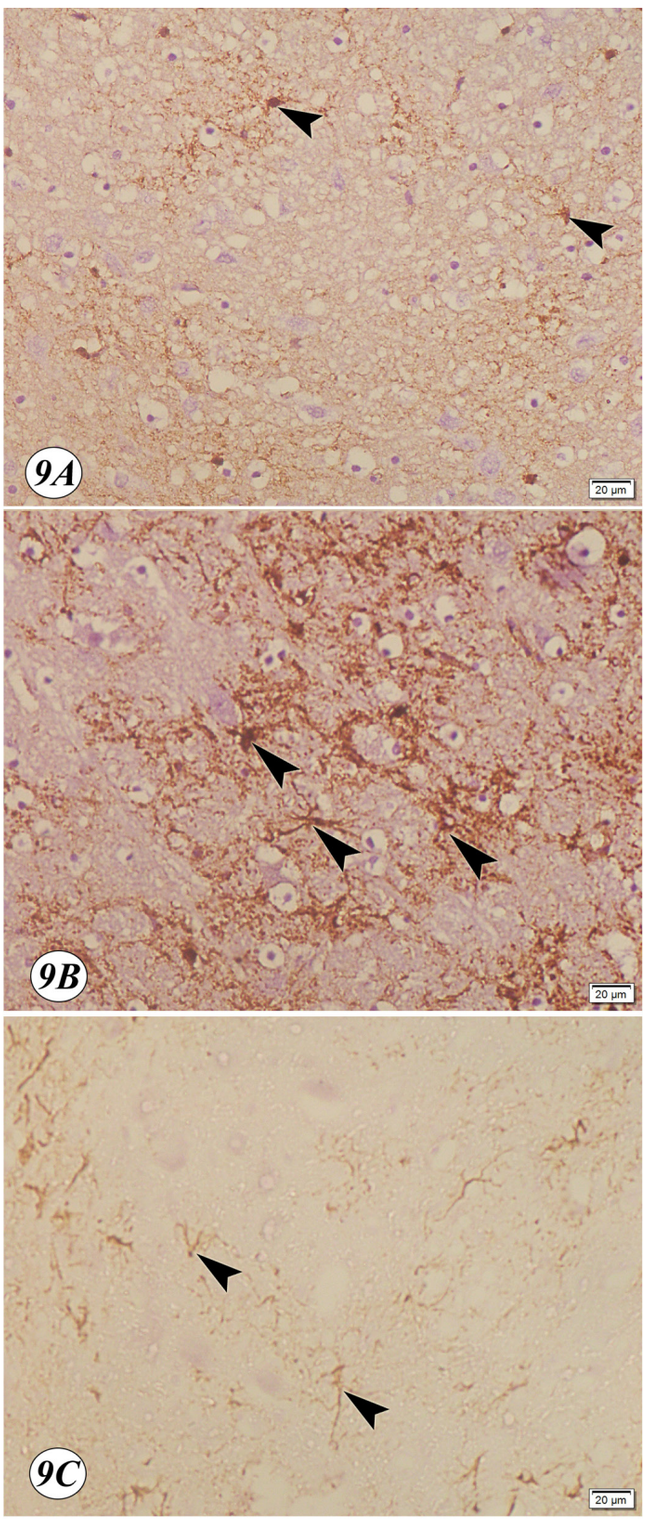
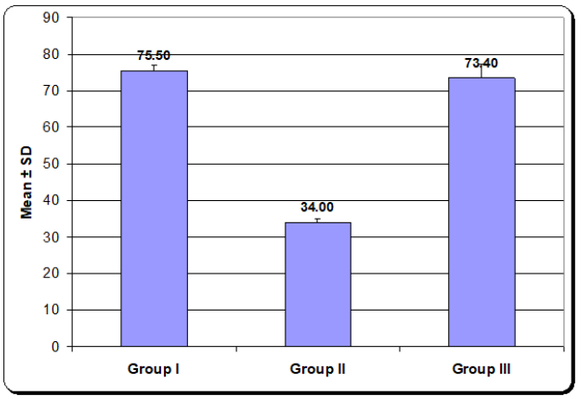


Fig. 9: Glial fibrillary acidic protein (GFAP) immunohistochemically stained sections in M part of the red nuclei of three months old rats .(9A) Control group: Showed few GFAP-positive cells with short thin processes (arrow heads) (9B) Acrylamide treated group: As compared with the control group, the detected GFAP positive cells appear to be large with long ,thick and branched processes (arrow heads) . (9C) Acrylamide / Lithium chloride -treated group: Reveal positive expression of GFAP that is nearly similar to that noticed in the control group (arrow heads).

Table 1: The mean number of the neurons in the magnocellular part of the red nucleus in the three months of different age groups; group I (control group) , group II (treated group) and group III (the Acrylamide / Lithium chloride -treated group)

	Group I	Group II	Group III	*P-value*1	*P-value*2	*P-value*3
Mean ± SD	75.50 ± 1.37	34.00 ± 1.00	73.40 ± 3.85	0.000*	0.241	0.000*
Range	74.0-77.0	33.0-35.0	69.0-79.0			

*P-value*1: Comparison between Group I & Group II
*P-value*2: Comparison between Group I & Group III
*P-value*3: Comparison between Group II & Group III
P- Value ≤ 0.05 was considered to be significant



Histogram 1: The mean number of the neurons in the magnocellular part of the red nucleus in the three months of different age groups; group I(control group) , group II(treated group) and group III(the Acrylamide / Lithium chloride -treated group)

DISCUSSION

The midbrain is a part of the extrapyramidal system, it plays a considerable role in the locomotor function, it has several neural connection with the motor cortex, brainstem nuclei and spinal cord[23]. Over many years the histological structure, function and clinical relation of the red nucleus have great attention[24].

Acrylamide (ACR) which is considered as an neurotoxic compound ,used in numerous branches of industry . The main route of the human exposure is through diet as ACR is found in carbohydrate-rich foods which was cooked at high temperature because of the reaction that occurred between the sugar and amino acids[6] . In spite of reporting the oxidative stress of ACR as regard the central nervous system[25], the effects of ACR on the neuronal development did not take great attention, although it was reported that ACR was able to cross the placental barrier[26;27] and could be excreted in milk[26]. Therefore, this study was designed to investigate the effect of ACR on the postnatal development of the red nucleus of the male albino rats and any possible protected role of concomitant lithium chloride administration.

Throughout this work, light microscopic examination showed that the red nuclei of the control rats at different age groups appeared as bilateral ovoid circumscribed collections of neurons on either side of the midline. They were subdivided into two parts; the magnocellular (M) and the parvocellular parts (P). The M part composed mainly of large, medium and small sized neurons. In accordance with that, Lemon, 2008[28] stated that the red nucleus was identified in all the vertebrates as distinct clusters of neurons that was present in the inferior and anterior part of the midbrain.

In harmony with this study, Liang *et al*. 2012[29] reported that the red nucleus was divided into ventrocaudal magnocellular and dorsorostral parvicellular parts with the presence of different size neurons in each part.

Cell processes could not be detected at the newborn but they well established with the progress of the age. This might be due to the further development of the neuronal axons and dendrites. This opinion of the present work was in agreement with the results of Zaki, 2009[30] who studied the postnatal development of the red nucleus at different age group in albino rats. He noticed that the nerve cells of the M part of the red nucleus had neural processes at the age of seven days . He added that these processes were clearly detected at the age of twenty one days and also in adult rats.

The present study demonstrated that the cells of the M part at the age of seven days were rich in Nissl's granules in comparison to the previous age. Furthermore ,the intensity of the Nissl's granules of the cells in the M part was increased as the age progressed from seven days till the age of three months . These results were coinciding with those finding of Zaki, 2009[30] who observed with the progress of age, the M part of the red nucleus displayed nerve cells with darkly stained cytoplasm as a result of increased amount of Nissl's granules with advanced ages . Essam *et al*.,2014[31] stated that adult nerve cells of the rat midbrain were rich with abundant amount of Nissl's granules.

In the current work ,sections stained with gallocyanine stain of the control group of three months rats displayed large well-differentiated nerve cells in the M part , mostly multipolar, some of them were pyramidal and fusiform. The cytoplasm of these cells were darkly stained as a result of increase the amount of Nissl's granules .Their nuclei appeared with the presence of prominent nucleoli. Aghoghovwia and Oorschot 2016[32] reported that the red nuclei were presented with neurons that had large rounded or ovoid nuclei with prominent nucleoli with Nissl bodies in their cytoplasm.

Electron microscopic examination of magnocellular part of the red nucleus of the control group revealed the presence of large and medium-sized cells. The large cells had heterochromatic rounded nuclei with peripheral chromatin condensation. As regard the medium-sized cells, they were presented with heterochromatic nuclei with indented nuclear envelope .The findings of the present study were in agreement with those described with Zaki, 2009[30] who observed the presence of large neurons within the red nucleus .Their cytoplasm was abundant with mitochondria, ribosomes and rough endoplasmic reticulum.

In the current work , the ACR treated newborns revealed that the red nuclei were hardly differentiated from the surrounding neural tissue. The two parts of the red nucleus were ill differentiated and presented with numerous degenerated neurons. The cellular architecture of the M part showed numerous degenerated neurons with darkly stained nuclei .In addition many patchy area of vacuolation were obvious. In accordance with the current work , Zeisel, 2004[33] found that ACR administration to the pregnant rats resulted in obvious decrease in the size of the cerebellum of the newborn rats. Dortaj, *et al*., 2018[34] noticed the marked sensitivity of the neonatal nervous tissues to the ACR treatment within lactation and pregnancy periods.

It has been reported that administration of ACR during pregnancy and period of lactation resulted in the suppression of antioxidant defense mechanism in the neonatal rats .These could explained the observed histological degenerative changes in the neonatal rats following ACR exposure during intrauterine life[35].

It has been reported that About 10%to 15% of the given ACR to the pregnant women through the diet were passed by the way of the placenta to the fetus[36] and about 8.18 mg/L passed by means of lactation[26]. The ACR toxicity during the prenatal periods might be due to the induction of oxidative stress and the biochemical changes which in turn resulted in the increased level of reactive oxygen species[37].

ACR treated seven day group showed disrupted architecture of the red nuclei with ill differentiated M and P parts . Noticeable darkly stained neurons of the M part were detected. The cellular architecture of the M part of the red nucleus was severely disrupted with the presence of numerous shrunken neurons with distorted lysed nuclei. The nerve cells seemed to be poor with Nissl's granules . Cells with darkly stained pyknotic nuclei were shown .Patchy areas of intercellular vacuolization were noticed. In harmony with the results of the present work Sörgel *et al*., 2002[26] reported that following treatment of the pregnant rat with ACR resulted in the passage of ACR and its byproduct (glycimide) to the placenta owing to their water solubility and therefore ,they could diffuse to fetal tissues. Shaheed *et al*., 2006[38] added that ACR administration resulted also in abnormal maternal behavior with subsequent poor lactation . So the treated ACR seven days rats suffered from both; ACR exposure , in addition to poor lactation and these two factors could explain the degenerative changes observed in the current work.

As regard the ACR treated twenty one days rats, apparent shrunken of the red nuclei in comparison with the control group was noticed . The M part had densely stained neurons that were surrounded with vacuolated areas. The cellular architecture of the M part showed shrunken neurons with rarified nuclei. Some neurons appeared as ghosts with absence of their nuclei . Scanty amount of Nissl's granules was noticed. The results of the present work were in agreement with, Al-Gholam etal., 2016[16] who studied the effects of ACR administration on the postnatal development of the spinal cord of rats. They noticed the presence of degenerated shrunken neurons with vacuolated neuropil and neurogliosis at the age of twenty one treated rats.

As regard ,the three months rats treated with ACR, sections stained with gallocyanine stain declared severely disruption of the architecture of the red nuclei .The M part of the red nucleus had numerous distorted neurons. Some neurons were irregular in shape and had densely stained pyknotic nuclei. Neurons that were surrounded with empty spaces could be detected. Furthermore numerous vacuolated areas were obvious. In the current work, the observed degenerative changes were in agreement with the results of the previous study done by Laag *et al*., 2014[39]. They reported the presence of neurodegenerative changes in the adult albino rat following ACR treatment ; Chromatolysis of Nissl granules and eccentric nuclei with an irregular outline. Wilson *et al*., 2006[40] added that ACR and its by product glycidamine played a major role in the mechanism of the ACR induced genotoxic. Both of them could react with enzymes and DNA with the occurrence of neurotoxicity.

Sections stained with toluidine blue of the adult rats that were received acrylamide displayed interrupted histological structure of the M part of the red nucleus .The large cell type had darkly stained nuclei and vacuolated cytoplasm . Numerous neuroglial cells were seen scattered in between the nerve cells . Nerve fibers with disrupted thick myelin sheath were noticed . The obtained results were supported by a previous study done by El –Sayyad *et al*., 2013[41]. They explained the occurrence of the neurological degeneration owing to ACR administration; ACR resulted in oxidative stress in different areas of the brain , that in turn increased the levels of 5-hydroxyindole acetic acid, dopamine and significant increase in the level of serotonin was noticed within the brainstem.

Obvious neurodegenerative deficits were noticed by the ultrastructure examination of the three months treated rats; the large cells appeared with shrunken nuclei with irregular nuclear membrane. The cytoplasm was rarified with ill-defined cell organelles and marked vacuolated areas. As regard the medium-sized cells, their nuclei showed peripheral chromatin condensation with marked

invagination of the nuclear envelope. The mechanism by which ACR induced neuronal injury might be due to its ability for the induction of apoptosis. It has been founded that even low dose of ACR could make the nerve cells enter the early stage of apoptosis . In addition ACR was able to prevent the axonal transport within the neurons[42]. ACR suppressed the actions of several enzymes within the nervous system ; neuron specific enolase and phosphofructokinase . Aa a result of this , rapid axonal transport and metabolism were inhibited with the occurrence of neuronal and axonal degeneration[42].

The current study declared apparent increase in the number and size of GFAP-positive cells in the treated group as compared with the control group. The detected GFAP positive cells appeared to be large with long ,thick and branched processes . In harmony with the results of the current work, Imam and Gadallah, 2019[43] reported that ACR administration produced obvious positive GFAP reaction . GFAP is considered as the major protein skeleton of the astrocytes and injury to the nervous tissues enhanced the GFAP upregulation in the reactive astrocytes . So GFAP is used as an evidence to the damage of the nervous system[44].

The results of the present work showed that Lithium Chloride (LC) was able to display neuroprotective effects in the rats treated with ACR in concomitant with LC. Some sort of protection was obviously noticed in the newly born rats received LC ; Gallocyanine stained sections revealed the red nucleus was in the tegmentum of the midbrain, dorsomedial to the substantia nigra . It was differentiated into two parts ;the M part in the caudal pole and the P part in the rostral pole of the nucleus . In addition ,the normal histological character of the red nucleus was conserved in the seven days old group . The M and P parts of the red nucleus were detected . Within the M part ,large neurons with well-defined nuclei and prominent nucleoli could be observed in addition to medium and small sized cells. The results of the present work confirmed the data of the previous study done by Yick, *et al*., 2004[17]. They found that treatment with LC promoted the axonal regeneration in the rat model following injury of the spinal cord . Wu *et al* ., 2014[45] stated that LC had considerable protective effects both in *vivo* and *vitro* studies on the neurons of different regions of the nervous system . They added that LC also had the ability of protection against numerous nervous insults' ; Ischemia , anticonvulsants drug. Zhang *et al* ., 2019[46] stated that ,during the process of nervous system development , LC could be used for the treatment of neuronal disorders as well as neurodegeneration disorders .

Previous strategies had shown that LC had both neuroprotective and neuroregenerative properties. As a neuroprotective factor, LC inhibited neuronal apoptosis . LC has the capacity of the induction of genes and different mechanisms which in turn protected cells against toxins and brain injury . It exerted its neuroregenerative action by stimulation of numerous genes for neuronal growth factor and cellular repair[47].

In the twenty one days group treated with AC and LC, the red nucleus architecture declared noticeable improvement. It seemed to be more or less comparable to the control group. The large neurons of the M part had more or less normal appearance; large vesicular nuclei with prominent nuclei with abundant amount of Nissl's granules. Few shrunken neurons with hardly identified nuclei could be identified . The current results were supported by the outcomes reported by Abdel-Hamid *et al*., 2017[48] who studied the role of lithium as a neuroprotective agent in the brain tissue of rat . In their study lithium administration reduced level of GABA and glycine (inhibitory neurotransmitters).

The gallocyanine stained sections of the three months rats showed that concomitant treatment with LC showed less obvious signs of degeneration in comparison with the treated group. The red nucleus was distinctly differentiated into M and P parts . Multipolar, pyramidal and fusiform neurons were present in the M part. These neurons had well-defined nuclei with abundant Nissl's granules. Few areas was presented with degenerated neurons. Sections stained with toluidine blue of the adult rats that were received ACR in concomitant with LC declared normal histological appearance to a great extent . In harmony with the results of the current study , subcutaneous administration of LC in a rat model of Huntington's disease exhibited a potent protective effects in the striatal neurons[49]. Previous studies suggested that LC stimulated a neuroprotective factor known as, brain-derived neurotrophic factor (BDNF), responsible for the neuronal survival and development[50].

In the present study the Acrylamide / Lithium chloride -treated adult rats revealed positive expression of GFAP that was nearly similar to that noticed in the control rats. In concomitant with this results, Ekici *et al*., 2014[47] noticed the obvious decrease of the GFAP positivity following LC administration. They added that LC could alleviated the secondary brain tissue damage and suppress the degeneration of the neurons.

In contrast to the results of the current work , Milutinović, 2016[51] reported that LC administration aggravated injury of the brain caused by 3-nitropropionic acid . This recorded difference in the results as regard the results of the present work might be due to the difference in the used dose of LC. In the study done by Milutinović, 2016[51] LC was used in a dose of 127 mg/kg. In the present study the used dose of LC was 85 mg/kg .The protocol of the current work was comparable to the protocol used by Yick,etal.,2004[17]. To support this point of view as regard the dose difference, Rang *et al* ., 2008[52] stated that LC was recorded to have a narrow range of therapy and it might be neurotoxic if used in a high dose. Yamantürk-Celik ,2012[53] added that intraperitoneal administration of LC a dose of 763.2 mg/kg resulted in the death of the treated rat.

CONCLUSION AND RECOMMENDATIONS

The data of the present work showed that Lithium chloride could be effective as a neuroprotective agent

against the acrylamide induced degenerative changes during the postnatal development of the midbrain red nucleus following maternal administration during pregnancy and lactating periods .So It is recommended for the supplementation of lithium chloride for the decrease of the acrylamide complications as regard the central nervous system . Further studies are recommended to be done using different species of animals and histological techniques .

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوجية وهستوكيميائية للتأثير الوقائى المحتمل لمادة اليثيوم كلوريد على السمية المسببة من مادة الأكريلاميد اثناء نمو النواة الحمراء فى المخ المتوسط فى مرحلة ما بعد الولادة لذكور الفئران البيضاء

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المقدمة: تعتبر مادة الأكريلاميد مركب صناعى كيميائى يستخدم فى جميع انحاء العالم . وقد تم توصيفه كعامل عصبى سام للانسان . ويعتبر قضية صحية عامة محل اهتمام.

الهدف من البحث: توضيح تأثير مادة الأكريلاميد على نمو النواه الحمراء في المخ المتوسط فى مرحلة ما بعد الولادة وذلك عقب تعاطى الام مادة الأكريلاميد اثناء فترات الحمل والرضاعة والتأثير الوقائى المحتمل لمادة اليثيوم كلوريد.

مواد وطرق البحث: تم اجراء هذه الدراسه على ثلاثين من اناث الفئران الحوامل. تم تقسيم الفئران الحوامل الى ثلاث مجموعات: المجموعةالاولى تم اعطائها ماء مقطر عن طريق الفم من اليوم السابع من الحمل حتى اليوم الثامن والعشرون بعد الولادة. المجموعة الثانية والثالثة: تم اعطائها مادة الاكريلاميد (١٠مجم / كجم / يوم) عن طريق الفم من اليوم السابع من الحمل حتى اليوم الثامن والعشرون بعد الولادة. المجموعة الثالثة: تم اعطائها يوميا بالتزامن مع مادة الاكريلاميد مادة اليثيوم كلوريد (٨٥مجم/كجم) بالحقن فى الغشاء البرتوني. وقد تم اخذ عينات للنواة الحمراء من الابناء الذكور عند العمار الاتية : حديث الولادة، اعمار سبعة ايام، واحد وعشرون يوم وثلاثة اشهر وتجهيزاها لدراستها هستولوجياً وهستوكيميائيا.

النتائج: اظهترت النتائج وجود الانوية الحمراء للمجموعات الضابطة فى الاعمار المختلفة كتجمعات محددة بيضاوية الشكل من الخلايا العصبيه على كل ناحية من المنتصف. وتتكون النواة الحمراء من جزئين: الجزء كبير الخلايا والجزء صغير الخلايا. وقد اضهرت الفئران التى عولجت بمادة الاكريلاميد خلل في تركيب الانوية الحمراء مع وجود خلايا عصبية داكنة الصبغة. وقد اضهر الفحص الهستوكيميائي للبروتين الحامضي الليفي زيادة عدد وحجم الخلايا الموجبة. اضهرت الدراسة الاحصائية اختلافا ملحوظا فى متوسط عدد الخلايا العصبية فى المجموعة المعالجة مقارتة بالمجموعة الضابطة. العلاج المتازمن مع مادة اليثيوم كلوريد اوضح وجود علامات للانحلال اقل مقارنة بالمجموعة المعالجة بمادة الاكريلاميد.

الإستنتاج: قد اظهرت النتائج ان مادة اليثيوم كلوريد من الممكن ان تكون عامل عصبى وقائى ضد الانحلال المسبب بمادة الاكريلاميد على نمو النواة الحمراء فى المخ المتوسط اثناء مرحلة ما بعد الولادة.