Original Article

Sodium Nitrite Induced Cerebellar Cortex Toxicity in Adult Male Albino Rat and the Possible Role of Metformin (Histological and Immunohistochemical Study)

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ABSTRACT

Introduction: Sodium nitrite (NaNO2) is one of the common food additives in preserving refrigerated meats. Humans are continuously exposed to NaNO2 from different sources especially vegetables and cured meat. Many cases of toxicities or even death in humans and animals from nitrate and nitrite were reported.

Aim of the Work: The aim of the present study was to analyze the role of metformin in reduction of toxicity induced by $NaNO_2$ administration in rat cerebellar cortex.

Materials and Methods: Forty adult male albino rats were used and were classified into four groups (10 rats in every group). Group I control. Group II: the animals were given metformin (200 mg/kg/day). Group III: the animals were given sodium nitrite (80 mg/kg/day) orally for 7 days. Finally, group IV was given sodium nitrite and metformin with same doses and duration. At end of the experiment cerebellar specimens were subjected to light microscopic, morphometric and immunohistochemical studies for expression of caspase-3, GFAP, iNOS, MAP2 and HIF-1α.

Results: Cerebellar cortex sections from sodium nitrite treated rats showed neurodegenerative changes in the three layers of rat cerebellar cortex particularly Purkinje cell layer. There was disturbed monolayer arrangement of Purkinje cells as well as patchy loss of some of them with significant decrease in their number ($P<0.01$), deformed and pyknotic Purkinje cells with empty spaces around them. Vacuolations in the neuropil of all layers of cerebellar cortex. The granular cells were dispersed and pyknotic. Significant increase in caspase-3, GFAP, iNOS, HIF-1α and significant decrease in MAP2 immunoreactivity were detected. However, administration of metformin improved the cerebellar cortex architecture.

Conclusion: The results revealed the neurological protective role of metformin therapy on hazardous effect of sodium nitrite of adult rat cerebellar cortex.

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Key Words: Cerebellar cortex, metformin, sodium nitrite.

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INTRODUCTION

Sodium nitrite ($NaNO_2$) is an inorganic salt which has harmful and beneficial effects in the food industry, it is named E250 and is utilized as preservative and color fixative for products of fish and meat[1]. It Inhibits the growing of spores of Clostridium botulinum in refrigerated meats which causes botulism[2] and postpones the occurrence of oxidative rancidity[3].

In addition, it is also used as intestinal relaxant vasodilator, bronchial dilator and antidote for cyanide poisoning[4].

Nitrite presents everywhere in the environment and can be formed from nitrogenous compound by microorganisms present in the water, soil, saliva and gastrointestinal tract[5].

On the other hand, sodium nitrite toxic effects were reported in mammalians, such as, deterioration of reproductive function, liver toxicity, deregulation of inflammatory responses, tissue injury, retarded growth, endocrine dysfunction and methemoglobinemia[6].

Furthermore, exposure to higher levels of nitrites increased rate of cancer in adults, brain tumors, leukemia, and tumors of nasopharynx in children due to formation of carcinogenic nitrosamines[7]. Additionally, it may lead to brain inflammation, ischemia and impairment of cerebral energy due to excessive free radicals' generation with impairment of balance between oxidant / antioxidant which lead to cellular brain damage[8].

Hypoxia is regulated by hypoxia inducible factor-1 (HIF-1). It is formed of oxygen sensitive subunit HIF 1α and HIF 1β. During hypoxia degradation of HIF-1 α is inhibited so it rapidly accumulates in the nucleus[9].

The effects of HIF 1 on ischemic outcomes may be dependent on duration of the Hypoxia-ischemia (H I), species and age of animal[10].

Moreover, microtubule-associated proteins (MAPs) are considered as the main group of cytoskeletal proteins which regulate neuronal morphogenesis. MAP2, is a neuron specific protein[11]. It is considered as the greatest protein of MAP family in the brain and the most abundant cytoskeletal protein mainly located in neuronal dendrites[12].

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Metformin (MTF), a dimethylbiguanide, is a hypoglycemic drug used in management of type II diabetes mellitus. It is a potent antioxidant agent with a direct scavenging effect against ROS. It decreases lipid peroxidation reducing oxidative stress markers and restores the antioxidant system[13]. Moreover, it has anti-tumor properties, anti-inflammatory and anti-apoptotic effects. In *vivo*, it cumulates in the brain after passing the blood-brain barrier. Nowadays, it has been documented to have neuroprotective effects on diseases of central nervous system by regulating oxidative stress, inflammatory response and neuronal apoptosis[14].

Owing to the wide use of $NaNO_2$ the purpose of this work was to study the role of metformin in reduction of $NaNO_2$ toxicity induced by its administration.

MATERIALS AND METHODS

Animals

40 adult male albino rats were used, their weight range was 180–200 g. The rats were selected from animal house present in the Faculty of Medicine, Menoufia University. They were supplied with standard food and unlimited water supply. The international guidelines for animal care were done and were endorsed by the experimental ethical committee Menoufia University.

Drugs and chemicals

The drugs and chemicals used in this study were purchased from Sigma-Aldrich Corp. (USA).

Sodium Nitrite: is a water soluble white odorless crystalline powder and stable in dry form at room temperature.

Metformin: Provided as powder, water soluble.

Experimental Design

The rats were separated into four groups at random way each of 10 rats. Drugs were dissolved in distilled water, freshly prepared and were given daily for 7 days by oral gavage.

Group I: kept as control group (received no treatment).

Group II: received metformin (200 mg/kg body weight per day dissolved in distilled water)[15].

Group III: received sodium nitrite (80mg/kg body weight per day dissolved in distilled water[16].

Group IV: received both metformin & sodium nitrite in the same doses and duration as pervious groups.

All animals were sacrificed by the end of the experiment, the cerebellum was subjected to Histological, immunohistochemical and morphometric studies.

Histological study

Specimens obtained from each animal were fixed in 10% formalin and processed. Paraffin sections 5 micron in thickness were obtained and were stained by hematoxylin and eosin (H&E)[17].

Immunohistochemical study

Paraffin sections were deparaffinized in xylene then rehydrated in descending grades of ethanol. Then put for 5 minutes in distilled water. Activity of endogenous peroxidase was blocked using 1% hydrogen peroxide for 30 minutes. The slides were washed in phosphate buffered saline (PBS). Antigen retrieval was done by boiling slides in citrate buffer solution (pH 6.0) in a microwave for 20 min. Then, sections were incubated in blocking solution (goat serum) to prevent the nonspecific background staining. The sections were incubated with the following primary anti-bodies: inducible nitric oxide synthase (iNOS) (Cat. #RB-9242-R7), Caspase-3 (Cat. # RB-1197-R7), glial fibrillary acidic protein (GFAP) (Cat. #MS-280-R7) Microtubule-Associated Protein (MAP2) (Cat#MS-250-P0), and hypoxia inducible factor (HIF -1α) (Cat. #MS-1164-P1ABX) for an hour at room temperature. Sections were rinsed with PBS, followed by incubation at room temperature with secondary biotinylated antibody for 20 min, then washed again in PBS, the sections were incubated with Streptavidin peroxidase complex for 10 min. Secondary antibody binding was visualized using 3,3-diaminobenzoicacid (DAB) to visualize antibody immunostaining areas. Finally, sections were rinsed with PBS, counterstained by hematoxylin and were washed by distilled water. At last, the slides were dehydrated, cleared then covered[18].

Morphometric study

Five randomly chosen sections stained with H&E and the immunostained (400×) from five rats from every group were analyzed for counting Purkinje cells number and number of immune positive cells of iNOS, Caspase-3, GFAP, HIF-1α. Area percentage of MAP2 was also calculated.

Statistical analysis

The collected data analysis was analyzed using SPSS program for windows version 23 (Inc., Chicago, IL, USA) and were presented as mean ± SD. The acquired data were analyzed by using one way-ANOVA followed by post hoc Bonferroni test. A *P-value* ≤ 0.05 was considered statistically significant[19].

RESULTS

Histological Results

Histological examination of the control group (group I) showed that adult rat cerebellar cortex was formed of three layers; outer molecular and inner granular cell layers with Purkinje cell layer in between. The outer molecular layer was composed of nerve fibers, neuroglial and few neurons. Purkinje cell layer was formed of one single continuous row of cells which are piriform in shape. They have vesicular nuclei, prominent nucleoli and a basophilic cytoplasm. The granular layer was formed of dense packed granule cells having granular chromatin in their nuclei and thin rim of cytoplasm separated by acellular areas (cerebellar

islands) (Figures 1,2). Metformin treated group (group II) showed the same histological appearance as control group.

H&E-stained sections of sodium nitrite treated group (group III) showed remarkable changes in the cerebellar cortex, especially in the Purkinje cell layer. Many deformed and pyknotic Purkinje cells were detected. Some Purkinje cells were irregular with fragmented nuclei and acidophilic cytoplasm, others had dark stained nuclei and cytoplasm. (Figures 3,4). They were surrounded by empty spaces (Figure 4). Purkinje cells were presented in many layers and often lacked proper organization. In other sections, Purkinje cells were disappeared leaving empty spaces (Figure 5). Vacuolations in the neuropil could be observed in the three layers of cerebellar cortex (Figures 3,5)

The granular cells were disorganized, pyknotic and widely separated in some sections and had dark stained nuclei (Figures 3,4). Dilated and congested blood vessels could be seen (Figure 3).

Sections from (group IV) showed restoration of shape, number and monolayer arrangement of the Purkinje cells. However, few Purkinje cells still have unidentified nuclei. Some granular cells are still loosely arranged (Figure 6).

Immunohistochemical results

Caspase 3 immunostained cerebellar sections of (group1) revealed negative immune reaction in cells of all layers of the cerebellar cortex (Figure 7A). Similarly, (group II) showed the same results. However, in (group III), a strong positive cytoplasmic reaction was observed mainly in Purkinje cells and in some granular cells (Figure 7B), while in (group IV) negative immune reaction was detected in cells of all layers of the cerebellar cortex similar to control group (Figure 7C).

Immunostained cerebellar sections of MAP-2 revealed strong positive reaction in molecular and granular layers in (group1) (Figure 8A), (group II) was identical to (group1). The expression was mild in sodium nitrite treated group (group III) (Figure 8B). On the other hand, strong positive reaction could be detected in (group IV) (Figure 8C).

GFAP-immunostained cerebellar sections of (group I) expressed mild positive GFAP reaction in the astrocytes and their processes in the three cerebellar cortical layers (Figure 9A). Same findings were present in (group II). In (group III) an intense GFAP immune-expression was observed in the astrocytes and their processes (Figure 9B) while (group IV) showed mild GFAP immune-expression (Figure 9C).

Immunohistochemical staining for iNOS of (group I) revealed negative iNOS immunoreactivity in cells of cerebellar cortical layers (Figure 10A) and also (group II). In (group III) strong positive immunoreaction was observed in Purkinje cells (Figure 10B) with mild immunoreactivity in granular and molecular cell layers. However, negative iNOS immunoreaction in cells of all layers of cerebellar cortex in (Group IV) was noticed (Figure 10C).

Immunohistochemical staining for HIF-1α of (group I) revealed negative HIF-1α immunoreactivity in cells of the three layers (Figure 11A). Similar findings were noticed in (group II). In (group III), a positive nuclear and cytoplasmic immunoreaction was observed in Purkinje cells (Figure 11B). However, negative immunoreactivity in cells of all cerebellar cortical layers was noticed in (Group IV) (Figure 11C).

By morphometric analysis

The mean number of Purkinje cells was significantly reduced in (group III) in comparison with (group I) (3.13 ± 0.64 vs 11.80 ± 0.68) on the other hand it was significantly increased in (Group IV) compared with (group III) (11.27 ± 1.28 vs 3.13 ± 0.64) ($P < 0.001$) (Table1, Histogram1).

This was accompanied by a significant increase in immunoreaction of caspase-3, apoptosis marker, in (group III) compared with the (group I) (27.40 ± 1.55 vs 1.0 ± 0.53) ($P < 0.001$). It was dramatically decreased in (group IV) (1.27 ± 1.39 vs 27.40 ± 1.55) compared to (group III) ($P < 0.001$) (Table2, Histogram 2).

Moreover, a significant decrease in MAP2 immunoreaction, a protein that improves the stability of the dendritic cytoskeleton, in (group III) compared with (group I) was observed (11.29 ± 1.11vs 23.0 ± 1.29) ($P < 0.001$). However significant increase in (group IV) (21.86 ± 1.07 vs 11.29 ± 1.11) compared to (group III) was detected ($P < 0.001$) (Table3, Histogram3).

Significant rise in immunoreaction of (GFAP), astrocyte marker, was observed in (group III) compared to (group I) (27.70 ± 0.95 vs 9.50 ± 0.71) ($P < 0.001$). On the other hand, a significant decrease in (group IV) (10.20 ± 1.13 vs 9.50 ± 0.71) compared to (group I) ($P < 0.001$) was noticed (Table2, Histogram 4).

Significant increase in (iNOS), oxidative stress marker, in (group III) compared with (group I) (24.47 ± 3.04vs 0.53 ± 0.52) ($P < 0.001$) was detected. On contrast, a significant decrease was observed in (group IV) compared with the (group III) (0.67 ± 0.49vs 24.47 ± 3.04) ($P < 0.001$) (Table2, Histogram 5).

This was accompanied by a significant increase in HIF-1α immunoreaction, a marker for hypoxia, in (group III) compared with (group I) (9.73 ± 0.46 vs 0.73 ± 0.46) ($P < 0.001$). However, significant decrease in (group IV) (0.60 ± 0.51vs9.73 ± 0.46) compared to (group III) was detected ($P < 0.001$) (Table2, Histogram6).

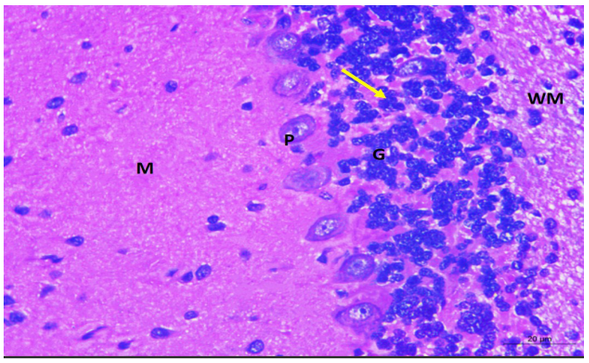


Fig. 1: A photomicrograph of a section of adult control rat cerebellar cortex showing the three layers; molecular layer (M) containing nerve fibers, neuroglia and few neurons. Purkinje layer has large Pyriform cells (P) with vesicular nuclei. The cells are arranged in one row. Granular layer(G)shows small closely packed cells (yellow arrow). Part of white matter can be seen (WM). (H&E X 400)

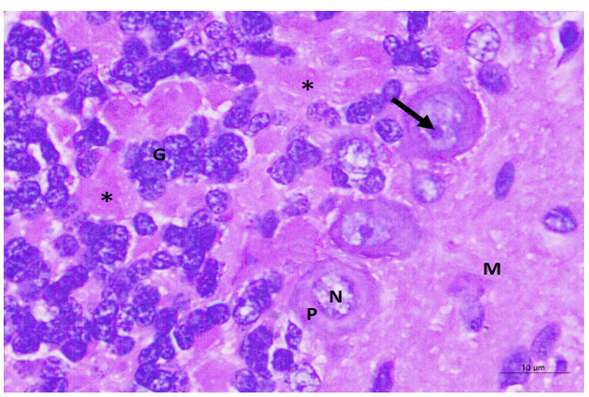


Fig. 2: A higher magnification of a section of adult control rat cerebellar cortex showing the three layers; molecular (M), Purkinje (P), and granular cell layers (G). Purkinje cells have large pale stained nuclei (N) and prominent nucleoli (black arrow). Granular layer formed of closely packed cells with granular chromatin in their nuclei and thin rim of cytoplasm. Notice the presence of non cellular areas in granular layer (cerebellar islands) (*). (H&E X 1000).

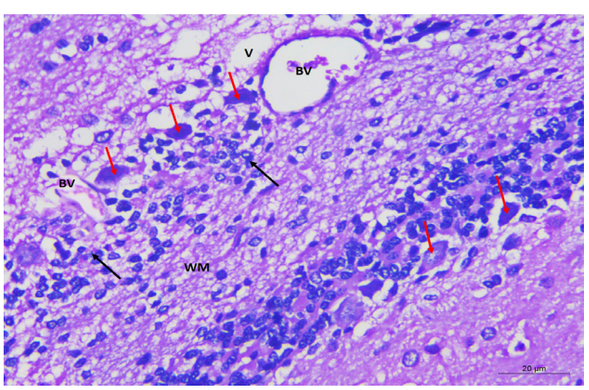


Fig. 3: A photomicrograph of a section in adult rat cerebellar cortex from (group III) showing vacuolations in the neuropil of the molecular layer(v). Purkinje cells are shrunken with dark stained cytoplasm and unidentified nuclei (red arrows) surrounded by empty spaces. Granular cells are widely separated and disorganized with many dark stained nuclei (black arrows). Dilated congested blood vessels (B.V) can be seen. Notice the presence of white matter (WM). (H&E X 400)

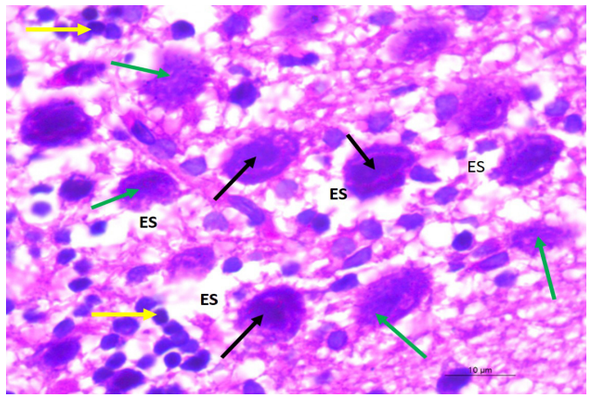


Fig. 4: A photomicrograph of a section of adult rat cerebellar cortex from (group III) showing multilayered arrangement of Purkinje cells (P). Some Purkinje cells are irregular with fragmented nuclei and acidophilic cytoplasm (green arrows), others have dark stained nuclei and cytoplasm (black arrows). Empty spaces (ES) can be seen around Purkinje cells. Granular cells are dispersed with dark stained nuclei (yellow arrows). (H&E X 1000).

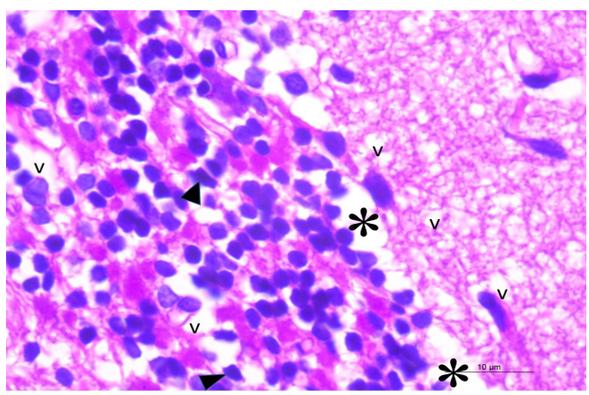


Fig. 5: A photomicrograph of a section in the cerebellar cortex from (group III) showing absence of Purkinje cells leaving empty spaces (*). Vacuolations(v) can be seen in neuropil of all layers. Loosely arranged granular cells with dark stained nuclei are detected(arrow heads). (H&E X 1000)

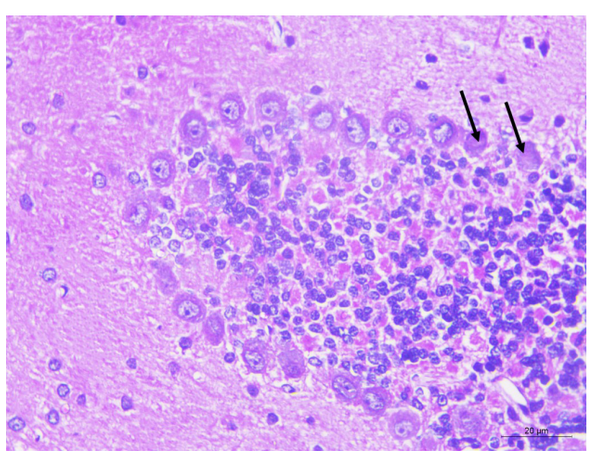


Fig. 6: A photomicrograph of a section in adult rat cerebellar cortex from (group IV) showing restoration of monolayer arrangement of Purkinje cells (P). Some Purkinje cells have unidentified nuclei (black arrow) .Some granular cells are still loosely arranged. (H&E. X400)

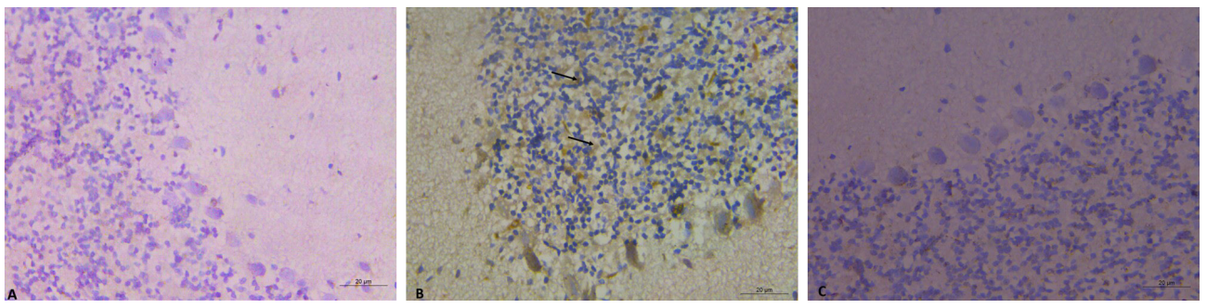


Fig. 7: A Photomicrograph of a section of adult rat cerebellar cortex stained with caspase-3 showing A)Negative immunoreaction in the cells of the three layers of the cerebellar cortex in control group. B) Group III with strong positive immunoreaction in the Purkinje cells and some granular cells(black arrows). (C) Group IV with negative reaction in the cells of the cerebellar cortex (Immunohistochemical stain of caspase-3 x400)

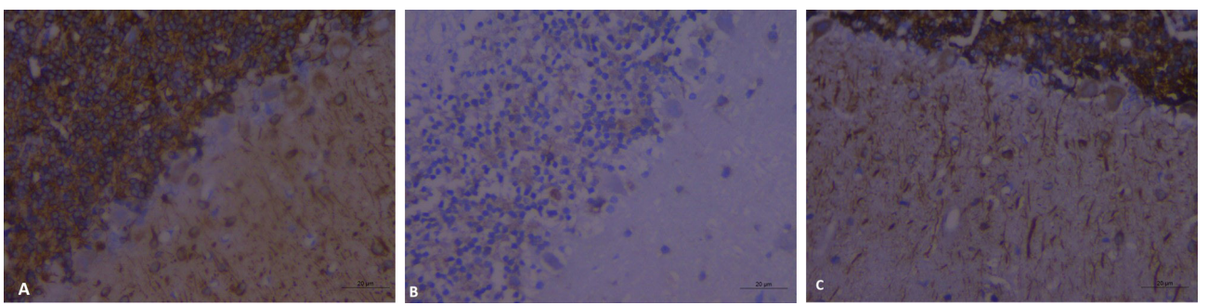


Fig. 8: A Photomicrograph of a section of adult rat cerebellar cortex stained with MAP-2 showing A) Strong positive reaction in molecular and granular layers in control group (G1). The expression was mild in (group III) (B) on the other hand strong positive reaction could be detected in (group IV) (C). (Immunohistochemical stain of MAP-2 x400)

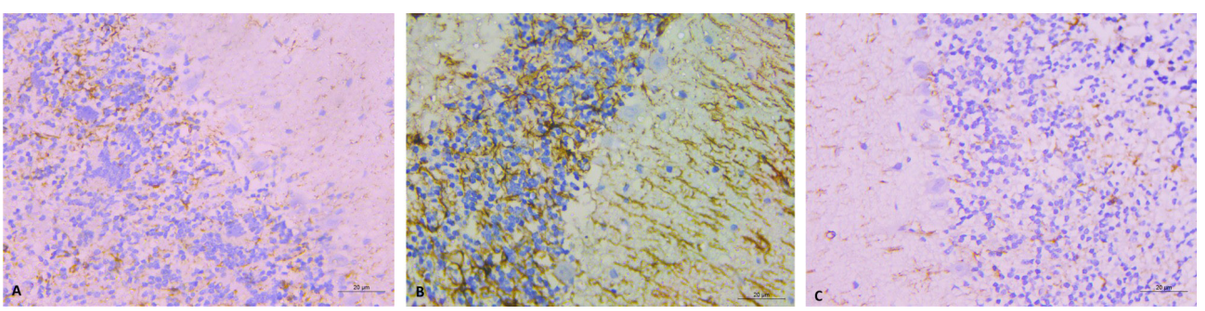


Fig. 9: A Photomicrograph of a section of adult rat cerebellar cortex stained with GFAP showing A) Control group with mild GFAP immune-expression in astrocytes and their processes of all layers of cerebellar cortex. B) Group III with intense GFAP immune expression in astrocytes and their processes in all layers. C) Group IV showing mild GFAP immune expression. (Immunohistochemical stain of GFAP x400)

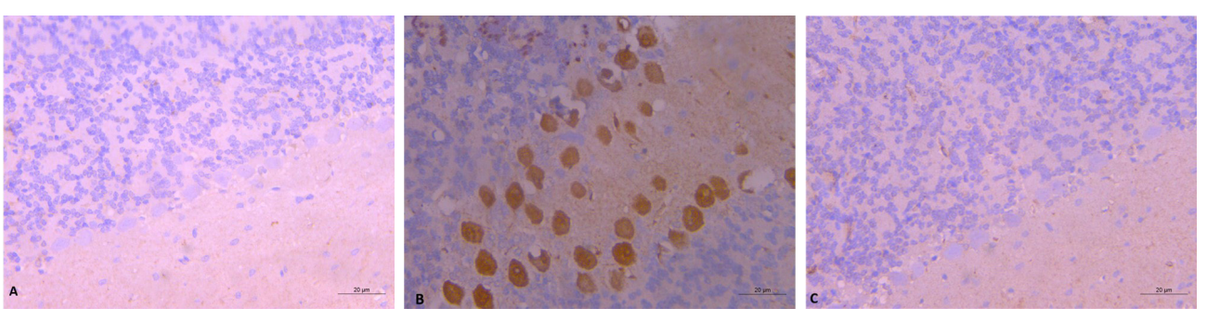


Fig. 10: A Photomicrograph of a section of adult rat cerebellar cortex stained with iNOS showing A) Control group(G1) negative immunoreaction of iNOS in cells of all cerebellar cortex layers. B) Group III showing strong positive reaction in Purkinje cells and some granular and molecular cell layers. C)Group IV showing negative immunoreaction in cells of all cerebellar cortex layers. (Immunohistochemical stain of iNOS x400)

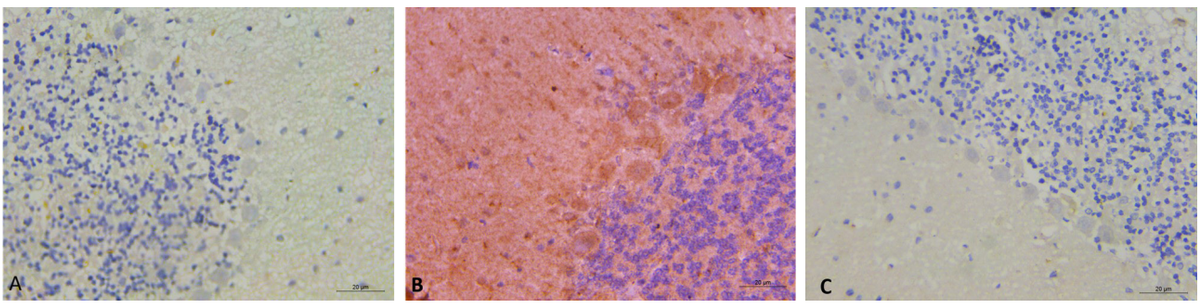


Fig. 11: A photomicrograph of a section in adult rat cerebellar cortex stained with HIF-1α showing A) Control group with negative immunoreaction in cells of the three layers of the cerebellar cortex.(B) Group III with a positive immunoreaction in the Purkinje cells. (C)Group IV with negative reaction in the Purkinje cells (Immunohistochemical stain of HIF-1α x 400)

Table 1: Comparing mean number of Purkinje cells of different groups. *P-value* either; non-significant (*) or highly significant (**)

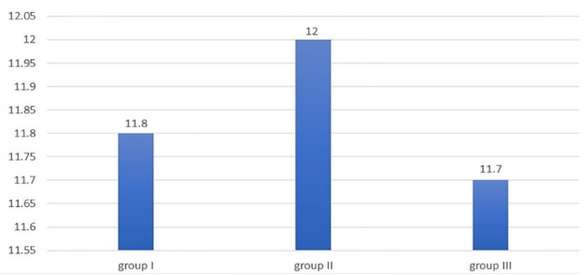
	G1	G III	G IV
Mean ± SD of no. Purkinje Cells	11.80 ± 0.68	3.13 ± 0.64	11.27 ± 1.28
Significance between groups			
Between Group I& III		0.001**	
Between Group III & IV		0.001**	
Between Group I & IV		0.448*	
P value		0.001**	

Table 2: Mean number of immunopositive cells in different groups. *P-value* either; non-significant (*) or highly significant (**)

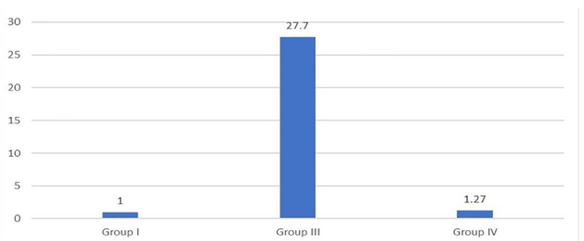
Mean ± SD (standard deviation) of	GI	G III	G IV
Caspase 3	1.0 ± 0.53	27.40 ± 1.55	1.27 ± 1.39
GFAP	9.50 ± 0.71	27.70 ± 0.95	10.20 ± 1.13
INOS	0.53 ± 0.52	24.47 ± 3.04	0.67 ± 0.49
HIF	0.67 ± 0.48	9.73 ± 0.46	0.73 ± 0.46
Significance between groups			
Between Group I & III for caspase, GFAP, INOS& HIF-1α		0.001**	
Between Group III &IV for caspase, GFAP INOS & HIF-1α		0.001**	
Between Group I & IV for caspase, GFAP INOS& HIF-1α		0.374*, 0.094*, 0.819* & 0.448*	
P		0.001**	

Table 3: Mean area percentage of MAP2 of different groups *P-value* either; non-significant (*) or highly significant (**)

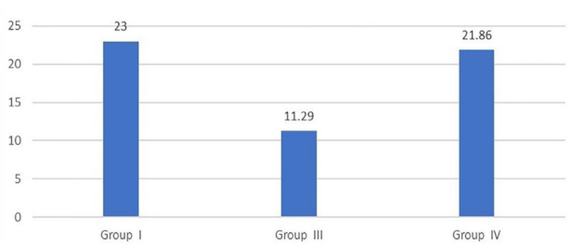
	G1	G III	G IV
Mean ± SD	23.0 ± 1.29	11.29 ± 1.11	21.86 ± 1.07
Significance between groups			
Between Group I& III		0.001**	
Between Group III & IV		0.001**	
Between Group I & IV		0.134*	
P		0.001**	



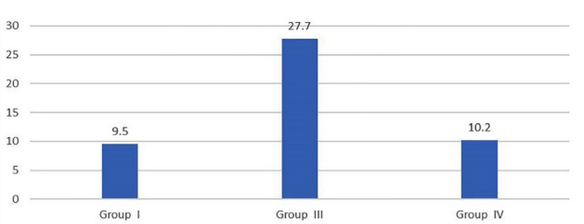
Histogram 1: Mean Number of Purkinje cells of different groups



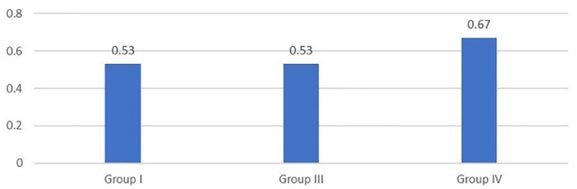
Histogram 2: Mean Number of caspase immuonreactive cells of different groups



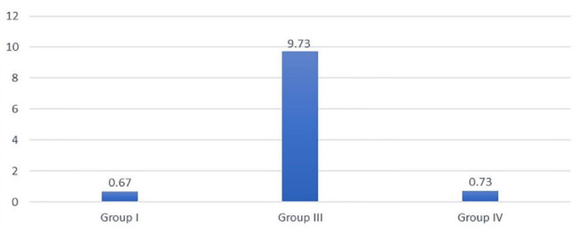
Histogram 3: Area percentage of Map-2 diferent groups



Histogram 4: Mean Number of immuonpositive GFAP cells of different groups



Histogram 5: Mean Number of iNOS immuonreactive cells of different groups



Histogram 6: Mean Number of HIF-1α immuonpositive cells of different groups

DISCUSSION

In the present study the arrangement of Purkinje cell layer in sections of cerebellar cortex treated with sodium nitrite group showed that some areas were disorganized as they appeared in many layers rather than the monolayer arrangement. This was in line with the results of Zaidi[8]. Previous researcher explained the crowding of Purkinje cell as adaptive mechanism in prolonged neuronal injury to reestablish synapsis with the other nerve cells to be able to achieve their functions[20].

On the other hand, other sections revealed patchy loss of Purkinje cells. This was approved morphometrically by the significant decline in the number of Purkinje cells in sodium nitrite treated group ($P < 0.001$) in comparison with the control group. The same results were reported by Zaidi[8] who postulated that serious damage to the Purkinje cells was induced by sodium nitrite administration due to hypoxia.

Moreover, deformed Purkinje cells with dark stained cytoplasm and unidentified nuclei were observed. This was evident in this study by significant increase in caspase3 immunoreaction in Purkinje neurons of sodium nitrite treated rats. Similar neurodegenerative changes were detected by previous investigators[21]. The same results were reported by Hamdan *et al*.,[22] and Jie *et al*.,[23] who found that after constant ischemia, apoptosis was developed in cerebellar neuronal cells[23].

Also, some authors considered Purkinje cells with dark stained cytoplasm as a sign for chromatolysis and gliosis[24]. Other researchers considered the dark shrunken neurons as a reflection for certain apoptotic phase[25].

In the present study the granular cells were widely separated and had small dark stained nuclei. This was in line with the results of Irmak *et al*.,[26].

Weber[27] reported that in neurons, ischemia damages mitochondria, followed by a shift to anaerobic metabolism with accumulation of intracellular K+, Na+ and Ca2+ which results in cell swelling. Reactive oxygens species (ROS) will be released from the damaged mitochondria and damage cellular membranes. The raised neuronal Ca2+ causes cell damage and death by several mechanisms such as calpains which result in permanent changes to cytoskeleton and membrane of cells of CNS. Nitric oxide, a free radical, is raised resulting in cell damage and apoptosis[28].

Vacuolations in the neuropil appeared in the three layers of cerebellar cortex in the group treated with sodium nitrite of the present study. Also, some authors described the formation of uneven small, apparently empty, microscopic vacuoles of different sizes in the neuropil[29,30]. Some investigators explained that the presence of vacuolations were due to loss of the cellular components within the cerebellar cortex[31].

This was confirmed immunohistochemically by the significant decrease of MAP2 expression in sodium nitrite treated group of the present study. Similar results were reported by Kuhn *et al*.,[32] who notice downregulation of MAP2 immunoreactivity after experimental ischemia in rat and gerbils. Decrease of MAP2 expression was considered to be not only a very early marker of ischemic neuronal injury[32] but also early indicator of neurodegeneration[33]. Reduction of MAP-2 during cerebral ischemia caused an abnormal microtubule gathering and neurotransmission[34] and finally, destruction of the microtubular structure and at last apoptosis of neurons[35].

In the current study, vascular changes appeared in the form of dilatation and congestion of blood vessels, this was in agreement with the results of Elsabagh[36] who found marked congestion of meningeal, cerebral and cerebellar blood vessels in sodium nitrite treated rats.

In addition, Hassan *et al*.,[37] reported that sodium nitrate induced nitric oxide (NO) formations caused relaxation of vascular smooth muscle with dilatation of their lumens and increase their blood flow. Furthermore, sodium nitrite stimulated angiogenesis in an NO-dependent manner[38].

The mechanism of sodium nitrite toxicity was explained by oxidative stress as evidenced by increase of serum MDA, decrease of plasma GSH, GST and SOD[39,40].

This gave explanation for the significant rise in iNOS expression, an oxidative stress marker, in sodium nitrite treated rats. This was in agreement with the findings Özena *et al*.,[41] and Fang *et al*.,[14] who declared that iNOS was increased in neonatal hypoxic-ischemic brain.

Nitric oxide is a physiological regulator of different functions in various tissues including acute and chronic hypoxia. Its formation is catalyzed by a family of nitric oxide synthases (NOS)[42]. The role of NO is based on its concentration. At a low concentration, it is included mainly in vasodilatation and neurotransmission. However, it is neurotoxic at higher concentrations[43].

Additionally, it was reported that sodium nitrite inside stomach interacts with amines in the food to produce nitrosamines and other free radicals which stimulate proteins fragmentation, and enzymes deactivation[44]. It induced remarkable decrease in the activity of mitochondrial cytochrome c oxidase enzyme inside mitochondria which is essential for producing aerobic ATP[22]. Many researches related this reduction to both reactive oxygen species and apoptosis[45,46].

Moreover, in blood $NaNO_2$ forms methemoglobin (MetHb), which is a potent agent increasing the formation of free radicals by reducing the ability of hemoglobin to carry oxygen causing ischemic hypoxia[47,48,49].

Hypoxia produced oxidative stress in rat brain by exacerbation of lipid peroxidation and reducing antioxidant enzymes activities[50]. Previous researches declared that oxidative stress is an early hallmark after exposure to hypoxia[51].

Hypoxia was confirmed in the present study by significant increase in HIF-1 α in sodium nitrite treated group. This coincides with the results of Molina *et al*.,[52], Juan *et al*.,[53] and Fadda *et al*.,[54] who declared that sodium nitrite caused a remarkable upregulation of HIF-1 level which was activated by production of iNOS during hypoxia. Both HIF-1α and iNOS were upregulated in hypoxic situations by the elevation of NO production[55]. This was in compliance with our results which revealed significant increase of both iNOS and HIF-1α in sodium nitrite treated rats.

In the present study a significant rise in GFAP positive cells was observed in sodium nitrite treated rats in relation to the control group. This was in accordance with the finding of Mohammed[56]. Researchers declared that in rats exposed to hypoxic ischemic injury, GFAP-positive cell density in cerebellar cortex was significantly increased[57]. furthermore, after perinatal hypoxic-ischemic insult, aggravation of reactive astrocytosis in the brains of human infants was documented[58]. This was previously explained by Pekny & Nilsson[59] and Biran *et al*.,[60] who stated that any chemical, degenerative or mechanical insults to the brain activate astrocyte proliferation with increase production of GFAP leading to vigorous astrogliosis. GFAP was produced by Glial cells as a response to the oxidative insult[61].

GFAP is considered as a specific marker for mature astrocyte. It is essential for normal construction of white matter and blood brain barrier[62]. It has a vital role in activation of astrocytes and glial scar which effectively limiting the damage[63]. On the other hand, detrimental effects might be resulting from over activated astrocytes insult by producing various neurotoxic substances[25].

In the current work, it was found that the administration of metformin improved the severity of pathological alterations, this was in line with the results of Li *et al*.,[64] and Zhu *et al*.,[65]. Metformin inhibits oxidation of haemoglobin by sodium nitrite in the blood. Moreover, it is neuronal antioxidant[66].

Furthermore, metformin decreased expression of caspase 3 in sodium nitrite treated rat in the present work. This was in accordance with Zhang *et al*.[67] and Chen *et al*.,[68].

The antiapoptotic effect of metformin may be due to its ability to protect mitochondrial integrity, especially opening of mitochondrial permeability transition pore (MPTP), decreased calcium overload and cell death[69].

Moreover, iNOS was down regulated by metformin in the present study. The same results were reported by Youssef[70] and Fang *et al*.,[14] who found that iNOS was remarkably decreased in neonatal hypoxic-ischemic brain injury of rat treated with the metformin. Metformin suppressed a fundamental signaling pathway for iNOS induction by reducing iNOS mRNA levels through both mRNA stabilization and promoter transactivation[71].

Furthermore, our study revealed that MAP2 was preserved by metformin treatment. This was in agreement with the finding of Zhu *et al*.,[72] and Fang *et al*.,[14] who declared that metformin treatment promoted remyelination and axonal restoration following hypoxia and ischemia in the neonatal rats. Metformin also promotes neurogenesis in the neurons of rodent and human by activating aPKC-CBP pathway, which was important for differentiation of neural precursors[16].

Furthermore, it was found that after metformin treatment there was a significant decrease in HIF-1α, this was in line with the reports of Liu and Zhang[73].

It was found that in ischemia, HIF induced the pro apoptotic molecules, which cause mitochondrial dysfunction and cell death[74]. Cellular damage and delayed cell death could be reduced by modulation of HIF-1 activity[75]. Moreover, an increase in (HIF-1α) was closely related to ischemic neuron damage[76].

Additionally, our experimental observation revealed a significant decrease in GFAP in metformin treated group. Similar results were recorded by Oliveira *et al*.,[77] by decreasing secretion of pro-inflammatory factors.

CONCLUSION

Metformin exerts neuroprotective effect against sodium nitrite induced histopathological changes in adult male rat cerebellar cortex.

CONFLICT OF INTERESTS

There are no Conflicts of Interest

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الملخص العربى

دور عقار المتفورمبن على التأتير السمى لقشرة المخيخ الناجم عن ما دة نترات الصوديوم في ذكور الجرذان البيضاء البالغين: دراسة هستولوجية وهستوكيميائية مناعية

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مقدمة البحث: نترات الصوديوم ماده تستخدم لحفظ اللحوم المصنعه لفترات طويله. وقد ذكرت دراسات سابقه مجموعة واسعة من أعراض التسمم لقشرة المخيخ الناتجة عن استخدام هذه الماده . و يعد دواء الميتفورمين أحد الأدويه التي تستخدم لعلاج مرض السكر وهو ايضا أحد مضادات الأكسدة المستخدمة للوقاية والعلاج في حالات مختلفة.

الهدف من العمل: تم إجراء هذا البحث لدراسة دور دواء الميتفورمين في التسمم لقشرة المخيخ الناجم عن مادة نترات الصوديوم باستخدام الدراسات الهستولوجية و الهستوكيمائيه المناعيه

المواد و الطرق: تم استخدام اربعون من ذكور الجرذان البيضاء البالغين حيث تم تقسيمهم الى اربع مجموعات: المجموعة الاولى: الضابطة، المجموعة الثانية المعالجة بعقار المتفورمين (بجرعة)٢٠٠ مج / كغ من وزن الجسم / يوم تعطى عن طريق الفم، المجموعة الثالثة المعالجة بنترات الصوديوم (بجرعة٨٠ ملجم / كجم من وزن الجسم تذاب في الماء وتعطي عن طريق الفم. المجموعة الرابعة تم علاجها بنترات الصوديوم بالاضافة إلى عقار المتفورمين . و قد أجريت التجربة لمدة ٧ أيام. و قد خضعت عينات قشرة المخيخ للدراسة باستخدام المجهر الضوئى والدراسات الكيميائية المناعية.

النتائج: أظهر الفحص المجهري انكماشا، وتشوها في خلايا بركينجي التي ظهرت داكنة الصبغة مع تغلظ في النوية، و عدم انتظام خلايا بيركنجي و زيادة الفراغات حولها . كما تسببت مادة نترات الصوديوم في زياده ذو دلاله إحصائية في الخلايا الايجابية لـكاسبس وعدد الخلايا النجمية الايجابية للبروتين الحامضى الليفى والعامل المحرض لنقص الأكسجة وانخفاض له دلالة احصائية في البروتين المرتبط بالأنابيب الدقيقة٢ . استخدام عقار المتفورمين يحسن التركيب الهستولجي لقشره المخيخ

الاستنتاج: استخدام عقار المتفورمين يخفف من التغيرات الباثولوجيه لنسيج قشرة المخيخ في ذكور الجرذان البيضاء الناتجة عن التأثير السمى الضار لما دة نترات الصوديوم.