Effects of Acyclovir on the Cerebellar Cortex of Adult Male Albino Rats And The Protective Effect Of Zinc Sulfate (Histological and Immunohistochemical Study)

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Abstract

Background: Acyclovir is an antiviral drug. for the treatment of systemic herpes infections and central nervous system infections. Zinc is a vital mineral that found in foods and consumed as a dietary supplement.

Objectives: to detect if the acyclovir affected the cerebellar cortical region, and a protective role for zinc sulfate when co-administered with it

Material and methods: sixty adult rats were used; they were divided into 4 groups: The control group received distilled water. Group II: received zinc sulfate syrup (30 mg/kg/day) by oral gavage for 2 weeks. Group 3: each rat received (432 mg/Kg) of Acyclovir suspended in distilled water by oral gavage once daily for 2 weeks. Group 4: Animals received acyclovir followed by zinc sulfate syrup. Cerebellar specimens taken and processed for histological and immunohistochemical examination.

Results: In the group treated with acyclovir, there were significant decrease in molecular thickness and Purkinje numbers compared to control group where (p value< 0.000),no significant difference between control group and zinc ,zinc &acyclovir treated group(p value >0.5), mild decrease in Purkinje number between control and zinc &acyclovir treated (p<0.012),there increase in area% of BAX,GFAP in acyclovir group (p<0.000), in zinc &acyclovir group (p<0.002) compared to control group.

Conclusion: The use of acyclovir can induce significant alterations in the cerebellar cortical area, and zinc sulfate may serve as a potential therapeutic counteract acyclovir-induced neurotoxicity**.**

Keywords: Cerebellum; Zinc sulfate; Acyclovir.

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Introduction

The cerebellum is a vital brain region that regulates motor activities and controls the balance of the body. **(Roostaei et al.,2014)**. It contributes to various cognitive tasks such as decision-making and speech, and it integrates input from both the spinal cord and other brain areas to fine-tune motor functions. **(Witter and De Zeeuw ,2015; Miall,2022)**

Acyclovir is an antiviral drug that mimics nucleosides and commonly prescribed for managing infections caused by herpes viruses, including chickenpox, shingles, genital herpes, and herpetic lesions in the brain, skin, and mucous membranes. **(Ericson et al.,2017).**

Over the past two decades, FDA-approved Acyclovir for the treatment of systemic herpes infections and central nervous system (CNS) herpes simplex virus (HSV) infections in both neonates and older patients. **(Hou et al.,2017).**

It is an antiviral activity through the inhibition of viral DNA, preventing further synthesis and viral replication. **(Ärlemalm et al.,2022)**. 9-carboxymethoxymethylguanine (CMMG) is the main metabolized component of acyclovir, which catalyzed by the alcohol dehydrogenase and aldehyde dehydrogenase enzymes; the accumulation of this metabolite leads to the appearance of neuropsychiatric symptoms in patients treated with acyclovir**. (Wang and Ji,2019).**

Zinc is a vital trace element that naturally found in various foods and often consumed as a dietary supplement. **(Nazarizadeh and Asri-Rezaie,2016).**

It plays a vital role in cellular processes, being essential for the function of multiple enzymes and involved in immune response, DNA, and protein synthesis, wound repair, cell communication, and division**. (Wessels et al.,2021).**

Zinc also contributes structurally and functionally to some proteins by stabilizing their structure and supporting enzymatic activity. **(Li et al.,2023).**

The neurological disorders are associated with alterations in zinc levels, including stroke, neurodegenerative diseases, and depression. Zinc deficiency can impair cognitive function and learning abilities and linked to elevated oxidative stress. Conversely, excessive zinc levels may lead to neurotoxicity and neuronal cell death. **(Ryu and Aydemir,2020).**

A decreased level of Zinc is associated with an increase in cytokine production, where it has a role in the reduction of pro-inflammatory signaling via the downregulation of nuclear transcription factor-κB (NF-κB), erythroid 2-related factor 2 (Nrf2), metallothioneins (MTs), and the reduction of oxidative stress. **(Olechnowicz et al.,2018).**

Zinc was implicated in astrocyte activation and had a role in controlling astrocytic pro-inflammatory processes, where zinc deficiency activates astrocytes, which affects neuronal development through astrocyte–neuron communication. **(Stanton et al.,2025).**

The current study aimed to detect if the acyclovir affected the cerebellar cortical region and the protective role of zinc sulfate through its antioxidant properties when used with it.

Materials and methods

Chemicals

Zovirax suspension. Each 5 ml contains 400 mg acyclovir. It is obtained from the pharmacy, it is manufactured for Smith Kline Beecham, for Glaxo Wellcome, Egypt

Sulfozinc syrup. Each 5 ml contains 20 mg zinc. It was manufactured for the Cid (Chemical Industries Development) company for HOLDIPHARMA, Egypt

Animals

60 adult male albino rats, weighing between 180-200 grams, were used in this study. The animals were housed individually in well-ventilated cages at the Animal House, Faculty of Medicine, Sohag University. They were maintained under controlled environmental conditions, including a standard light/dark cycle and appropriate temperature, with free access to food and water for two weeks.

Experimental design

They divided randomly into 4groups; 15 in each group.

Duration:2 weeks

Group 1: The control group received distilled water orally by oral gavage once daily for 2 weeks.

Group 2: Zinc-treated group; animals received zinc sulfate syrup (30 mg/kg/day) by oral gavage for 2 weeks. **(Nazarizadeh and Asri-Rezaie,2016).**

Group 3: Acyclovir-treated group; Acyclovir each rat received (432 mg/Kg) of Acyclovir suspended in distilled water by oral gavage once daily for 2 weeks. **(Bassam et al.,2008).**

Group 4: Acyclovir and Zinc treated group, animals received acyclovir (432mg/kg), then followed by zinc sulfate syrup (30 mg/kg/day) by oral gavage for 2 weeks.

Following two weeks of experimentation, animals from all groups were anesthetized using ether and subsequently euthanized. The cerebellum was carefully extracted and sagittal sectioned for microscopic evaluation.

Histological process

The tissue specimens were immersed in 10% neutral buffered formalin for fixation. Paraffin-embedded serial sections, each 5 µm thick, were prepared and mounted on charged glass slides. Sections underwent deparaffinization and dehydration, followed by staining with Hematoxylin and Eosin (H&E) to assess the structure of the cerebellar cortex. **(Suvarna et al.,2013).**

Immunohistochemical analysis

Additional paraffin sections of the same thickness were mounted on positively charged slides. These were dewaxed, rehydrated, and washed with phosphate-buffered saline (PBS) to expose antigenic sites, then processed using the appropriate immunohistochemical stain. Immunohistochemical staining will be performed using antibodies against:

(a) Glial fibrillary acidic protein (GFAP): a protein which is considered the skeleton of the astrocytes, stained with rabbit polyclonal anti-GFAP antibody (mouse monoclonal antibody for GFAP, dilution 1/100, Sigma, St Louis, Missouri, USA), the sections were then rinsed and incubated with appropriate rabbit-specific secondary antibodies**.** Positive reaction for GFAP appears as brown star-shaped cells present within the molecular, granular, and Purkinje cell layers. (**Mekkawy et al.,2020).**

(b) BAX (Bcl-2 Associated X-protein) is a protein that helps control cell death and belongs to the Bcl-2 family. The tissue sections were treated with a mouse monoclonal anti-rat BAX antibody. A positive result appeared as brown coloring in the cytoplasm or nuclei of cells in all layers of the cerebellar cortex. **(Reed,2006).**

Ethical consideration: Animal ethical considerations were fully

justified according to the guidelines of the Sohag University Committee for Animal Care and Use with approval certificate number [sohage-5-12-10/2024-1].

Morphometric studies

Quantitative analysis was conducted by selecting 10 non-overlapping fields from each section. Measurements were carried out using ImageJ software (version 1.51k; Wayne Rasband, NIH, USA). The results were collected from H. and E.and immunohistochemistry sections. The following parameters were calculated for quantitative evaluation:

1-Mean thickness of molecular cell layer. with H& E.-stained sections (x 200). **(Celik et al.,2018).**

2- Mean number of Purkinje cells with H&E-stained sections (x 400). **(Ibrahim et al.,2021).**

3- Mean Area% of GFAP immuno-positive reaction in immuno-stained sections (×200). **(Ibrahim et al.,2021).**

4- Mean Area% of BAX immuno-positive reaction in immuno-stained sections (×200). **(Zare et al.,2022).**

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical evaluation was performed using SPSS software (version 16.0), and intergroup differences were assessed using post-hoc tests. A P-value of ≤ 0.05 was considered indicative of statistical significance.

Results

General observations

Through 2 weeks, animals showed normal behavior and appetite, as well as no sensory-motor dysfunction, and there was no significant change in their weight.

Histopathological results

Hematoxylin and eosin (H&E) staining in the cerebellum of control and zinc-treated groups showed similar trilaminar organization of cerebellar cortex; molecular, Purkinje, and granular layers. The molecular layer is formed of fibers and small stellate cells located superficially, but basket cells were observed in deeper regions adjacent to the second layer, the Purkinje cell layer, in which Purkinje cells were arranged in a distinct single row between the molecular and granular layers. Purkinje cell exhibits a characteristic pear-shaped morphology with central vesicular nuclei, basophilic cytoplasm, and prominent nucleolus. The third layer is the granular layer, which consists of densely packed, small, rounded cells with intensely stained nuclei (granule cells); also, it has clear acidophilic areas in between these cells (cerebellar glomerulus) (**Fig. 1-A, B**).

While in the acyclovir-treated group, the molecular cell layer shows decreased cellularity. The stellate cells and basket cells of the molecular layer appeared darkly stained with pre-neural space around them, while the Purkinje cells were no longer arranged in their usual monolayer pattern. Purkinje cells are crowded in some areas and are lost in other areas. Purkinje cells appeared with irregular outlines, dark-stained cytoplasm, and invisible nuclei. In the granular layer, granular cells within the granular layer showed reduced density, dark nuclei, and numerous intercellular vacuoles; more abundant cerebellar glomeruli **(Fig.1-C).**

In acyclovir and zinc treated group the stellate cells and basket cell of molecular layer appeared normal with no perineural spaces around them, Purkinje cells layer showed some Purkinje cells with regular shaped bodies, they have pale basophilic cytoplasm, vesicular nuclei and prominent nucleoli, while other cells

appear irregular shaped with homogenous cytoplasm and hardly identified nuclei near normal but still area of vacuolation appeared between cell, the granular cells density is restored and it appeared crowded again with normal pattern of distribution, Granular cerebellar glomerulus is less present in between them **(Fig.1-D).**

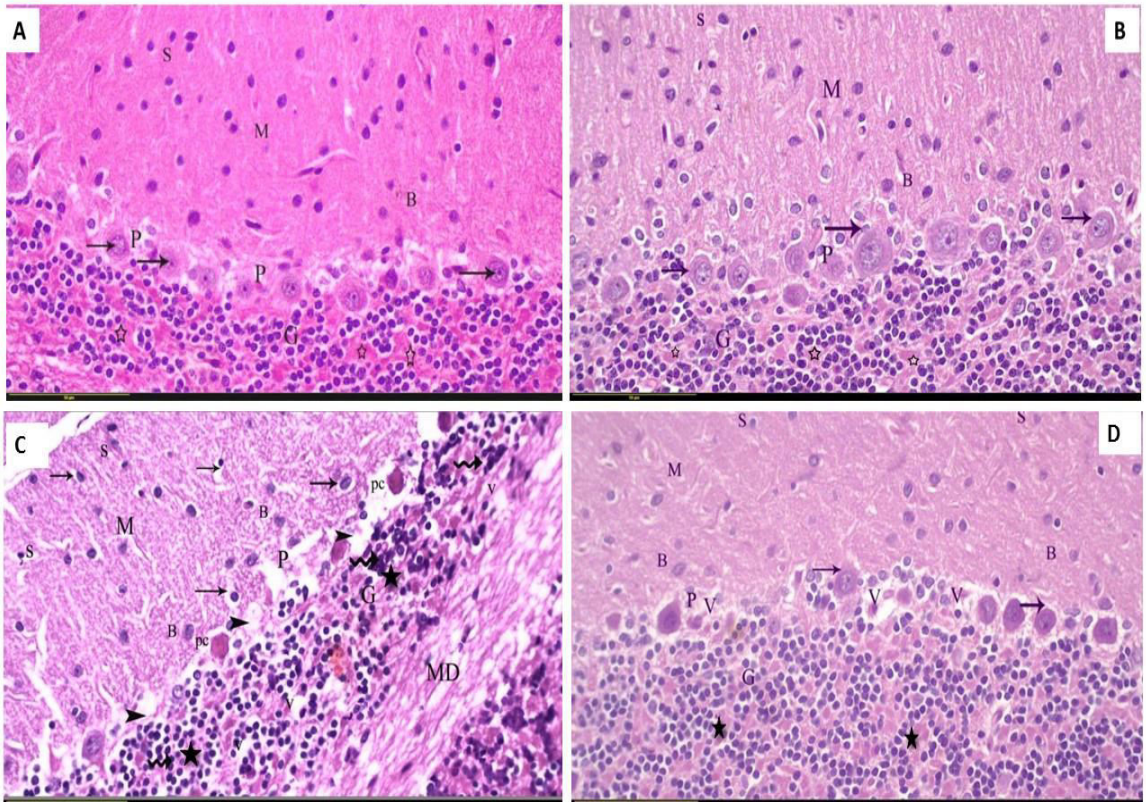


Fig.1. Photomicrographic sections **(A, B):** in the cerebellar cortex of control group and zinc treated rate shows the molecular layer **(M)** with stellate cells **(s)** and basket cell**(B)** present deep**,** Purkinje cell layer **(P)** Purkinje cell is pear shaped cell with large vesicular nucleus and prominent nucleolus **(arrow).** Granular layer **(G)** shows small, rounded, and densely packed cells with deeply stained nucleoli and cerebellar glomeruli in between cells **(star). (C):** Acyclovir treated group shows marked decreased cellularity with deeply stained stellate cells (**S**) and basket cells (**B),** it appears with pre neural space around them (**thin arrow**), Purkinje cells show loss its arrangement, irregularity in their shape, also it shows homogenized darkly stained cytoplasm and ill-defined nuclei (**PC**) and empty spaces are observed in between cells (**arrowhead**). Granular cell layer shows granule cells (**irregular arrow**), less crowded, numerous vacuolation present (**V**), and more abundant cerebellar glomeruli in between cells **(star). (D):** Acyclovir & zinc treated the molecular layer (**M**) with normal stellate cells (**S**) and basket cells (**B**). Purkinje cells (**PC**) show normally shaped cells (arrow), and others appear with dusky-stained nucleus **(arrow)**, but there are some areas of vacuolation in between cells **(V),** the granular cell layer appears with normal granular cells and cerebellar glomeruli (**star**). **(H&E×400, scale bar =50** μm).

Immunohistochemical results

GFAP stain: In control rats' cerebellar cortex, it shows minimal reactivity to GFAP stain appeared in the granular layer with few astrocytes **(Fig.2 -A)**, while in the zinc-treated group, there was a mild reaction to GFAP, appearing as a few astrocytes in the molecular and granular layer **(Fig.2-B)**.In an acyclovir-treated rat, there was a highly positive reaction to GFAP, where intensely stained astrocytes of different sizes were scattered in the three layers**,** brownish star-shaped astrocytes were abundant in the granular layer (**Fig. 2-C**). In the Acyclovir and zinc-treated rats, there was a moderate positive GFAP reaction, where astrocytes with less intensity than those in acyclovir-treated rats were observed in the three layers. **(Fig. 2-D).**

In BAX stain: Cerebellar sections of control and zinc-treated rats, which were stained by Bax, showed very weak immunoreaction to Bax in the cerebellar cortex **(Fig.3-A, B).** While in acyclovir-treated rats' cerebellar sections, which were stained by Bax, show a strong positive cytoplasmic immune reaction to BAX. **(Fig.3-C).** While in acyclovir and zinc-treated rats, cerebellar sections, which were stained by Bax, show a mild cytoplasmic immune reaction (**Fig.3-D).**

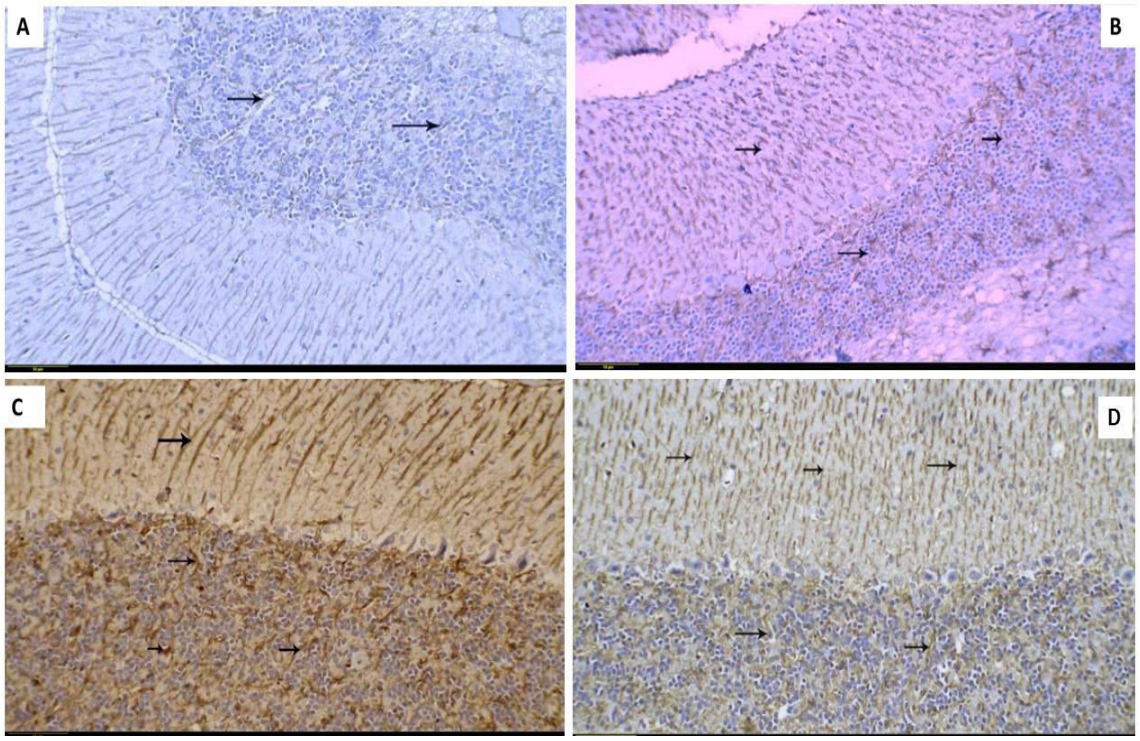


Fig. 2. An immunohistochemical sections in the rat cerebellar cortex of **(A):** the control group show a very weak reaction to GFAP**,** minimal astrocytes are present in the granular layer (**arrow). (B):** in the zinc-treated group, there is mild GFAP reaction, where mild astrocytes are present in the molecular &granular layer (**arrow**). **(C)**The acyclovir-treated group shows strong positive brownish immunoreaction to GFAP, which appears more in the molecular and granular layer (**arrows)**. **(D)**: acyclovir & zinc-treated group shows moderate cytoplasmic immunoreaction to GFAP in the molecular layer and granular layer **(arrow). (GFAP ×200, scale bar =50um).**

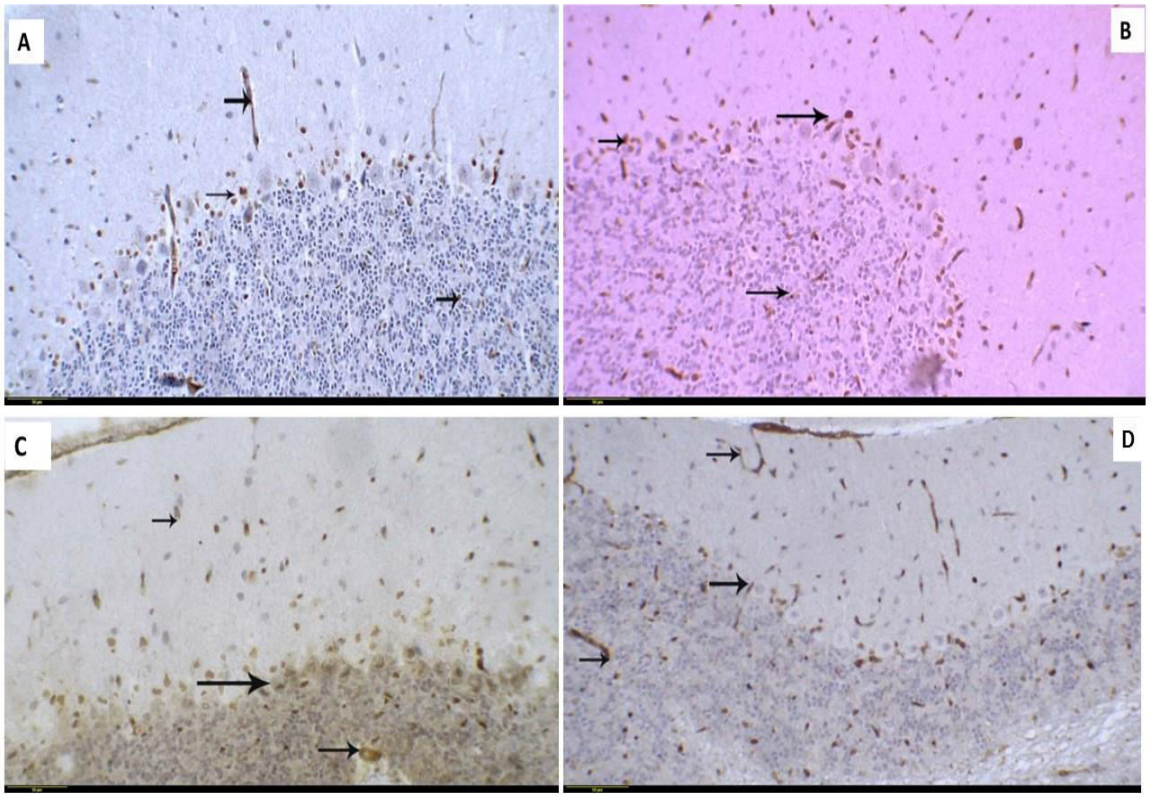


Fig. 3. An immunohistochemical sections in the rat cerebellar cortex of the rat **(A):** control group show minimal immunoreaction to Bax in Purkinje cells and molecular cell layer, but very weak reaction in the granular cell layer. **(B):** The zinc-treated group shows mild immunoreaction to Bax appears in molecular layers, between **Purkinje** cells, and in granular layers **(arrow). (C):** The acyclovir-treated group shows positive immunoreaction to BAX, more abundant in Purkinje cells and the granular cell layer **(arrow).** (D)The acyclovir & zinc-treated group shows a moderate positive reaction to BAX stain in the molecular, Purkinje cell layer, and granular cell layer **(arrow). (Bax ×200, scale bar =50um).**

Morphometric and statistical results

****Molecular thickness in Hematoxylin & Eosine :** There was no significant change in molecular layer thickness between the control group and the treated group, and between the control and acyclovir & zinc-treated group. While molecular layer thickness showed a significant decrease in the acyclovir-treated group compared to the control, zinc and zinc & acyclovir-treated groups. (**Table.1, histogram 1).**

****Purkinje number in Hematoxylin & Eosin stain:** There was no change in Purkinje cell number between the control group and zinc-treated group, and a mild decrease in number when compared between the control group and acyclovir & zinc-treated group. While there was a highly significant decrease in the number of Purkinje cells in the acyclovir group when compared to the control group, there was no significant difference between the zinc and zinc & acyclovir-treated groups. **(Table.1, histogram2).**

****Area% of GFAP in immunohistochemical stain:** There was no significant difference in GFAP expression between the control and zinc-treated group; otherwise, there was a

highly significant difference in GFAP expression between the acyclovir group in comparison with the control group, the zinc, acyclovir & zinc-treated group, and a significant difference between the control and acyclovir & zinc-treated group. **(Table.2, histogram 3)**

****Area% of BAX in immunohistochemical stain:** There was no change in BAX expression in the control group when compared to the zinc-treated group. While there was a very highly significant increase in BAX expression between the acyclovir group in comparison with the control group, zinc, acyclovir & zinc-treated groups. And there was a highly significant increase in BAX expression in the acyclovir & zinc-treated group in comparison with the control group**. (Table.2, histogram 3).**

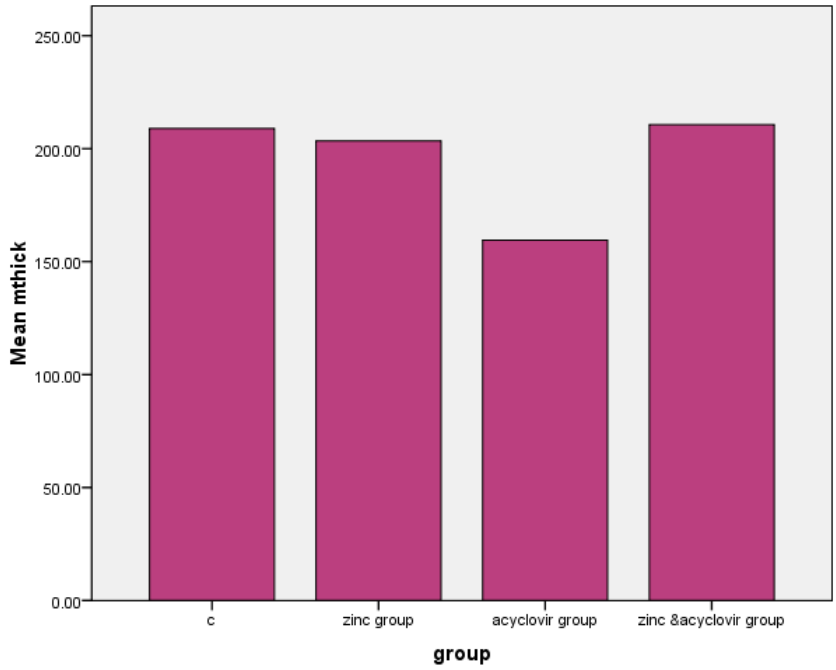
Table 1. The mean value± standard deviation of the molecular layer thickening and number of Purkinje cell in both control and treated groups

Variables	Control	Zinc treated	Acyclovir treated	Acyclovir and zinc treated	P 1	P2	P3	P4	P5
Molecular thickness	208.9± 33.5	203.3±36.8	159.4± 29.32	210.7± 31.02	.6	.000	.8	.001	.000
Purkinje number	10.4±1.35	10.8±1.4	6±1.3	9.2±1.08	.3	.000	.012	0.00	.000

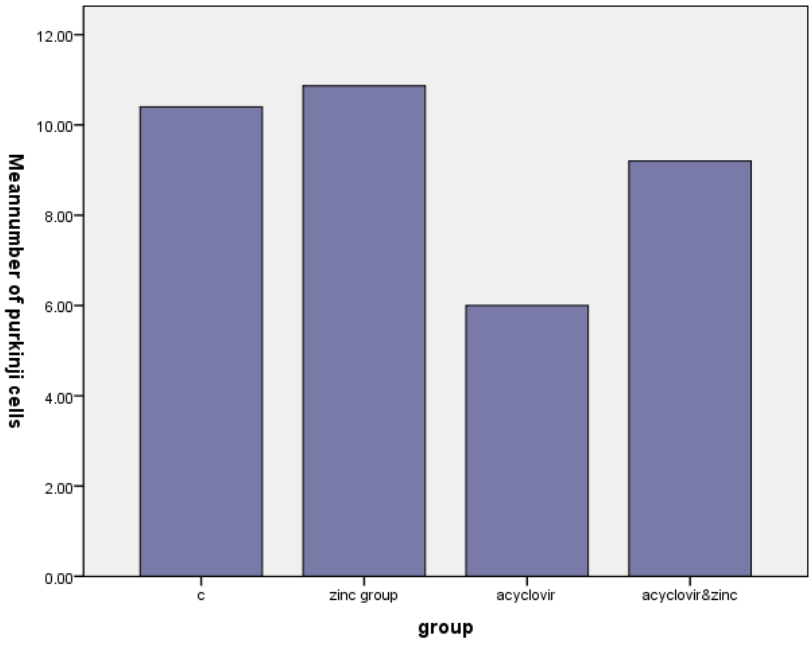
p1: difference between control and zinc treated group. p2: difference between control and acyclovir treated group, p3: difference between control group and acyclovir & zinc treated group, p4: difference between acyclovir treated group and zinc treated group. P5: between acyclovir and acyclovir &zinc treated group.

Table 2. The mean value± standard deviation of the area percentage of BAX and GFAP stain in both control and treated groups

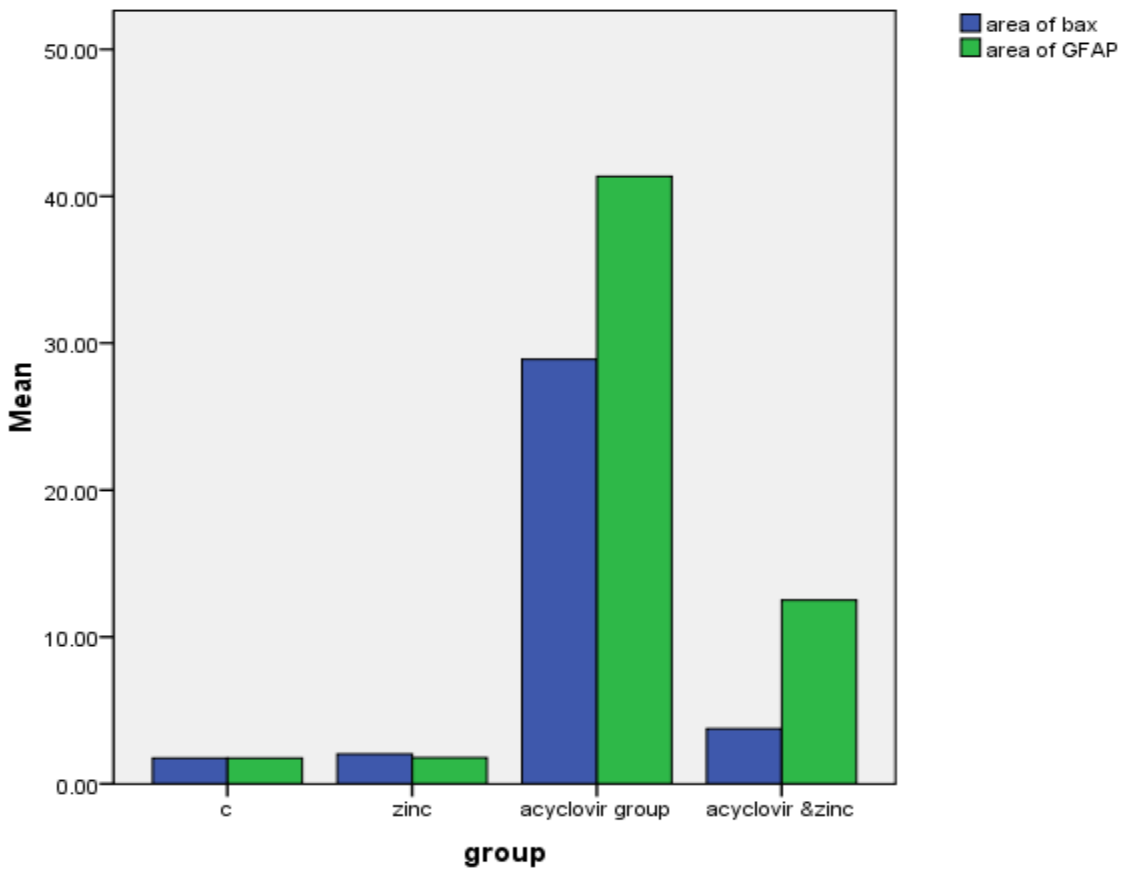
Group	Control group	Zinc treated group	Acyclovir treated	Acyclovir and zinc treated	P1	P2	P3	P4	P5
Area % of BAX	1.75±1.44	2.03±0.63	28.9±5.36	3.75±1.7	.50	.000	.002	.000	.000
Area% of GFAP	1.75±0.78	1.78±1.13	41.36±7.28	12.5±1.73	.95	.000	.000	.000	.000



Histogram (1). The mean thickening of molecular layer in control and treated group.



Histogram (2). The mean number of Purkinje cells of control and treated group.



Histogram (3). The mean area %percentage of GFAP &BAX immunohistochemical stain in both control and treated group.

Discussion

Acyclovir is a purine nucleoside analogue that exhibits potent antiviral activity against human herpesvirus infections, its action achieved by inhibition of (deoxyribonucleic acid) DNA synthesis. **(Paintsil and Cheng, 2019).** High concentrations of acyclovir are required for the treatment of severe central nervous system (CNS) infections to enhance its antiviral efficacy and broaden its therapeutic spectrum **(Gurgel et al.,2021).** Both acyclovir and its prodrug valacyclovir are widely used for prophylaxis and treatment of HSV infections **(Kłysik et al.,2020).**

Zinc is an essential nutrient that has multiple fundamental functions in the developing and adult nervous system. **(Krall et al.,2021).** Zinc is a structural or functional component of many proteins; it is an essential cofactor in protein structure stabilization and enzyme catalysis, such as DNA synthesis, brain development, and neurotransmission**. (Li et al.,2023).**

In the present study, Histological examination of the cerebellar cortex in the acyclovir-treated group revealed marked structural changes. Stellate and basket cells in the molecular layer appeared deeply stained and were surrounded by widened perineuronal spaces. The number of Purkinje cells was reduced, and the remaining cells showed irregular outlines, darkly stained cytoplasm, and indistinct nuclei. In the granular layer, a decrease in cell density was observed along with hyperchromatic nuclei and prominent intercellular

vacuolation, suggesting degenerative alterations. **Purcell et al. (2018)** approved that vacuolations are a common histopathological finding that can be associated with neurotoxicity as a result of neuronal injury and cell death. Also, **Soliman and Ali (2021)** accepted that disturbed organization of Purkinje cells in cerebellar sections is considered a sign of neural insult, which is triggered by an agent that activates oxidative stress. Also, **Brandariz-Nuñez et al. (2021)** said that acyclovir and its derivative valacyclovir can produce rare neurological adverse effect as toxic symptoms including confusion, altered level of consciousness, hallucinations, agitation, and dysarthria, after 3 days of treatment and reverse completely after 7 days or less, especially in patients with old age or those with impaired renal function.

Also, **lindström et al. (2019)** demonstrated the correlation between the accumulation of the metabolite 9-Carboxymethoxymethylguanine (CMMG) produced during the metabolism of acyclovir by the enzyme's alcohol dehydrogenase and aldehyde dehydrogenase and the emergence of neuropsychiatric symptoms in patients receiving acyclovir or valacyclovir treatment.

Elseady et al. (2022) explained that nucleotide analogues can induce mitochondrial damage, leading to the release of large amounts of reactive oxygen species (ROS), which act as signaling molecules that contribute to the injury of normal tissues.

Ibrahim et al. (2021) observed similar cerebellar damage following sofosbuvir administration, including reduced Purkinje cell count and alterations in granular cells. Similarly, **Peter et al. (2017)** reported degenerative changes in cerebellar cells following lamivudine treatment for HIV and hepatitis B.

In the present study, there were strong positive reactions to GFAP in Acyclovir-treated sections of the cerebellar cortex layers, and an increase in the area percentage of GFAP.

According to **Sofroniew (2015)**, astrocytes play an important role in preventing toxic substances from passing through the brain barrier. Also, they increase in size and number in response to nervous tissue injury. Also, these results corroborate with **Peter et al. (2017)**, who found elevated GFAP expression and reduced molecular layer cell count following lamivudine treatment, which indicates neuroinflammation and neuronal injury. Also, **Hui et al. (2020)** observed that acyclovir inhibits microglial but not astrocyte activation when used in the treatment of the herpes virus. Similarly, **Ibrahim et al. (2021)** found increased GFAP expression indicative of astrogliosis in rats treated with sofosbuvir, reflecting a compensatory astrocytic response to neurodegeneration.

Also, the increased level of GFAP is a marker of astrocytosis, which is produced by damaged astrocytes, indicating neurodegenerative disorders**. (Oeckl et al.,2019).**

In this study, BAX protein was detected in Acyclovir-treated specimens as a brownish immunoreaction, which denotes the apoptosis process, especially in Purkinje and granular cell layers.

BAX protein is a pro-apoptotic protein of the Bcl-2 family, which translocates to mitochondrial membranes during the apoptosis process**. (Means and Katz, 2022),** Also**, Kaya et al. (2022)** found significant increases in

Bcl-2 expression in the brain tissue, which is a sign of destruction of the cerebellum when using the antiviral drug favipiravir.

In our study zinc treated group showed normal structure of cerebral cortex While in acyclovir and zinc treated group cerebellar showed slight preservation of normal cerebellar cortex architecture; where the stellate cells and basket cell of molecular layer appeared normal, Purkinje cells layers appeared near normal but still area of vacuolation appeared between cell while the granular cells appeared near normal with preserved cerebellar glomeruli. Mild increase in BAX and GFAP expression compared to the control and zinc-treated group.

Zinc elevates antioxidant enzymes such as Superoxide Dismutase (SOD) and Catalase (CAT), slows down important pro-oxidant enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthetase (iNOS), and regulates oxidant production, which is expressed in the hippocampus, cerebral cortex, and cerebellar cortex. **(Lee et al., 2020).**

Zinc is essential for proper brain development and function. It acts as a cofactor for key enzymes such as DNA and RNA polymerases, histone catalases, and DNA ligase. As a result, zinc plays a crucial role in protein synthesis and gene expression processes within the central nervous system**. (Skrajnowska and Bobrowska-Korczak, 2019).**

This aligns with **Ibrahim et al. (2015)**, who demonstrated the neuroprotective effects of zinc oxide against lithium-induced brain toxicity, attributed to zinc's antioxidant properties. Similarly, research reveals that Zn plays a role in decreasing oxidative stress, inhibiting inflammation, and improving lipid and glucose metabolism chiefly in the brain**.** **(**Akintoye **et al.,2023).**

Warsito et al. (2025). Reported that the use of zinc sulfate had a protective effect by modifying oxidative damage and apoptotic signaling in lead-induced reproductive toxicity.

Also, **Ali and Aziz (2022)** showed that the use of zinc sulfate against oxaliplatin treated rat produce a decrease in GFAP expression in brain tissue by reducing the inflammatory reactions via decreasing NF-κB, TNF-α, and IL-1β activation.

Conclusion

Acyclovir is a key antiviral agent in the management of viral encephalitis; however, the present findings suggest it may exert undesirable effects on cerebellar cortex layers. Co-administration of zinc exhibited a protective influence, potentially mitigating these structural changes. Further research is needed to reveal the underlying mechanisms of acyclovir-induced neurotoxicity.

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