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ORIGINAL ARTICE

Age-Related Changes of Purkinje Cells and Astrocytes in Rat Cerebellar Cortex: Histomorphometric, Immunohistochemical and Biochemical Study

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ABSTRACT

Background: The cerebellar cortex is responsible for coordinating movement, adapting to special conditions, and is involved in storing memories. This cortex undergoes age-related pathological changes in the form of declined cortical thickness, neuronal loss particularly Purkinje cells, hypertrophy, and hyperplasia of the astrocytes as well as alterations in oxidative status. These changes are responsible for various senile disorders. The aim of work is to evaluate histological changes in cerebellar Purkinje cells and astrocytes and to determine the alteration in Malonaldehyde (MDA) and Glutathione (GSH) in relation to age in albino rats, and to find a probable correlation between the cellular changes and the oxidative status.
Methods: Two age groups of albino rats (3-6 months & 22-26 months) were sacrificed, and their cerebella were excised and divided into three parts. 1st part was sectioned and stained with Haematoxylin and Eosin, Silver and Cresyl Violet stains. 2nd part was sectioned and prepared for immunohistochemical study using Glial Fibrillary Acid Protein (GFAP) antibodies then examined by light microscope and morphometric measurements were performed. 3rd part was biochemically processed to measure the MDA and GSH levels. All data were statistically analysed.
Results: The obtained data showed that the most pronounced age-related changes were the decreased thickness of cerebellar cortex, decreased Purkinje cell number with increased degenerated cells, significant increase in astrocytes immune reactivity to GFAP and insignificant change in MDA and GSH levels.
Conclusions: Cerebellar cortex of senile rats showed pathological changes in Purkinje cells and astrocytes. These changes were not solely relevant to the oxidative status. Perhaps, other factors contributed as well.
Key Words: Aging; Cerebellar cortex; Purkinje cells; GFAP; Oxidative status.

INTRODUCTION

The cerebellum is referred to as the little brain or as a neuronal machine; it coordinates motor behaviour, muscle tone, eye movement, adaptation, respiration, and cognition. It also mediates sensory discrepancy, time perception, storing memories and plays a principal role in nociception [1] [2].

The cerebellar cortex consists of three layers from outside inwards; molecular, Purkinje and granular. The molecular layer consists of few basket cells. Purkinje cell layer is formed of large neurones with flask-shaped cell bodies and multiple dendrites. As for the granular layer, many small neurones (granule cells) and astrocytes that modulate the activity of Purkinje cells. The granule cells receive incoming impulses from other parts of the central nervous system and send axons into the molecular layer where they branch in a T- shaped manner, so that the axons contact the dendrites of several Purkinje and basket cells. The large dendrites of Purkinje neurons form nearly two-dimensional layer through which parallel fibres from the deeper-layers pass. Each cell has multiple dendrites and a single axon (nerve fibre) that represents the beginning of the outflow from the cerebellum [3].

Purkinje cells' pathology results in cerebellar ataxias and autism. The genetic conditions ataxia telangiectasia and Niemann Pick disease type C, as well as cerebellar essential tremor, involve the progressive loss of Purkinje cells. In Alzheimer's disease, loss of dendritic branches of the Purkinje cells was noticed [4].

Cerebellar astrocytes, a particular glial cell type, can be classified based on their morphologies and layering into three main types, comprising Bergmann glia, granular layer astrocytes in the cerebellar cortex and fibrous astrocytes in the cerebellar white matter. Astrocytes play key roles in regulating the pathophysiology of neuronal functions and are essential for proper cerebellar development and functioning [5]**.** Moreover, astrocytes are important for synaptogenesis, maturation and maintenance of synaptic activity and plasticity [6]. ***Verkhratsky and Nedergaard*** [7] declared that astrocytes are crucial for regulation of cerebellar tissue homeostasis.

Pertusa et al. [8] reported that astrocytes may partially lose their neuroprotective ability during aging and suggested that aged astrocytes may contribute to exacerbating neuronal injury in age-related neurodegenerative processes. Astrocytes participate in the inflammatory response and play a key role in the progression of neurodegenerative diseases [9].

Glial Fibrillary Acid Protein (GFAP) is a specific marker for astrocytes. GFAP immunoreactive astrocytes are sensitive to aging and display age-related proliferation, hypertrophy, and up-regulation of GFAP [10]**.**

Inspection of the elderly's brain at the cellular and molecular levels reveals several hallmarks of aging. These hallmarks comprise**:** mitochondrial dysfunction; imbalance between free radicals and antioxidant i.e., oxidative stress; genetic disorder; dysregulated energy metabolism; impaired cellular "waste disposal" mechanisms (autophagy lysosome and proteasome functionality); impaired adaptive stress response signalling; compromised DNA repair; aberrant neuronal network activity; dysregulated neuronal Ca^{2+} handling; stem cell exhaustion; and inflammation [11].

Oxidative stress is one of the commonly accepted hallmarks. Nearly 20% of the total oxygen inspired is consumed by the brain. The presence of high level of polyunsaturated fatty acids (PUFAs) in the brain serves as a major target for reactive oxygen species [12]. Lipid peroxidation contributes to the damage of PUFAs in membrane phospholipids and has a significant role in cell and tissue damage. Malonaldehyde (MDA) is the most abundant aldehyde emerging from lipid peroxidation [13].

Endogenous antioxidants, for example the non-enzymatic scavenger glutathione (GSH) and antioxidant enzymes; superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are the first line of defense and function by scavenging potentially harmful moieties of free radical [12].

Santulli et al. [14] stated that aging has several conflicting factors that play a role in evaluating the degree of associated tissue damage. ***Aditi et al.*** [15] added that the combined effects of these multiple factors should be understood to create causal connections between aging and oxidative stress.

The aim of the current study is to elucidate the changes in the morphological criteria of Purkinje

cells, determine the mean area percentage of reactive astrocytes to GFAP, measure the MDA and GSH content in the cerebellum of young adult and senile rats, and to find a probable correlation between the cellular changes and the oxidative status.

METHODS

Twenty male albino rats were used in this study. They were obtained from the animal house of Fayoum University. The rats were used in accordance with ethics of animal care and use committee (ACUC) of Fayoum University. The experiment complied with the ARRIVE guidelines and carried out in accordance with the U.K. animals. Rats were divided into two age groups 10 rats each. Group I: Young adult rats (3-6 months) & Group II: Senile rats (22-26 months).

All rats were sacrificed after anaesthetisation with 50ml/kg subcutaneous injection of thiopental sodium and the cerebella were excised and prepared for the following studies: ***Light microscopic study***: The cerebellum specimens were fixed in formalin solution and processed for paraffin blocks. Sections were cut into 7 µm thickness and stained with Haematoxylin and Eosin [16], Silver [17] and Cresyl Violet [18] stains. ***Immunohistochemistry*** using glial fibrillary acidic protein (GFAP) Rabbit polyclonal antibody (PA5-85109) (Thermo Fischer, Labvision, USA) to demonstrate immunoreactive astrocytes in the granular layer [10]. ***Biochemical study:*** Parts of cerebellum specimen were stored at 20º C, homogenized then processed for determination of MDA and GSH levels. Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) test. The MDA concentration was calculated from the absorption at 532 nm by use of a molar extinction coefficient of 1.56×10^5 mol^{-1} cm^{-1}, and also recalculated from MDA standards produced by the acid hydrolysis of 1,1,3,3-tetramethoxypropane [19]. The GSH level was determined by a modified Ellman method, after centrifugation and treating with sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$), supernatants were reacted with 5, 5-dithiobis (2-nitrobenzoic acid). Absorbance was read using the spectrophotometer at 412 nm. The GSH level was calculated using an extinction coefficient of13.000 mol^{-1} cm^{-1} [20]. ***Histomorphometric study and Statistical analysis:*** The data was obtained using the image analyser computer system (Leica Qwin 500). The collected data was organized, tabulated, and statistically analysed using SPSS software statistical computer package version 22 (SPSS Inc. USA). The average number of the Purkinje cells for 10 non-overlapping fields for each group using binary mode were counted in magnification power of 400. Fields comprising degenerated Purkinje cell were presented as number and percentages; Fisher exact test was used as a test of significance. For degenerated Purkinje cells within fields and tissue contents of MDA and GSH, the mean and standard deviation (SD) were calculated, and Mann-Whitney U test was used as a test of significant. The area % of GFAP immunoexpression was measured in 10 high power fields for each group using binary mode (a magnification power of 400). Independent t-test was used to test the difference between mean values of GFAP area % among groups. For interpretation of results of tests of significance, significance was adopted at $P \leq 0.05$.

RESULTS

Histological results

Sections of cerebellar cortex stained with **Haematoxylin and Eosin** stain of the young adult group showed that the outermost layer; the molecular layer, is highly stained with eosin as it presents few neuronal cell bodies of basket cells, widely separated from each other with unmyelinated nerve fibres. The middle layer consisted of Purkinje cells arranged in one row and have pyriform cell bodies with large pale vesicular nucleus and clear nucleolus and possess long axons that arborize in the molecular layer. The inner granular layer showed a large number of small deeply stained cells with few cerebellar islands in between **(Figs.1, 2& 3).** Sections of cerebellar cortex of senile group

revealed marked decrease in the thickness of both molecular and Purkinje cell layers, increase in thickness of granular layer and total decrease in the whole cortical thickness in many specimens. The molecular layer displayed vacuolated nerve fibres. The Purkinje cells lost their pyriform shape and were swollen, exhibiting irregular condensed large nucleus, and widely displaced in a markedly disturbed layer, some Purkinje cells were vacuolated, and others were shrunken with pyknotic and karyolytic nuclei and most of them lost their axons. The granular layer showed extensive dark stained small cells with wide cerebellar islands in between. **(Figs.4, 5&6).**

In sections stained with **silver stain,** the young adult group visualized a single layer of pyriform shaped Purkinje cells with large nucleus and single axon extending into the molecular layer **(Fig.7)**, while the senile group visualized loss of the pyriform shape of Purkinje cells with irregular large nuclei. Some cells were swollen, and others were degenerated with irregular shape and loss of axon **(Fig.8).**

In sections stained with **Cresyl Violet stain,** the young adult group showed extensive basophilic Nissl bodies scattered around the nucleus of Purkinje cells **(Fig.9)**, whereas the senile group showed shrunken and degenerated Purkinje cells with increased intensity of Nissl bodies around the nucleus **(Fig.10).**

Immunohistochemical results

Sections of cerebellar cortex of the young adult rats revealed weak GFAP positive astrocytes and radial glial fibres in the granular layer **(Fig.11)**, while senile rats' sections revealed strong GFAP positive astrocytes of different sizes in the granular layer. The processes of adjacent astrocytes were interwoven with each other **(Fig.12).** The intensity of GFAP immunoreaction was markedly increased in the senile group which is an indication of astrocytes hypertrophy and hyperplasia.

Biochemical study

The mean values ± SD of MDA and GSH were (7.18 ± 1) and (0.91 ±0.19) in young adult group and (7.23 ± 2.11) and (0.87± 0.27) in senile group.

Statistical analysis of data

The average values of the Purkinje cells in 10 non-overlapping fields were 11±1 and 10±1 for group I and II, respectively. All fields in group II showed degenerated Purkinje cells compared to 3 fields only in group I, which was statistically significant, p=0.003. As well, there was a statistically significant increases in degenerated Purkinje cells in rats of group II compared to rats of the group I (2.60± 0.70 VS 0.33±0.48) , p<0.0001 **(Tables 1&2 and figures 13 &14).**

GFAP Cerebellum area % was statistically significantly higher in group II as compared with group I (3.97 ± 0.93 VS 1.01 ± 0.35), p =0.001 **(Table 3 and figure 15).**

The cerebellar content of MDA and GSH was statistically insignificant when comparing the two groups $p>0.05$**. (Table 4 and figure 16).**

Table 1: Illustrating fields that showed degenerated Purkinje cell of both groups.

	Group I		Group II		P-value
	N	%	N	%	
Fields showed degenerated Purkinje cell					
Yes	3	30.0%	10	100.0%	**0.003**
No	7	70.0%	0	0.0%	**(S)**

(S)= Significant

Table 2: Illustrating the mean number of degenerated Purkinje cells ± SD of both groups.

	Group I		Group II		P-value
	Mean	SD	Mean	SD	
Degenerated Purkinje cell	0.3	0.48	2.6	0.7	**<0.0001 (S)**

(S)= Significant

Table 3: Illustrating Area% for GFAP of both groups.

	Group I (young adult)		Group II (senile)		P-value
	Mean	SD	Mean	SD	
GFAP Cerebellum Area%	1.01	0.35	3.97	0.93	0.001 (S)

(S)= Significant

Table 4: Illustrating the mean value of MDA and GSH ± SD of both groups

	Group I (young adult)		Group II (senile)		P-value
	Mean	SD	Mean	SD	
MDA (nmol/g tissue)	7.18	1	7.23	2.11	>0.05 (IS)
GSH (µmol/g tissue)	0.91	0.19	0.87	o.27	>0.05 (IS)

(IS)= insignificant

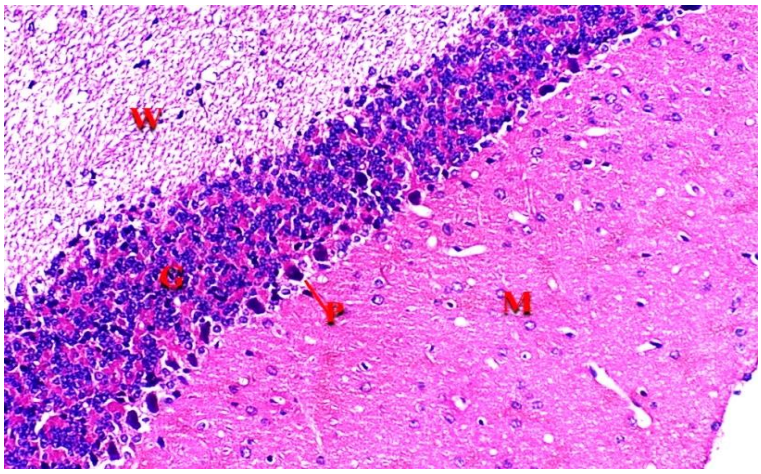

Figure 1: A photomicrograph of a section in group **I** (Young adult rat 3-6 months age) showing normal layers of cerebellar cortex from outside inwards; molecular layer (M), Purkinje cell layer (P) and granular layer (G). The white matter (W) could be observed.

(H&E X100)

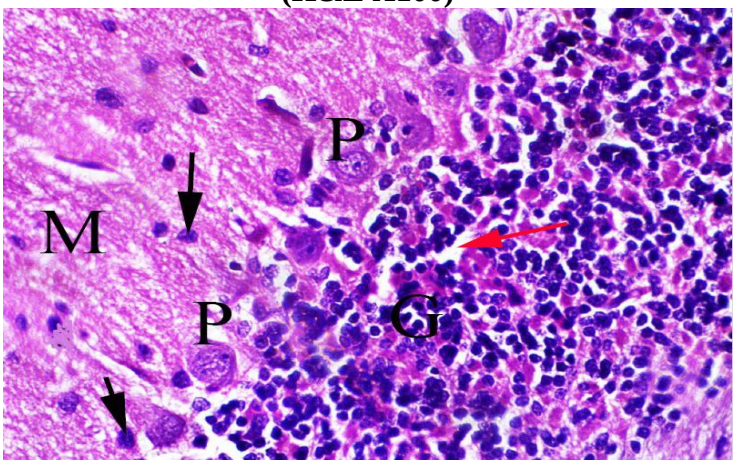

Figure 2: A photomicrograph of a section in the cerebellar cortex of a rat in group I showing the three layers of the cerebellar cortex: the molecular layer (M) formed mainly of unmyelinated fibres and few basket cells (black arrows), Purkinje cells (P) formed of pyriform shaped cells arranged in one row

with pale vesicular nuclei and clear nucleoli, and the granular layer (G) with a large number of small deeply stained cells with few cerebella islands in between (red arrow).

(H&E X400)

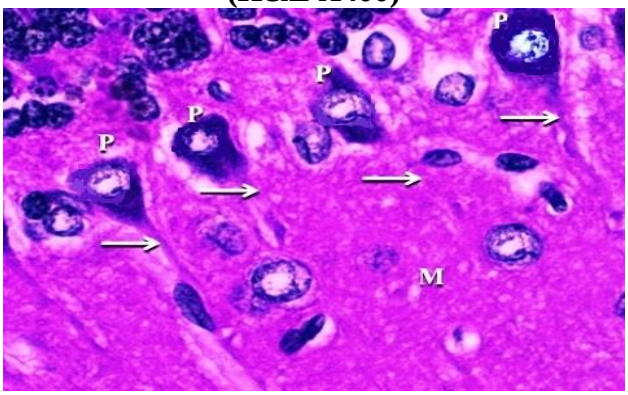

Figure 3: A photomicrograph of a section in the cerebellar cortex of a rat in group I, showing the pyriform Purkinje cells **(P)** arranged in one layer with large prominent vesicular nuclei and single axon **(arrows)** extending into the molecular layer (M**).**

(H&E X1000)

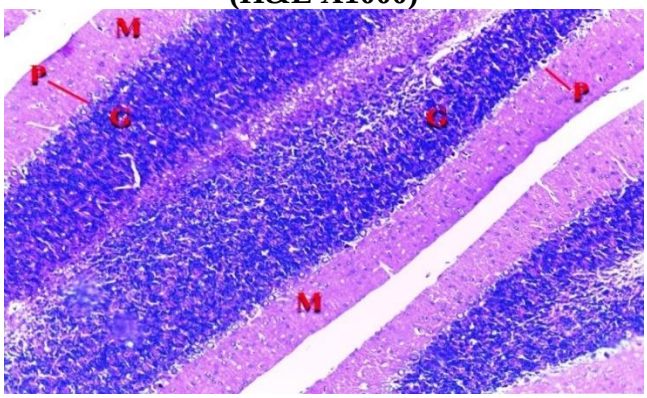

Figure 4: A photomicrograph of a section in group II (Senile rats 22-26 months age) showing layers of cerebellar cortex from outside inwards; molecular layer (M), Purkinje cell layer (P) and granular layer (G). Note marked decrease in molecular and Purkinje cell layers, increase in granular layer and total decrease in cerebellar cortex.

(H&E X100)

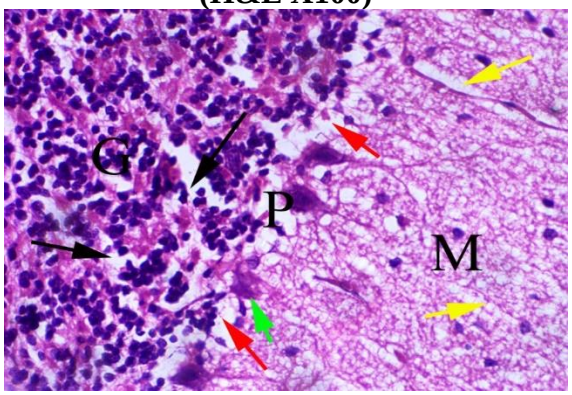

Figure 5: A photomicrograph of a section in the cerebellar cortex of a rat in group II, showing the molecular layer (M) displaying vacuolated nerve fibres (yellow arrows), Purkinje cells layer (P) widely displaced with dark nuclei and markedly disturbed layer, some Purkinje cells are vacuolated (red arrows) and others are shrunken with pyknotic nuclei and loss their axons (green arrow) and

granular layer (G) containing extensive dark stained small cells with wide cerebellar islands in between (black arrows).

(H&E X400)

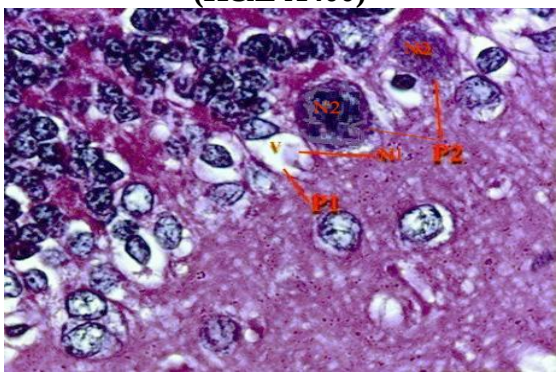

Figure 6: A photomicrograph of a section in group II, showing Purkinje cell **(P1)** with vacuolated cytoplasm **(V)** and karyolytic shrunken nucleus **(N1)** and other Purkinje cells **(P2**) lost their pyriform shape with ill-defined axon and exhibited irregular condensed large nucleus **(N2).**

(H&E X1000)

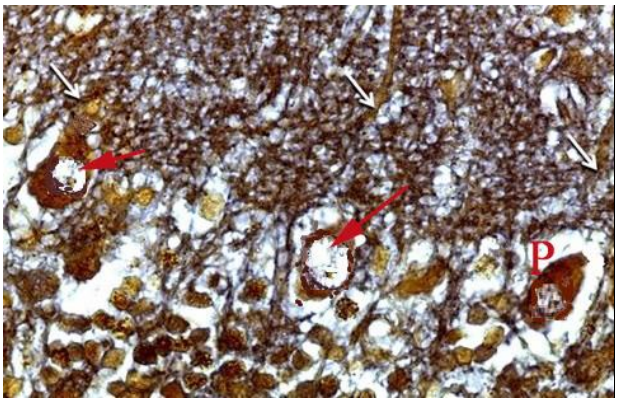

Figure 7: A photomicrograph of a section in the cerebellar cortex of a rat in group I, showing pyriform Purkinje cells **(P)** arranged in one layer with vesicular nuclei **(red arrows)** and single axon **(white arrows)** extending into the molecular layer.

(Silver X1000)

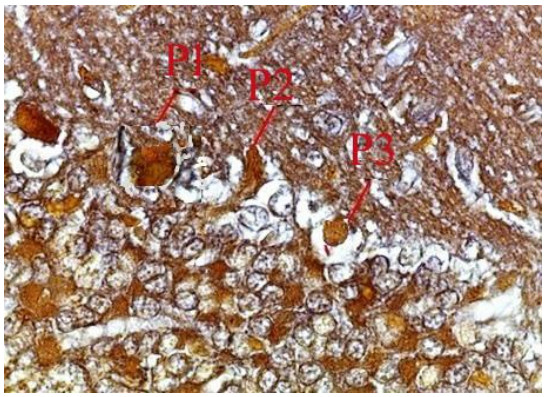

Figure 8: A photomicrograph of a section in the cerebellar cortex of a rat of group II, showing Purkinje cells loss their pyriform shape (P1), some of them appear shrunken with loss of axon (P2) and others appear vacuolated (P3).

(Silver X1000).

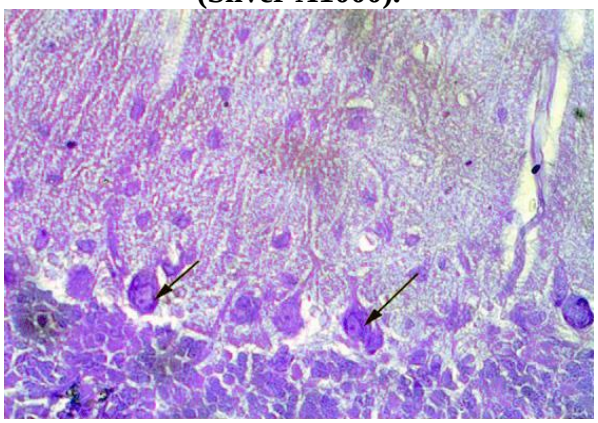

Figure 9: A photomicrograph of a section in the cerebellar cortex of a rat in group I, showing pyriform shaped Purkinje cells with Nissl bodies around the nucleus (arrows).
(Cresyl Violet X 400)

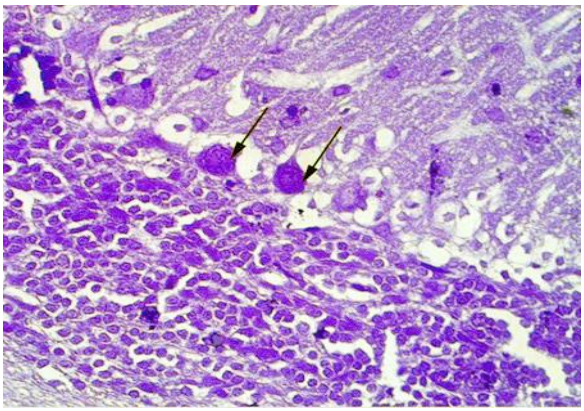

Figure 10: A photomicrograph of a section in the cerebellar cortex of a rat in group II, showing widely displaced degenerated Purkinje cells with dark nucleus and increased intensity of Nissl bodies around the nucleus (arrows).
(Cresyl Violet X400)

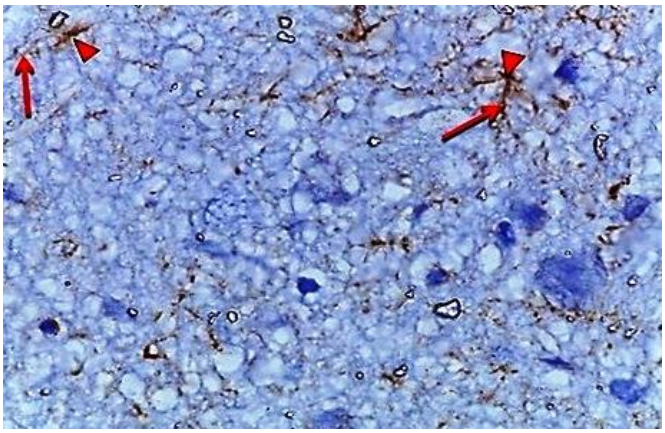

Figure 11: A photomicrograph of a section in the cerebellar cortex of a rat in group I, displaying weak GFAP positive stain in astrocytes in granular layer (arrowheads) with weak positive glial fibres (arrows).

(GFAP immunostaining X400)

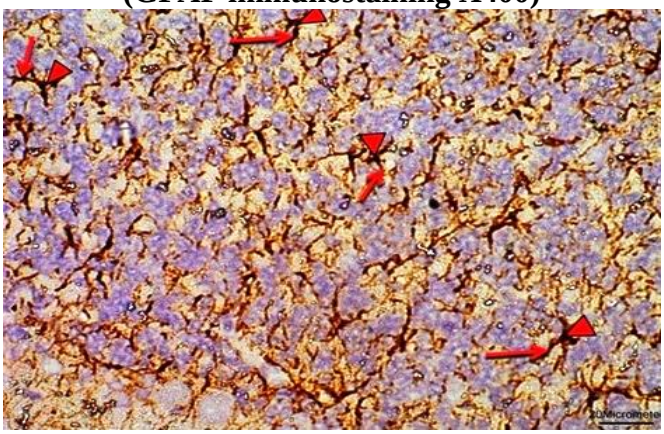

Figure 12: A photomicrograph of a section in group II showing a strong GFAP positive stained astrocytes (arrowheads), the processes of adjacent astrocytes were interwoven with each other with strong positive nerve fibres (arrows).

(GFAP X400)

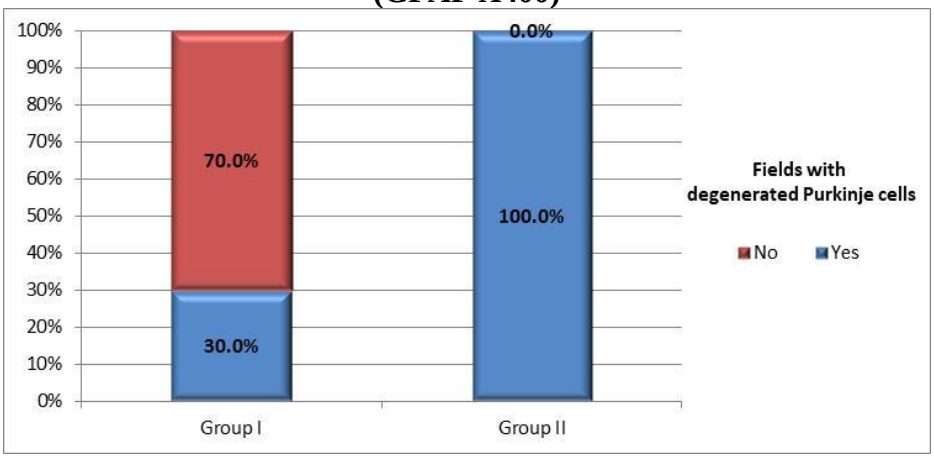

Fig 13: Histogram illustrating fields that showed degenerated Purkinje cell of both groups.

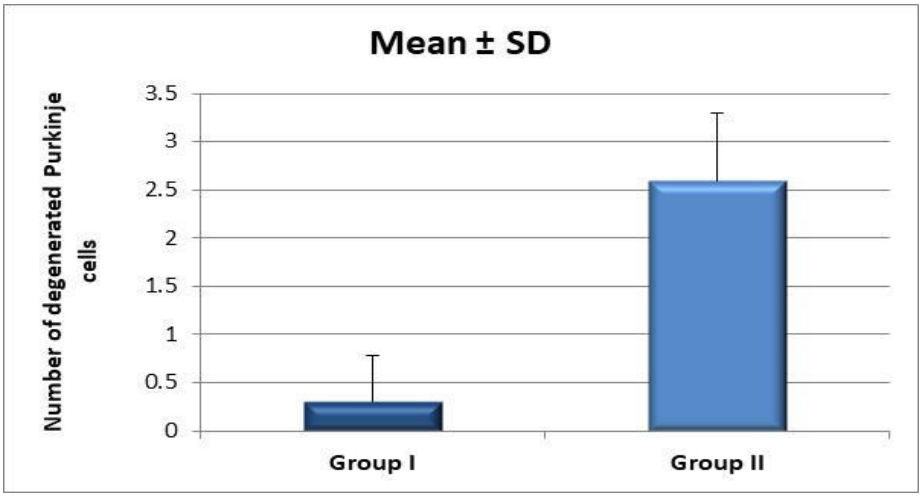

Fig 14: Histogram illustrating the mean number of degenerated Purkinje cells ± SD of both groups.

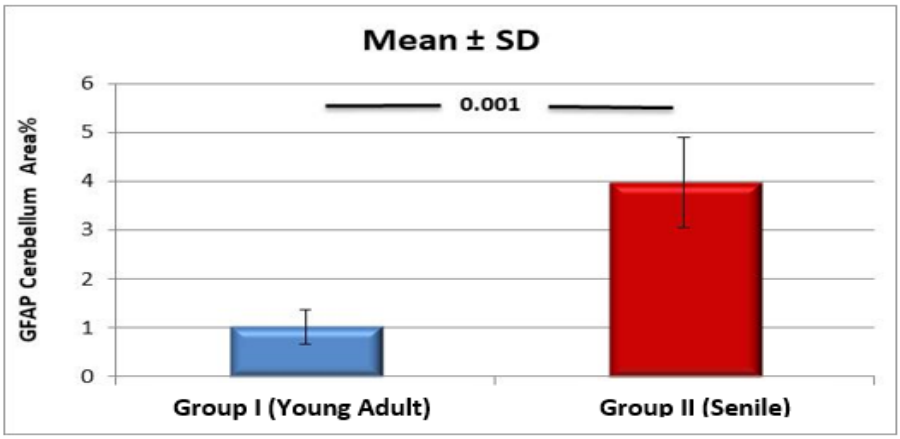

Fig 15: Histogram illustrating Area % for GFAP of both groups.

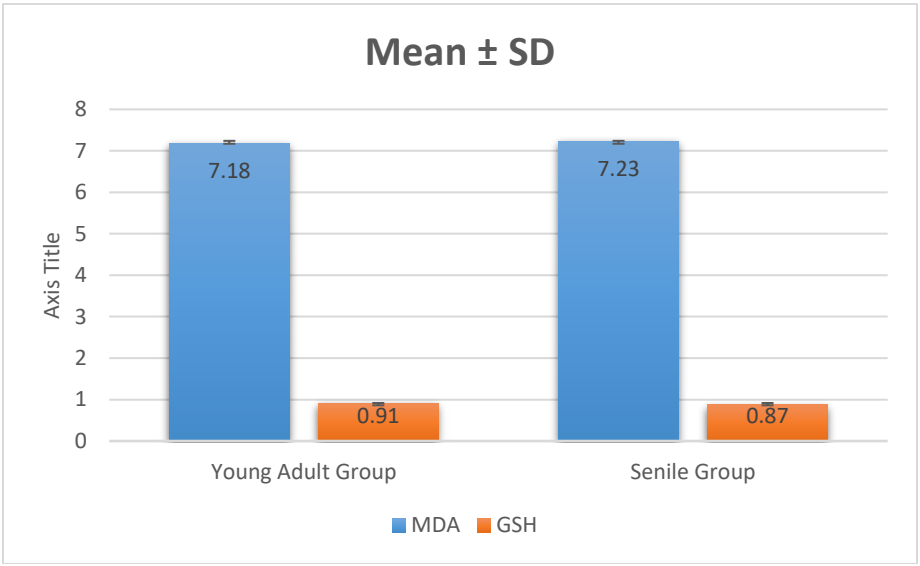

Fig 16: Histogram illustrating mean value of MDA and GSH contents in both groups.

DISCUSSION

The cerebellum is associated with fine control of movements and memory. As age advances deterioration is noticed in the cerebellum. Studying the changes in the Purkinje cells and astrocytes of the cerebellar cortex enable clinical correlation with age related cerebellar dysfunction. In the present work, microscopic examination of senile group cerebellum revealed a decrease in the thickness of the molecular and Purkinje cell layers which was largely caused by the progressive degeneration of Purkinje cells, most of them lost their axons, and acquired an irregular swollen shape. They showed disrupted plasma membrane and marked cytoplasmic vacuolation. Similar findings were reported by ***Zhang et al.*** [21] who declared that there was a loss of neurones and decrease in number of dendrites of the Purkinje cells in aged cerebellar cortex, which might underlie the reduction in afferent efficacy and integration of information in the senescent cerebellum.

Castejon [22] attributed the decreased number of Purkinje neurones to the process of cell aging and degeneration and added that neuronal loss or shrinkage is not the only mechanism by which age may affect the cerebellum, a reduction in the dendritic branches of Purkinje cells may also interfere with information exchange in spite of the presence of surviving cell bodies. Likewise, ***Fan et al.*** [23] reported that the loss of arborization of Purkinje cells could directly cut down the amount of

information input, decline afferent efficacy and signal transmission in the aged cerebellum. These changes would lead to a reduction in motor control and accurate motor coordination.

Paschen and Frandsen [24] revealed that aging was associated with disturbed endoplasmic reticulum functions (decrease of ER calcium pump activity with age) which induce release of cytochrome C from mitochondria and activation of caspase 3 which is a pathological process seen as hallmarks of programmed cell death**.** In addition, ***Zhang et al.*** [25] reported that aging was associated with lysosomal dysfunction which led to abnormal protein degradation and deposition in neurones, and this might be the primary contributor to age-related neurodegeneration. ***Baloyannis*** [26] attributed aging of cerebellar cells to dysfunction of cell organelles mainly smooth and rough endoplasmic reticulum, mitochondria, and Golgi apparatus due to accumulation of malformed proteins over the years in the human cerebellum.

In the current work, Purkinje cells of aged rats lost their axons and exhibited an extensive nuclear damage in the form of pyknosis, karyolysis and abnormally large nuclei. According to ***Martini et al.*** [27], the pyknotic nuclei were mostly because of a decrease in DNA transcription and gene expression due to lysosomal dysfunctional defects with subsequent reduction of protein synthesis which affects various functional activities of the neurons. ***Guo et al.*** [28] reported that axonal loss is caused by hyperphosphorylation of Tau protein; a microtubule responsible for maintaining neuronal morphology and suggested that this would affect the axonal transportation and impaired the neuronal functions.

Concerning Nissl bodies, Cresyl Violet stain in our study demonstrated that the intensity of Nissl bodies in Purkinje cells increased with age and this may result from the shrinkage and decreased in size of Purkinje cells with aging. This result coincides with that of ***Khalatbari et al.*** [29]. On the other side, this result conflicts with that of ***Angela and Abraham*** [30], who reported that Nissl bodies in Purkinje cells decrease with aging and this may explain the deterioration in function of Purkinje cells with aging.

GFAP is a special marker for astrocytes. The current study showed that there was a significant increase in the area % of GFAP immune response in the cerebellar cortex of senile group. Likewise, ***Davis et al.*** [31] reported that the number and size of GFAP-immunoreactive astrocytes in the central nervous system increase following certain types of brain injury and in various pathological conditions. ***Zhang et al.*** [21] declared that the age-dependent enhancement of the activity of the astrocytes may exert a protective effect on neurons in the aged cerebellum**.** ***Li et al.*** [9] stated that reactive astrocytes play a complex role in the process of neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, amyotrophic lateral syndrome, and multiple sclerosis); astrocytes become responsive and dramatically alter their phenotypes. Similarly, ***Siracusa et al.*** [10] reported that astrocytes are sensitive to aging and display age-related proliferation, which is usually described as up-regulation of GFAP.

Baydas et al. [32] explained the increased GFAP expression in astrocytes to be related to oxidative stress due to reduction in circulating melatonin concentrations with aging. On the other hand, ***Godbout and Johnson*** [33] reported that chronic activated astrocytes can induce brain damage by releasing highly toxic products, such as reactive oxygen intermediates, inflammatory cytokines and complementary factors. The elevated neuroinflammatory response may lead to more severe long-lasting behavioral and cognitive deficits.

The cellular redox status, described by ***Castagne et al.*** [34] as the balance between intracellular oxidants and antioxidants, is not within an optimal cell survival range; it can be suggested that age-related variations in antioxidant defences are the cause of increased susceptibility to disease related to redox imbalance. In the current study, the cerebellum MDA and GSH levels changed insignificantly

with age. Likewise no changes have been reported in the levels of oxidant stress and antioxidant capability in previous studies conducted on the rat model [35]**.** ***Francine and Jean*** [36] stated that, lipid peroxidation, expressed in term of MDA formation, decreases in all the organs of the aged rats. Therefore the age-related rise in the MDA content suggested as a criterion of the aging process should be interpreted with caution. ***Akbulut et al.*** [37] found that the levels of MDA were similar in various parts of the brain in the younger rats. On the other hand, in aged rats the cerebellar MDA levels were significantly lower than that of the frontal and occipital cortex. On the contrary, increased lipid peroxidation and decreased antioxidant ability have been recorded in aged animals [38]**.**

In previous research conducted by ***Zuhal and Nedret*** [39], they found that cerebellum GSH level was significantly higher in the young rats when compared to the middle-aged rats. Whereas, the levels of cerebellum MDA did not change with age. However, experimental findings clearly indicate that the preservation of a sufficient balance of antioxidants/pro-oxidants plays a significant role in the maintenance of the health of the aged animal.

It could be concluded that histopathological changes of the cerebellar cortex were obvious in aging, manifested as significant increase in degenerated Purkinje cells and in astrocytes immunoreactivity to GFAP. These changes were not solely relevant to the oxidative status. Perhaps, other factors contributed as well. These cellular changes could be responsible for various senile movement and memory disorders, in the light of the increase of the incidence of geriatric diseases like Alzheimer's.

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The authors report no conflicts of interest.

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