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Possible Role of Oxidative Stress and Nrf2/HO-1 Pathway in Pentylenetetrazole-induced Epilepsy in Aged Rats

Walaa Obydah*[1], Ahmed Fathi Abouelnaga[2], Marwa Abass[3], Somaya Saad[1], Asmaa Yehia[1], Omar Abd-Alhakem Ammar[4], Alaa Mohamed Badawy[5], Mohie Mahmoud Ibrahim[5], Abdelaziz Mohamed Hussein[1]

Abstract

Background: To examine the impact of aging on the response of rats to pentylenetetrazole (PTZ)-induction of epilepsy and the possible role of oxidative stress and nuclear factor erythroid 2-related factor 2 (Nrf2)/ heme oxygenase (HO)-1 pathway in this response.

Methods: Forty male albino rats were equally allocated into 4 groups; 1) Young control (YC) group, aged 8-12 weeks, 2) Old control (OC) group, aged 24 months, 3) PTZ-Young group: young rats received PTZ (50 mg/Kg, i.p. every other day) for 2 weeks and 4) PTZ-Old group: as group 3 but rats were old. The seizure score stage and latency to the first jerk were recorded in rats. Redox state markers in brain tissues including malondialdehyde (MDA), catalase and total antioxidant capacity (TAC) were evaluated. Also, the expression of Nrf2 and HO-1 genes were measured in the brain tissues.

Results: Old rats showed an early and a significant rise in the seizure score with PTZ administration and a significant drop in the seizure latency compared to young rats (P <0.01). Also, old rats showed a significantly higher MDA concentration and a significantly lower TAC and catalase activity than young rats (P <0.01). Moreover, the expression of Nrf2 and HO-1 was significantly lowered in old rats compared to young rats with PTZ administration (P < 0.01).

Conclusions: Aging increases the vulnerability of rats to PTZ-induced epilepsy. An effect might come down to the up-regulation of oxidative stress and the down regulation of antioxidant pathways including Nrf2 and HO-1.

Keywords: Aging, Epilepsy, HO-1, Nrf2, Oxidative stress, Pentylenetetrazole (PTZ).

Introduction

The prevalence of epilepsy, a chronic neurodegenerative disease, ranges from 0.5% to 2% worldwide (1). In a study by Zack and Kobau, (2), they reported that the prevalence of epilepsy rose by 24% between 2010 and 2015 in the USA. Additionally, among people over 60, the prevalence of epilepsy and seizures has dramatically risen (3). A-community-based research that looked at the age-specific incidence of epilepsy found that it rose with age, with the following incidence per 100,000 persons: 10.6 in the 45–59 age range, 25.8 in the 60–74 age range, and 101.1 in the 75–89 age range (4). With advancing age, the frequency of severe symptomatic seizures also rises. Elderly people are more likely to

1: Department of Medical Physiology, Faculty of Medicine, Mansoura University, Mansoura (35516), Egypt.
2: Department of Animal Husbandry and Development of animal wealth, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt.
3: Department of Surgery, Anesthesiology, and Radiology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt.
4: Basic Science Department, Delta University for Science and Technology, Gamasa, Egypt.
5: Department of Anatomy and Embryology, Faculty of Medicine, Mansoura University, Mansoura, 35516, Egypt.
***Corresponding author: Walaa Obydah; Tel: +20 1002421140; E-mail: menhag@mans.edu.eg.**
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experience their first spontaneous seizure with an incidence rate ranged from 52 to 59 per 100,000 persons in the 40 to 59 age group and rose to 127 per 100,000 in the 60 and later age group (5). The older population also had a higher recurrence incidence following the initial seizure, at 79% in the first year and 83% in the following three years (6). Finally, the prevalence of status epilepticus (SE) is 86 per 100,000 people older than 60 years, which is 2-5 times higher in the elderly than in young peoples (7). Therefore, a thorough understanding of the pathophysiological mechanisms underlying epilepsy in elderly is imperative for a tailored and a proper management.

During epileptogenesis, the brain goes through several structural changes such as neuronal losses, astrocytosis or overgrowth of astrocytes, mossy fibers overgrowth, the reorganization of synapses, and neurogenesis (8). Oxidative stress plays a significant role in the onset and progression of epilepsy in addition to inducing an original brain injury (9). In addition to immune, inflammatory, and blood-brain barrier dysfunction, an excess of reactive oxygen radicals (ROS) during convulsions causes oxidative stress in neurons (10, 11). Regarding epilepsy in elderly, there is no current strong experimental evidence supporting the notion of variable molecular underpinnings of seizures according to the patient's age. Unfortunately, to date, age has not been considered as an independent variable in studies addressing seizures underlying mechanisms However, few studies investigated the effect of aging on epileptogenesis such as Dawson et al., (12) who found an increase in aspartate, glutamate, and norepinephrine levels, while Shetty et al., (13) reported reduction of brain-derived neurotrophic factor (BDNF) in brain tissues of aged rats treated with kainate.

The transcription factor nuclear factor 2 erythroid-related factor-2 (Nrf2) encourages the production of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and heme oxygenase-1 (HO-1), rendering cells resistant to oxidative damage (14, 15). So, activation of Nrf2 transcription factor in the brain tissues in case of epilepsy is considered as an endogenous adaptive mechanism that protects the brain against the insult caused by oxidative stress to the neurons (16). In the instance of temporal lobe epilepsy, glutamate-induced oxidative injury was found to be diminished Nrf2/ARE-dependent HO-1/NQO-1 production. In the case of uncontrollable epilepsy, activation of the Nrf2/Ho-1 pathway can be viewed as a possible therapeutic target (14, 17). Recently, we showed that the PTZ-induced epileptic rodents' brain tissues had decreased Nrf2/HO1-pathway activity (18). We hypothesized that aging increases the sensitivity of rats to PTZ via down-regulation of the antioxidant system Nrf2/HO-1 and enhancement of oxidative stress. So, in the current study we examined the effect of aging on PTZ-induced epilepsy and to explore the role of Nrf2/HO1 and oxidative stress in such effect.

Materials and Methods

Laboratory Animals

At the medical experimental research facility, Mansoura Faculty of Medicine, Egypt, forty male Sprague-Dawely rats weighing 170-190 g were housed in ordinary cages. Rats given a conventional feed and unlimited access to water. The Care and Use of Laboratory Animals was followed when caring for the animals (1996; National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA). All experimental protocols were authorised by the ethical-committee, MU-ACUC (VM.R.23.02.56).

Study design

Rats were allocated into 4 groups, each 10 rats; 1) Normal young control (YC) group; rats aged 8-12 weeks and received 0.2 mL saline (intraperitoneal injection; i.p. for 2 weeks), 2) Normal old control (OC) group: as YC group but rats aged 24 months, 3) PTZ-Young group: young rats received PTZ (50 mg/Kg) (Sigma Aldrich, USA) in 0.2 mL saline (i.p., every other day for 2 weeks and 4) PTZ-Old group: as group 3 but using old rats (19).

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Animal Model

Rats were kept in translucent Plexiglas cages after each PTZ administration, so that a video camera could capture their convulsive behaviour for 30 minutes after PTZ injection for two weeks, or seven records, or trials (every other day). Seizure score and latency to seizure start (sec) were observed. According to Racine's scale (0 = non-epileptic activity, 1 = oral and facial movements, grooming, hyperactivity, sniffing, scratching and wet dog shakes, 2 = head nodding, and tremors, 3 = forelimb extension and clonus, 4 = rearing, salivating, tonic colonic activity, and 5 = falling, status epilepticus), the severity of the seizures was scored (20).

Harvesting of brain tissues

Rat's sacrifice was conducted at the end of the experimental study using a high dose of Na^+ thiopental (120 mg/kg i.p.). The brains of six rats in each group had been collected for biochemical and molecular studies after perfusion with 100 mL of heparinized saline using a cardiac catheter; the brain of the other four rats were collected for histopathological and immunohistochemical studies using a cardiac catheter perfusion of 150 mL of formalin (10%) (19).

Measurement of catalase activity, MDA and total antioxidant capacity in rats' brain

The brain's hippocampal tissues were blended in 1-2 mL of cold, 50 mM-phosphate buffered saline (PBS) with 1 mM EDTA at pH 7.5. Then, centrifuged for 15 minutes, at 900 G and 4 °C. According to the manufacturer's instructions, colorimetric assays of catalase enzyme, MDA, a marker of lipid peroxidation, and total antioxidant capacity were performed using commercially available kits (Bio-Diagnostics, Giza, Egypt).

Real time PCR for the expression of Nrf2 and HO-1 genes in brain tissues

Real-time PCR was used to find the mRNAs for the transcription factor Nrf2 and heme oxygenase (HO)-1 in brain regions. We extracted total RNA from brain tissue samples using commercially available kits according to their manufacturer's instructions. RNA was measured spectrophotometrically, and agarose gel electrophoresis and ethidium bromide staining were used to assess its purity. One µg of total RNA was used to create cDNA, which was then diluted in 25 µL of water, then 25 µL of cDNA was then diluted in a 100 µL total amount. All details of the primers, amplification, and detection of tested genes (Nrf2, HO-1, and GAPDH) were mentioned in previous studies (21, 22).

Hematoxylin and Eosin staining

The brain specimens were promptly fixed in 10% neutral buffered formalin for 24 hours, cleaned in xylol, dehydrated with alcohol of increasing strength, and then embedded in paraffin. To examine the histological makeup of the rat hippocampus, 5 µm thick sections were cut, prepped, and stained with hematoxylin and eosin (H&E). The different regions of hippocampus, dentate gyrus, CA1,2,3 and 4, were examined for neuronal loss such as pyknotic cells, apoptotic cells and perineuronal vacuolations, and normal vesicular pyramidal cells (PC). Also, the cell count of normal PC was measured in CA1, CA3 and CA4 in 5 high power fields (HPF) and the mean was calculated.

Statistical analysis

GraphPad Prism version 5.0 was used for statistical analysis. The behavioral parameters were tested for statistical significance by independent T test, while other parameters were tested using one-Way ANOVA with Tukey's posthoc test. $P < 0.05$ was considered significant.

Results

Effects of aging on seizure score and latency in PTZ-epileptic rats

Rats of old-PTZ group displayed significant increase in the PTZ-induced seizure score in all trials except trial 2 and 3 compared to young group ($P < 0.01$) (Fig. 1A). Also, the seizure latency displayed significantly lower values in the old-PTZ group compared to the PTZ-young group in all trials ($P < 0.05$) (Fig. 1B).

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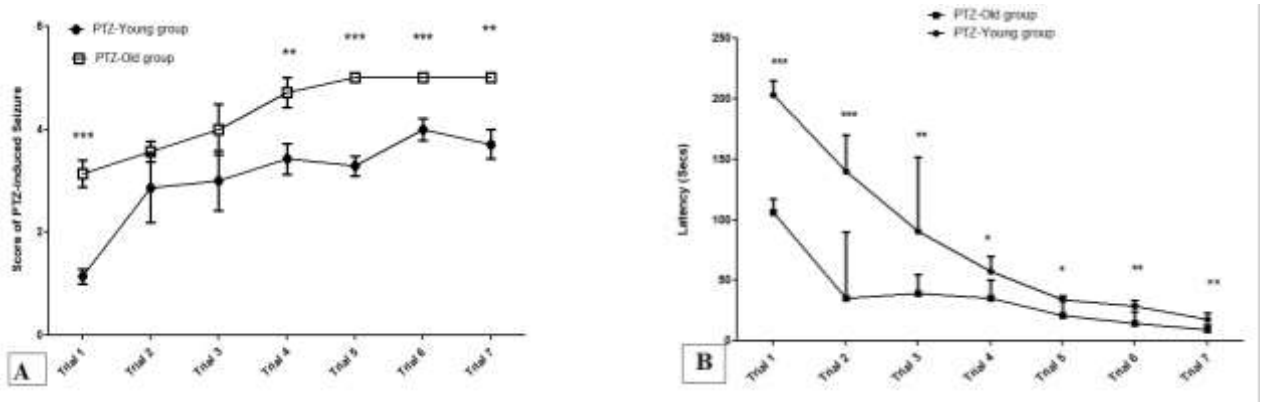

Fig. 1. Effects of aging on Seizure score (A) and = seizure latency (sec) (B) in PTZ-induced epilepsy. Independent-sample T test. * Significant vs PTZ-young group, *** P < 0.001 and ** P < 0.01 and * P < 0.05

Effect of aging on oxidative stress markers (MDA, CAT and TAC) in rat's brain

A significant increase in the concentration of MDA (lipid peroxidation marker) in brain tissues of PTZ-kindled young rats compared to PTZ-treated young rats (P < 0.05) was found as well as a more significant rise in its concentration in old rats treated with PTZ compared to PTZ-treated young rats and non-PTZ treated young and old rats (P < 0.05). On the other hands, the activity of CAT enzyme and TAC showed a significant reduction in old rats compared to young rats (P < 0.01) and in PTZ-treated young rats compared to non-treated young rats and in PTZ-treated old rats compared to young and old non-PTZ treated rats (P < 0.01) (Table 1).

Table 1. Oxidative stress markers (CAT, MDA and TAC) in brain tissues of different groups.

	Young control (YC)	Old control (OC)	PTZ-Young	PTZ-Old
MDA concentration (nmol/g brain tissues)	22.08± 2.89	30.5 ± 1.55	42.28 ± 5.89$^{\$}$	66.36 ± 3.15$^{\$\$\$,\#\#,**}$
CAT enzyme activity (U/g brain tissues)	71.83 ± 3.26	49.83 ± 3.57$^{\$\$\$}$	35.67 ± 3.43$^{\$\$\$,\#}$	32.87 ± 1.37$^{\$\$\$,\#\#}$
Total antioxidant capacity (TAC)	4.81 ± 0.427	2.88 ± 0.53$^{\$\$}$	1.138 ± 0.21$^{\$\$\$,\#}$	0.86 ± 0.16$^{\$\$\$,\#\#}$

Data were expressed as mean ± SEM. One-way ANOVA with Tukey's posthoc test. $ significant vs YC group, # significant vs OC group and * significant vs PTZ-young group. P < 0.05 is considered significant. ### P < 0.001, ### P < 0.01 and # P < 0.05.

Effects of aging on the expression of Nrf2 and HO-1 in PTZ-kindled rats

The expression of Nrf2 and HO-1 in brain tissues of old rats was significantly reduced compared to young rats (*P* < 0.01). Moreover, there was a non-significant rise in t heir expression in young rats treated with PTZ compared to young non-treated rats and a significant reduction in their expression in old rats treated with PTZ compared to non-treated counterparts and both treated and non-treated young rats (*P* < 0.01) (Fig. 2).

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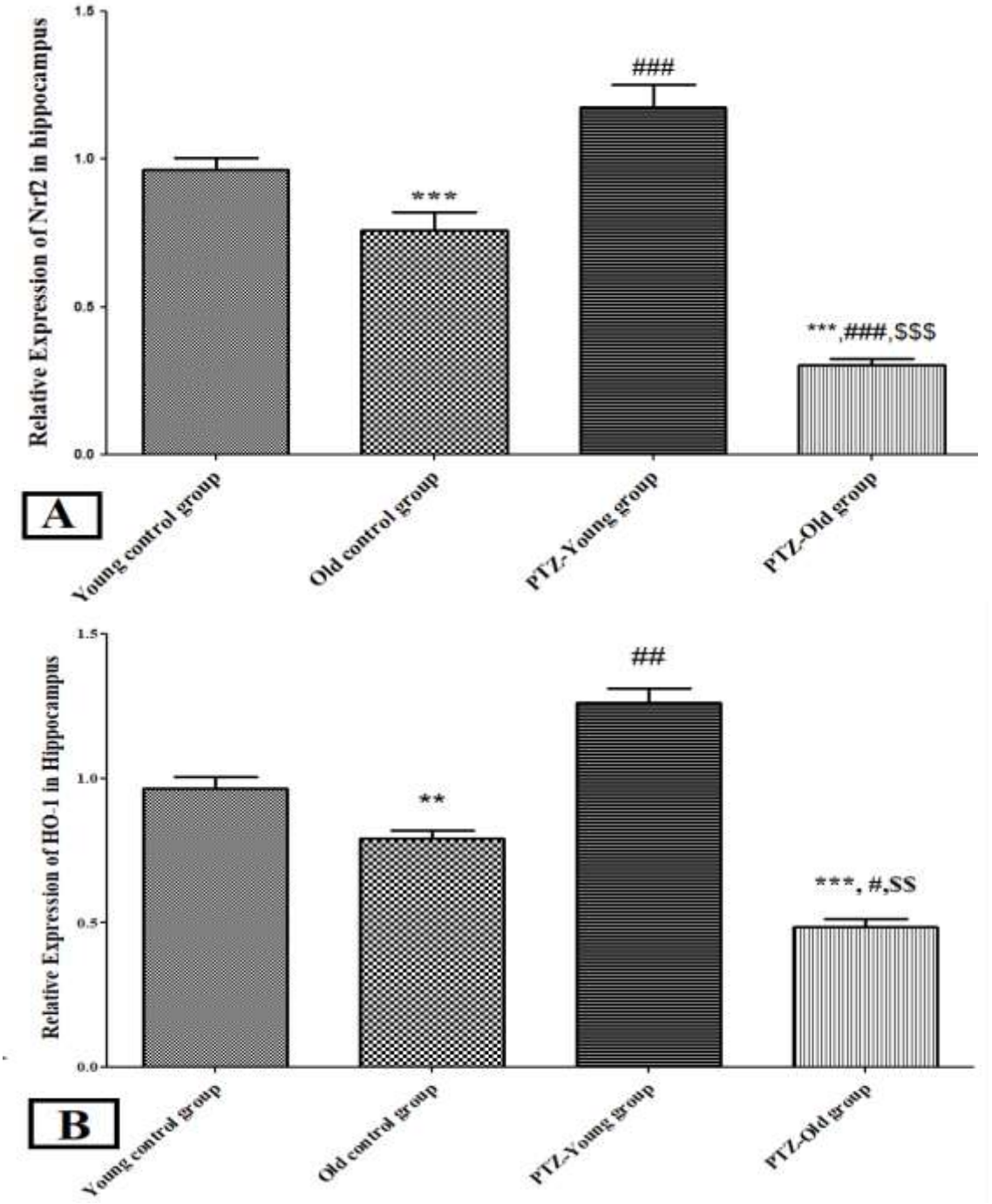

Fig. 2. Effects of aging on the expression of antioxidant genes in PTZ-induced seizures. A: Nrf2 and B: heme oxygenase (HO)-1 in brain tissues. One-way ANOVA with Tukey-posthoc test. * Significant vs YC group, # significant vs OC group and $ significant vs PTZ-young group. *** P < 0.001 and ** P < 0.01 and * P < 0.05.

Effect of aging on hippocampal histopathology

The PC count showed a significant drop in the different areas of CA4 and CA3 but not CA1 of the old non-treated group compared to the young group ($p \leq 0.001$, 0.001 and > 0.05). Also, the PC count was significantly reduced in the PTZ- treated young rats compared to young control rats in CA4, CA3 and CA1 ($p \leq 0.001$, 0.001 and < 0.05). Moreover, the PC count was significantly reduced in the PTZ-treated old rats compared to old-non treated rats, young PTZ-treated and non-treated rats in CA4, CA3 and CA1 ($P \leq 0.001$, 0.001 and < 0.001) (Fig. 3).

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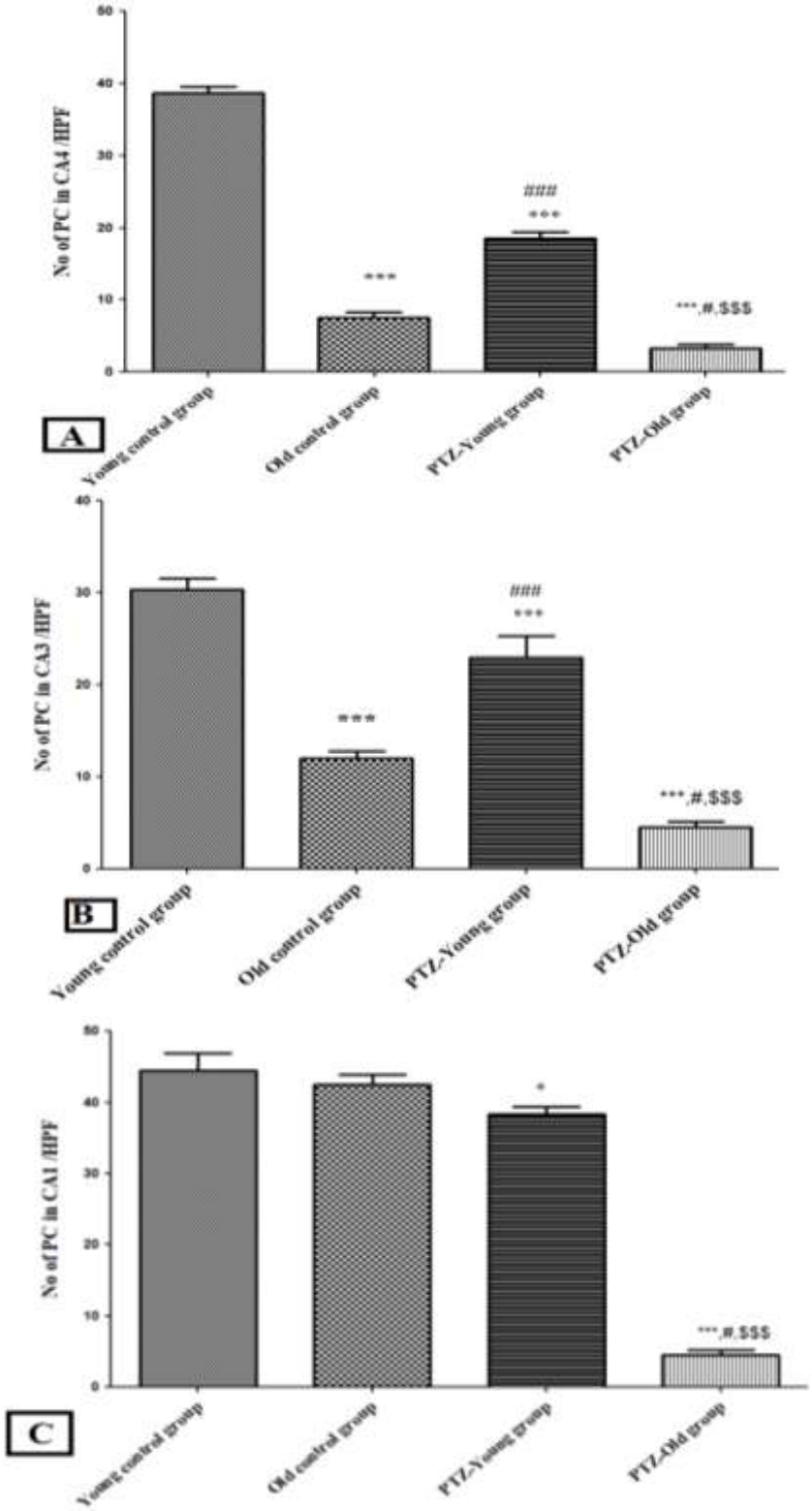

Fig. 3. Effect of aging on cell count of pyramidal cells in different hippocampal regions; A: CA4 region, B: CA3 and C: CA1 regions. One-way ANOVA with Tukey-posthoc test. * Significant vs YC group, # significant vs OC group and $ significant vs PTZ-young group, *** $P < 0.001$ and ** $P < 0.01$ and * $P < 0.05$.

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Brain tissue samples from young control rats, displaying the CA4, CA3, and CA1 areas of the hippocampus. They comprised from three strata i) pyramidal cell layer (PCL), the primary cell layer, contains small, densely packed pyramidal cells in CA1 and large, sparsely packed pyramidal cells in CA3 and CA4 (triangular and have large, rounded, vesicular nuclei with prominent nucleoli), ii) Polymorphic layer (POL), which contains either deeply or lightly stained glial cells. Small glial cells with rounded to oval nuclei and the distinctive perinuclear halo were seen within the PCL, and iii) molecular layer (ML), which contains PCL's branched apical dendrites and widely separated cells, was also observed (Fig. 4).

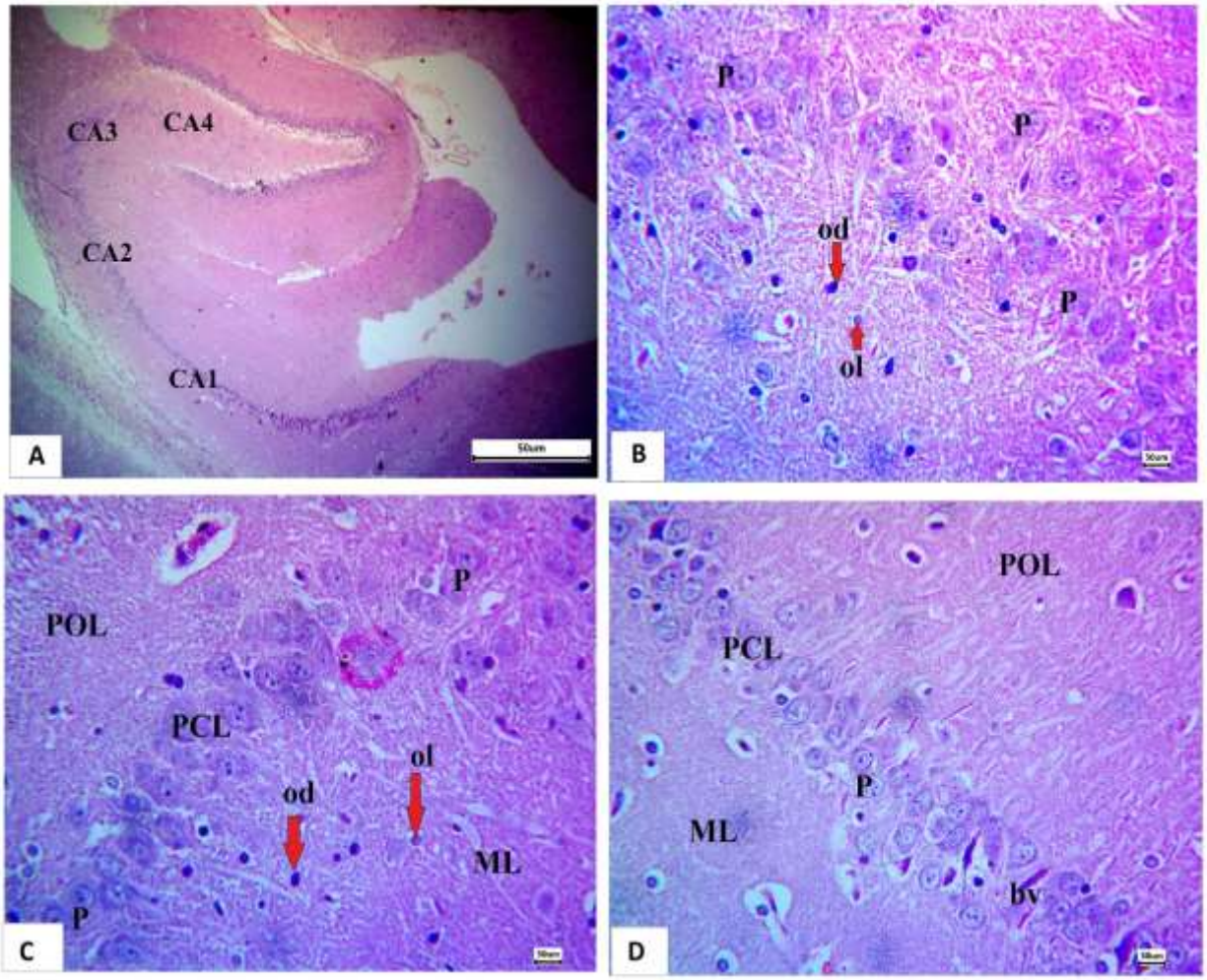

Fig. 4. Photomicrographs of hippocampal regions from young control rats. A: different parts of cornu of ammonis (CA), CA4, CA3, CA2, and CA1 at low power 40x. CA4 shows normal pyramidal cells (PC) which are triangular and had large rounded vesicular nuclei with prominent nucleoli (P) and small glial cells with either deeply (od) or lightly (ol) stained rounded to oval nuclei and characteristic perinuclear halo (B, 400x). Also, CA3 region consists of 3 layers; i) polymorphic layer (POL), ii) pyramidal cell layer (PCL) (show PC which had large vesicular rounded nuclei with prominent nucleoli) and iii) molecular layer (ML) (show small glial cells with either deeply (od) or lightly (ol) stained rounded to oval nuclei (C, 400x). The same for CA1 region (D, 400x).

On the other hand, specimens from brain tissues of old non-PTZ treated rats show a few numbers of normal pyramidal cells in CA4 and CA3 regions with a big number of degenerated PC (apoptotic and pyknotic nuclei) and perineural vacuolation. However, CA1 region shows large number of normal PC (Fig. 5).

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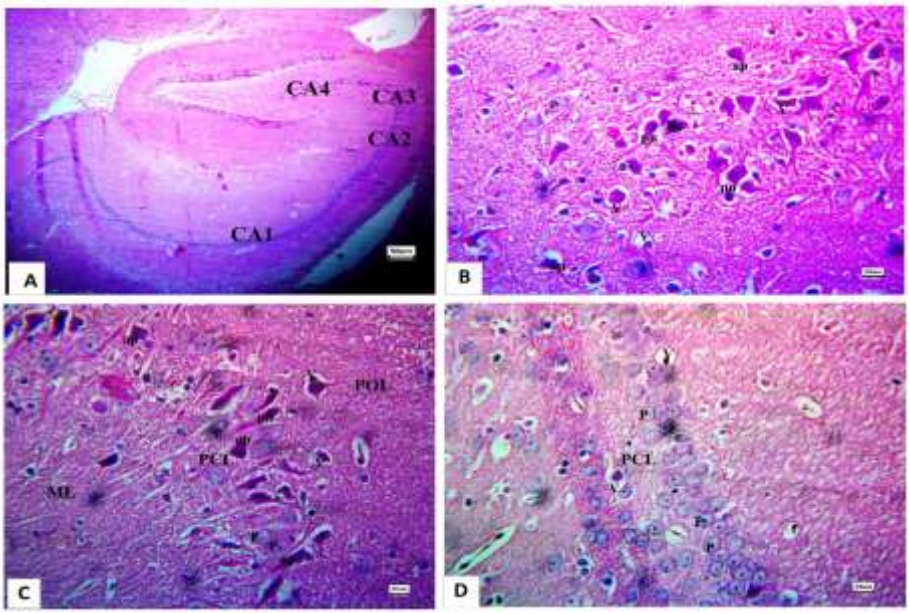

Fig. 5. Photomicrographs of hippocampal regions from old control rats. A: shows different parts of cornu of ammonis (CA), CA4, CA3, CA2, and CA1 at low power 40x. CA4 shows a few numbers of normal pyramidal cells with vesicular nuclei (P) and a large number of degenerated pyramidal cells with densely stained nuclei; apoptotic (ap) and pyknotic nuclei (pn), (B: 400x). Also, CA3 region few numbers of normal pyramidal cells (P) with large number of degenerated pyramidal cells densely stained nuclei; apoptotic (ap) and pyknotic nuclei (pn), and perineural vaculation (C: 400x). On the other hand, CA1 region shows normal structure (D: 400x).

Also, specimens from brain tissues of young-PTZ treated rats show a few numbers of normal pyramidal cells in CA4 regions with a big number of degenerated PC (apoptotic and pyknotic nuclei) and perineural vaculation, while CA3 and CA1 regions show a big number of normal PC (Fig. 6). Finally, specimens from brain tissues of old-PTZ treated rats show a few numbers of normal pyramidal cells in CA4, CA3 and CA1 regions with a big number of degenerated PC (apoptotic and pyknotic nuclei) and perineural vaculation (Fig. 7).

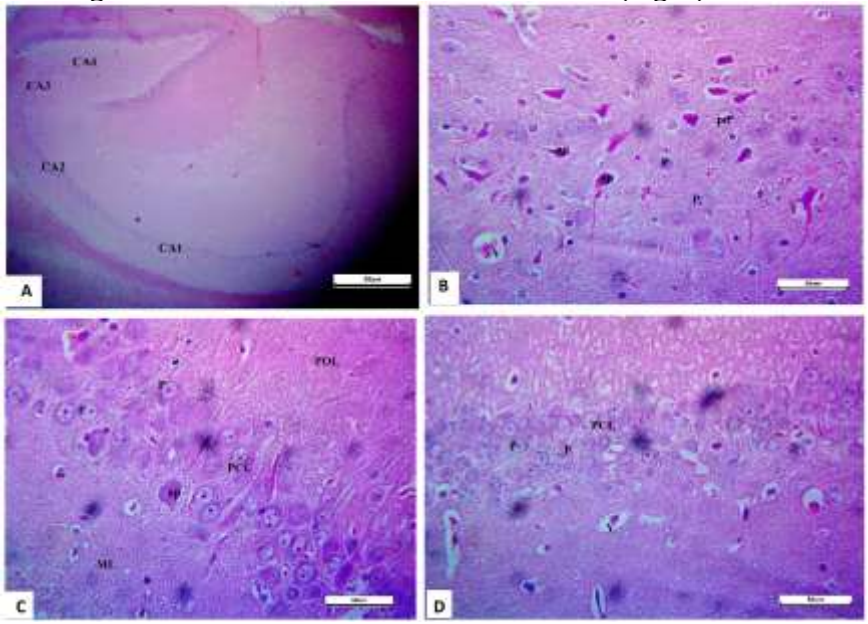

Fig. 6. Photomicrographs of hippocampal regions from Young-PTZ rats. A: different parts of cornu of ammonis (CA), CA4, CA3, CA2, and CA1 at low power 40x. CA4 shows a few numbers of normal pyramidal cells with vesicular nuclei (P) and large number of degenerated pyramidal cells with densely stained nuclei; apoptotic (ap) and pyknotic nuclei (pn), (B, 400x). CA3 and CA1 regions show a big number of normal pyramidal cells (P) with few numbers of degenerated pyramidal cells densely stained nuclei; (apoptotic (ap) and pyknotic nuclei (pn) and perineural vaculation (C, D: 400x).

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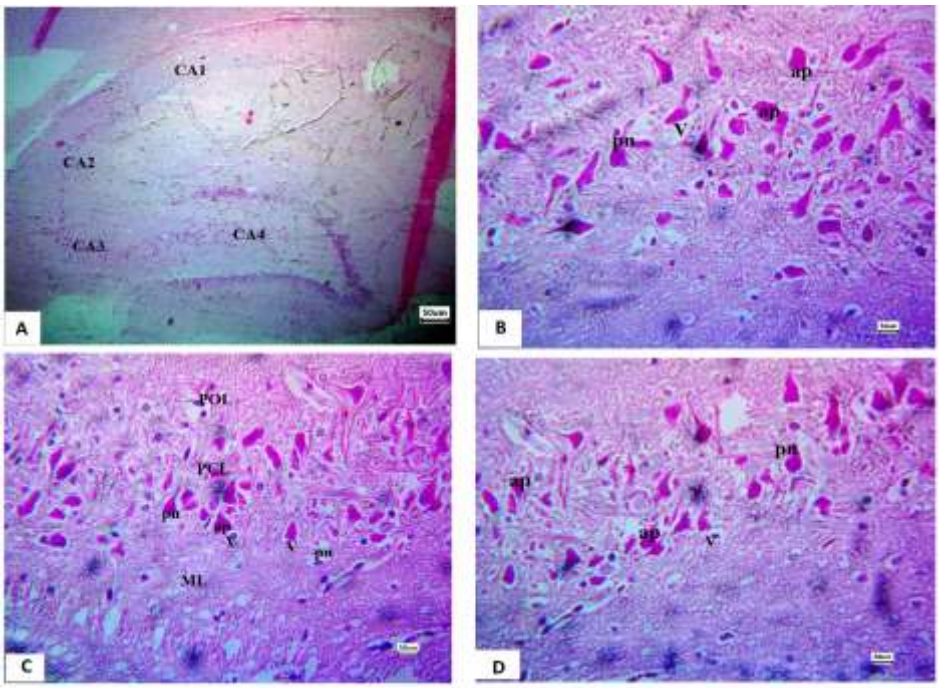

Fig. 7. Photomicrographs of hippocampal regions from Old-PTZ rats. Fig. 7A shows different parts of cornu of ammonis (CA), CA4, CA3, CA2, and CA1 at low power 40x. CA4 shows a big number of degenerated pyramidal cells with densely stained nuclei (apoptotic (ap) and pyknotic nuclei (pn) (B, 400x). Also, CA3 and CA1 regions show a big number of degenerated pyramidal cells densely stained nuclei; apoptotic (ap) and pyknotic nuclei (pn), and perineural vacuolation (C, D, 400x).

Discussion

Researchers have employed aged animals to learn more about the variations between the young and the aged brain, as well as how these variations may shed light on the processes underlying epilepsy and acute seizures in elderly. The modelling of acute seizures and epilepsy in old, healthy animals has received comparatively little attention to date. In the current study we adopted a well-known animal model of kindled epileptic rats; PTZ-induced epilepsy. In the current study, we found that administration of PTZ every alternate day was associated with marked increase in the PTZ-induced seizures stage and decreased latencies values in PTZ-old rats when compared to PTZ young rats for two weeks. These findings suggest hypersensitivity of the old rats to PTZ-induced seizures than young rats. Also, they confirmed the findings reported by previous studies such as Wozniak et al., (23), and Liang et al., (24) who reported that young Sprague-Dawley rats (5–6 months) were unaffected by low doses of kainic acid, but older animals (22–25 months) experienced status epilepticus, neuronal damage, and death. On the other hand, Okamoto et al., (25) depicted a delay and shortening of nicotine-induced convulsions in 24-month-old Wistar rats despite the fact that the half-life of nicotine was greater in older animals.

The hippocampus was implicated in the process of epipleptogenesis and the process of aging. So, in the current study we examined the morphology of the hippocampus in young and old rats. In the current study we found neurodegeneration of different hippocampal regions especially CA3 and CA4 in aged rats compared to young rats and the degree of affection with PTZ became more marked and extended to CA1 region. These findings suggest high susptability of PC to degeneration by PTZ in old rats than young rats. In agreement with these findings, Liang et al., (24) demonstrated that kainic acid injection led to a rise in seizure susceptibility, and hippocampal pyramidal cell loss in the aged Sprague-Dawley rats only. Also, McCord et al., (26) and Benkovic et al., (27) demonstrated

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greater degree of hippocampal degeneration in old rats compared to young rat, a finding, in line with our study.

Cells can be severely harmed by oxidative stress, which is known as an imbalance between pro- and antioxidants and results in cell damage and death. In 1954, the first proof that oxygen species cause tissue damage was published (28). In overabundance, free radicals can nitrate or oxidize fatty acids, proteins, and DNA, altering their structure and function in the process. Gene mutations result from the cross-linking of base pairs in the DNA strand (29). The three most significant characteristics of epileptogenesis are known to be ROS, neurodegeneration, and a reduction of the seizure threshold (30). As shown in Table 1, PTZ aged rats in our research had significantly higher levels of MDA and lower levels of TAC and catalase activities than PTZ young rats. In the mouse model of temporal lobe epilepsy brought on by Theiler's murine encephalomyelitis virus, there was a proof of a tight connection between two epileptogenesis hallmarks (neuroinflammation and redox status imbalance). Astrogliosis and microgliosis, marking these cells extreme activation, are signs of neuroinflammation and oxidative stress. The activated astrocytes are stimulated by the cytoplasmic transcription factor nuclear factor erythroid regulatory factor 2 (Nrf2) (31).

The transcription factor Nrf2 activates a battery of antioxidant enzymes including HO-1 and glutathione peroxidases (14). A 14-day alternate-day 50 mg/kg PTZ injection in the current research revealed no appreciable difference in the expression of Nrf2 and HO-1 between the PTZ young rats and the control young rats. Moreover, the current study demonstrated significant attenuation in the expression of the Nrf2/HO-1 pathway genes in old rats compared to young rats with more significant attenuation with PTZ administration. Moreover, we found upregulation of Nrf2/HO-1 in young rats after PTZ administration. In line with our findings, Li et al. (30) found that giving rodents daily doses of 37 mg/kg PTZ for 40 days reduced the expression of Nrf2 and HO-1. Nrf2 is triggered in epilepsy following oxidative stress as an endogenous adaptive mechanism to defend against the neuronal oxidant insult. On the other hand, PTZ elderly rats displayed decreased Nrf2-HO-1 pathway expression in neural tissue, which may help explain why old rats are more susceptible to the onset of epileptogenesis. So, we propose that the activation of Nrf2 and its antioxidant genes could be potential therapeutic targets for epileptic seizures in aged rats. In consistence with this proposition, Wang et al. (14) demonstrated that the use of sulforaphane (potent activator of Nrf2) ameliorated the effect of a 15-day amygdala-kindling rat paradigm using repeated sub-convulsive electric stimulation.

In conclusion, ageing makes rats more susceptible to PTZ-induced seizures. This might result from neurodegeneration of hippocampal PC which could be attributed to oxidative stress and downregulation of the antioxidant genes Nrf2 and HO-1.

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Authors declared that nobody is acknowledged to this work.

Ethics
This work was approved by ethical committee for animal ethics and care, Mansoura faculty of veterinary medicine with approval code: MU-ACUC (VM.R.23.02.56).

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Conflict of Interest
All authors declared that there is no conflict of interest.

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