THE POSSIBLE EFFECT OF A MIXTURE OF POMEGRANATES AND GRAPES JUICE ON THE HIPPOCAMPUS OF ADULT AND SENILE MALE ALBINO RATS

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ABSTRACT

Background: Aging is a natural, multifaceted, progressive and inevitable phenomenon, adversely affecting the structural integrity and biological functions of the brain generally and the hippocampus specifically. It is a major risk factor for several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and dementia

Aim and objectives: Study the effect of aging on the hippocampal-dependent learning ability and spatial memory in the adult and senile male albino rats by applying the behavioral test (Morris water maze test).

Patients and Methods: The current work was carried out on 60 male albino rats from three different age groups (I: Adolescent, II: Adult and III: Senile). In each age group (n=20), rats were divided equally into two groups A and B.

Results: there was a statistically significant difference between the studied groups as regard Comparisons between the mean numbers of platform-site crossovers in all experimental groups (n:10) in the probe trial except Adolescent IA in Adolescent + J IB, Adolescent + J IB in Adult + J IIB and Adult IIA in Senile + J IIIB, mean area percent of GFAP immune reactivity except Adolescent IA in Senile + IIIB, the mean area percent of GFAP immune reactivity except Adolescent IA in Senile + IIIB and the mean level of BDNF gene expression except Adolescent + JIB in Adolescent + J IB.

Conclusion: Regular intake of functional food such as pomegranates and grapes juice is necessary for the maintenance of normal cognitive functions required for healthy aging.

Key words: Pomegranates, Grapes Juice, Hippocampus, Senile Male Albino Rats.

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INTRODUCTION

Aging is a progressive, complex, multifactorial, universal process affecting whole body systems especially the brain. It is manifested by physiological, sensorimotor and cognitive deterioration, ultimately leading to death. It is a major risk factor for a variety of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and dementia **(1)**.

The mammalian hippocampus has become the focus of attention in the past few years, because of its key role in all aspects of memory processing; formation, storage and recall, learning, emotional behavior and spatial navigation **(2)**.

The hippocampus is a unique, complex and mysterious area of the brain, it comprises the hippocampus proper, dentate gyrus & subiculum. In most brain regions neurogenesis is completed by birth, nevertheless, the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) contains a niche of neural stem cells that contributes to adult neurogenesis throughout life. The ongoing formation of functional adult-born new neurons in the DG is severely affected by aging **(3)**.

The current treatments for neurodegenerative disorders are limited, largely ineffective, with many side effects besides the high cost of the medications and caregiving **(4)**. Loss of individual's functionality and lack of independence of these patients, have obliged neuroscientists to direct their research to an alternative/complementary nature-based anti-aging neutraceuticals; that aim to reduce the severity and the socioeconomic burden of aging disorders, and to improve geriatric mental health, in order to achieve better life quality in elderly population **(5)**.

The present study was assigned to: Study the effect of aging on the hippocampal-dependent learning ability and spatial memory in the adult and senile male albino rats by applying the behavioral test (Morris water maze test). Demonstrate the influence of aging on the hippocampus in adult and senile male albino rats by using: Histological study, Immunohistochemical study and Quantifying the hippocampal expression of α-Synuclein and BDNF (brain-derived neurotrophic factor) genes. Evaluate the possible effect of a mixture of pomegranates and grapes juice on the hippocampus of adult and senile rats regarding the previous parameters.

MATERIAL AND METHODS

Material:

Fruits: Fresh pomegranates (Punica Granatum, L.) and red grapes containing seeds (Vitis vinifera, L.) were obtained from a local market, 6 October city, El Giza Governorate, Egypt.

Preparation of pomegranates and grapes juice mixture: Samples of fruits free from evidence of insect infestation and objectionable materials were cleaned and washed. Pomegranate peels, in particular, were removed manually, then homogenized with their seeds using commercial blender (Braun, Germany).

Experimental animals: Sixty apparently healthy male albino rats of Wistar strain were utilized in this study. They were obtained from the Animal House, Faculty of Medicine, Cairo University.

Thus, the experimental rats were categorized into six groups, (10 rats each), as follows: Group IA (Adolescent): ranging in age from three to five months weighing about (100-150 g), they received standard diet only, Group IB (Adolescent PGJ-treated): the same age and weight of group IA. They were given PGJ orally, Group IIA (Adult): ranging in age from fifteen to eighteen months weighing about (200-250g), received standard diet only, Group IIB (Adult PGJ-treated): the same age and weight of group IIA. Rats received oral PGJ, Group IIIA (Senile): aging ≧24 months, weighing ≧350 g, received standard diet only and Group IIIB (Senile PGJ-treated): the same age and weight of group IIIA. Rats were given PGJ **(6)**.

Technique of gastric gavage (Fig. VII):

The PGJ was delivered directly into the stomach of rats via a bulb-tipped stainless steel gastric gavage needle attached to a syringe. The inserted needle length was adjusted to the distance from the mouth to just beyond the last rib.

The rats were gently grasped by the loose skin of the neck and back to immobilize the head without causing distress to them. Rats were maintained in an upright (vertical) position and the gavage needle was passed along the side of the mouth; following the roof of the mouth, then it was advanced into the esophagus and toward the stomach **(7)**.

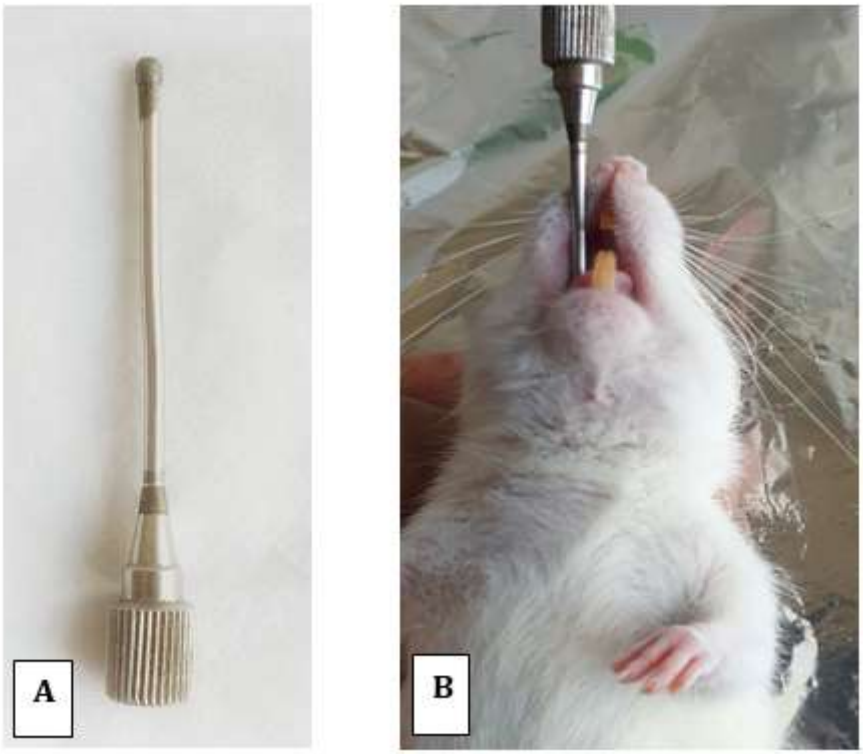

Figure 1: A: Gastric gavage needle**, B:** insertion of the needle into rat's mouth

Methods:

Learning and memory test (Morris Water Maze test (MWMT)), Perfusion fixation technique, Brain extraction and hippocampal dissection, Histo-morphometric study and Gene expression study by quantitative real time Polymerase Chain Reaction (qRT-PCR) for (Alpha/ α-Synuclein (α-Syn) and BDNF (Brain derived neurotrophic factor)

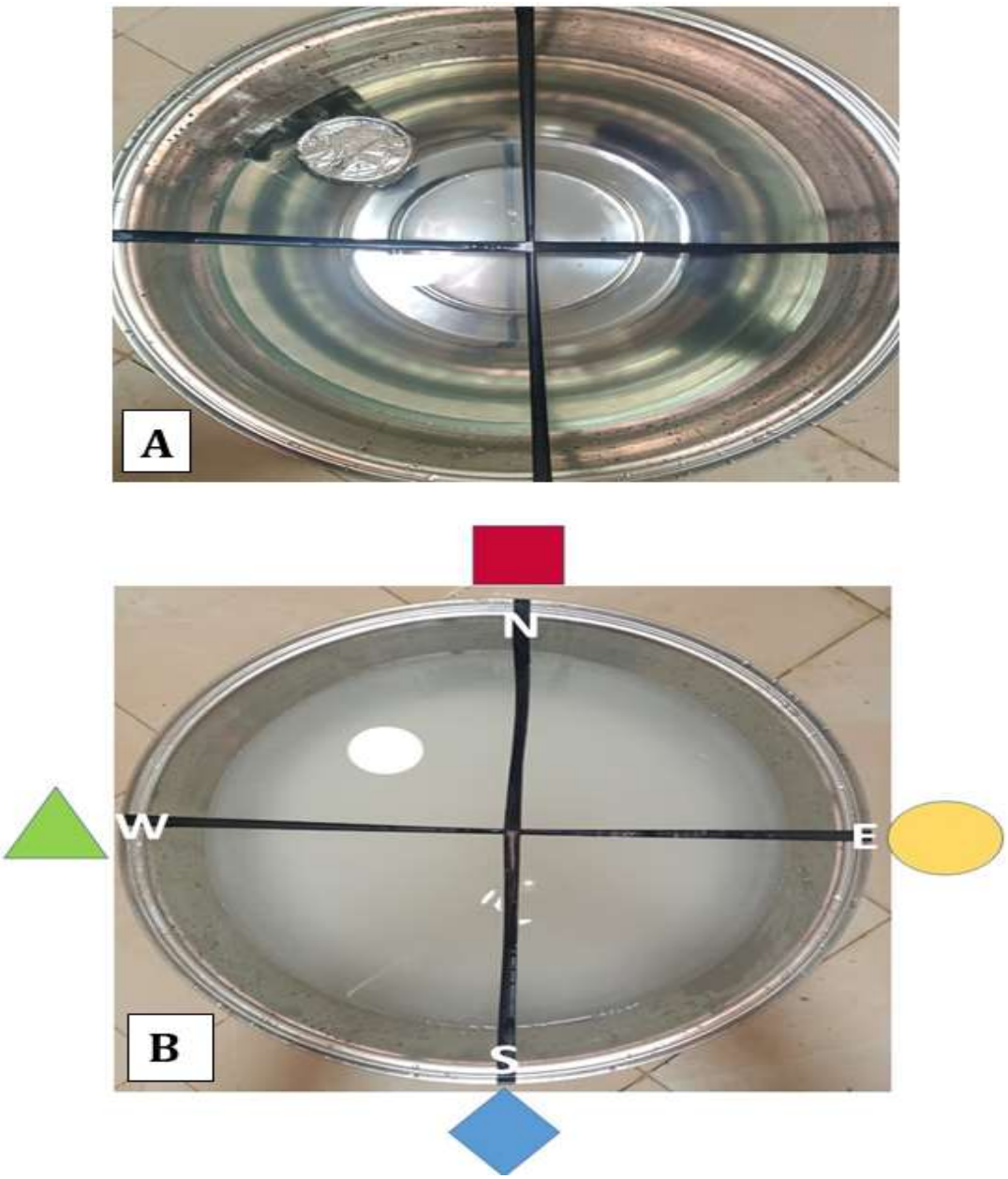

Figure 2: A: A Photo of the circular tank of MWMT, divided into four quadrants, with the circular platform in the northwest quadrant. **B:** is showing the tank filled with opaque water and the coloured wall papers; **N**: red square, **S**: blue diamond, **E:** yellow circle, **W:** green triangle. **White circle**: the hidden platform site.

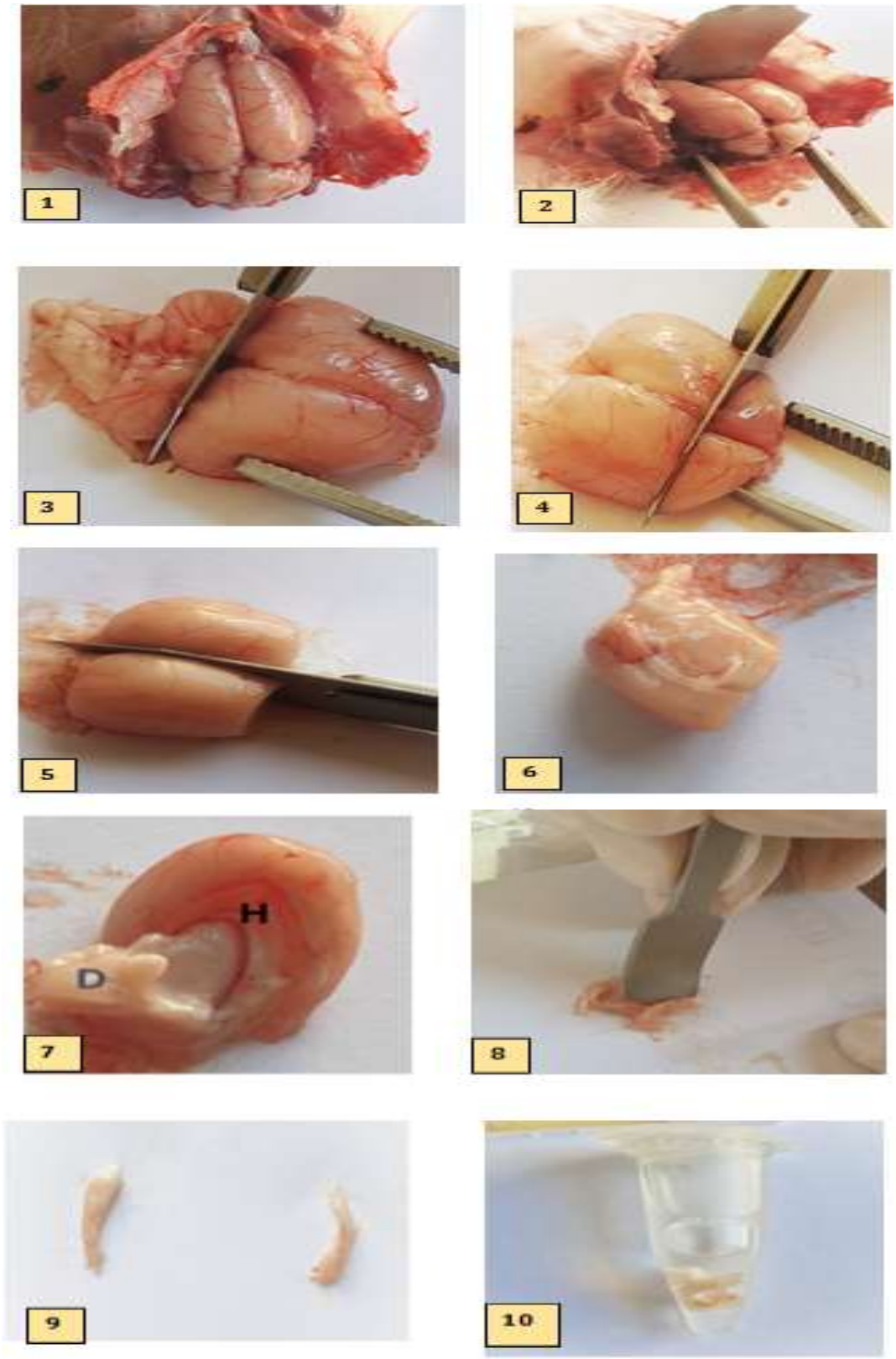

Figure 3: Steps of hippocampal dissection. **1:** skull opening**, 2:** brain extraction, **3:** cerebellum removal, **4:** frontal lobe removal, **5:** sagittal section in the brain, **6:** frontal cut section is facing down, **7:** removal of the diencephalon **(D)** to expose the medial surface of the hippocampus **(H), 8:** separation of the hippocampus from the cortex by the spatula, **9:** isolated hippocampi, **10:** hippocampus in eppendorf tube containing RNA lysis solution.

Light Microscopic Procedures: Preparation of paraffin blocks and sections (Hematoxylin and Eosin (Hx & E) stain and Immunohistochemical studies)

Statistical analysis:

Numerical data of MWMT calculated parameters, histomorphometric and gene expression studies were statistically analyzed using SPSS program (Statistical Package for Social Sciences) version 29. Results were presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was employed to compare means between groups. Bonferroni Post Hoc test was used to detect pairwise significance between every two individual groups. The standard deviation and the p-value were calculated for each experimental group and compared among the different groups. Significance cut-off value was considered when p-value was $\leq$ 0.05, and highly significant when P value $\leq$ 0.001. P value > 0.05

Was considered non-significant. The data were examined by the Kolmogorov-Smirnov test for normality, data were represented in tables, line and bar charts.

RESULTS

Table (1): Comparisons between the mean numbers of platform-site crossovers in all experimental groups (n:10) in the probe trial

Groups	Mean ± SD	Versus groups	P-value
Adolescent IA	11.4000 ± 1.14018	----------	----------
Adolescent + J IB	12.2000 ± 1.92354	Adolescent IA	1.000
Adult IIA	8.0000 ± 1.50740	Adolescent IA	0.002*
		Adolescent + J IB	<0.001**
Adult + J IIB	10.6000 ± 0.54772	Adolescent IA	1.000
		Adolescent + J IB	0.626
		Adult IIA	0.028*
Senile IIIA	3.4000 ± 1.15216	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	<0.001**
		Adult + J IIB	<0.001**
Senile + J IIIB	6.2000 ± 0.83666	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	0.352
		Adult + J IIB	0.002*
		Senile IIIA	0.014*

J: pomegranates and grapes juice-treated group

This table showed that there was a statistically significant difference between the studied groups as regard Comparisons between the mean numbers of platform-site crossovers in all experimental groups (n:10) in the probe trial except Adolescent IA in Adolescent + J IB, Adolescent + J IB in Adult + J IIB and Adult IIA in Senile + J IIIB.

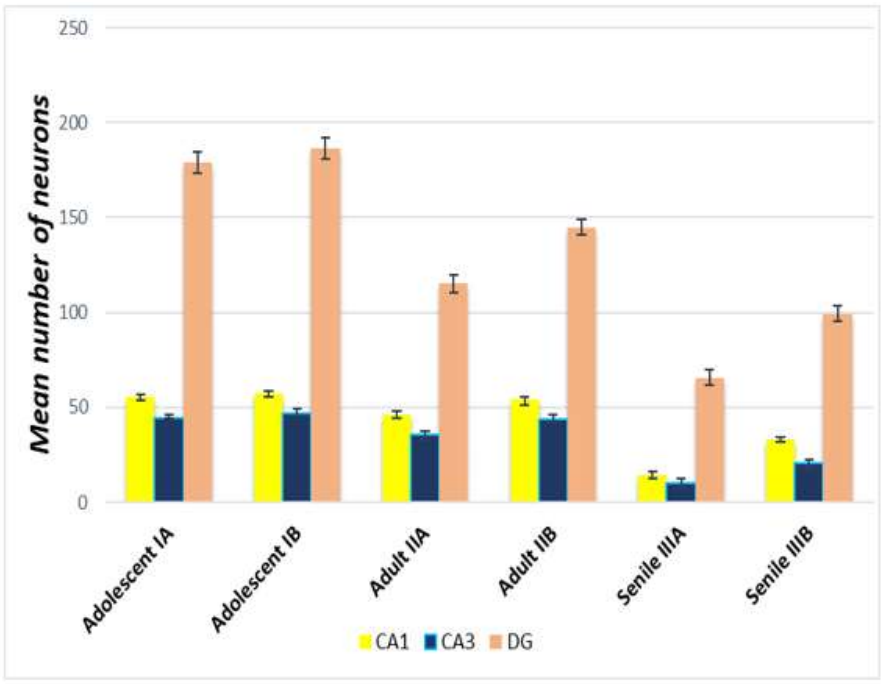

Figure 4: Bar chart showing the mean number of hippocampal pyramidal neurons in CA1 & CA3 regions and granular neurons in DG in all experimental groups (n:10).

Table (2): Comparisons between the mean area percent of GFAP immune reactivity in all experimental groups (n:10)

	Mean ± SD	**Versus groups**	**P-value**
Adolescent IA	1.8200 ± 0.64576	----------	--------
Adolescent + J IB	0.8800 ± 0.34928	Adolescent IA	1.000
Adult IIA	8.7000 ± 1.15758	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
Adult + J IIB	6.2600 ± 0.72319	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	0.02*
Senile IIIA	16.0300 ± 0.93338	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	<0.001**
		Adult + J IIB	<0.001**
Senile + J IIIB	8.4180 ± 0.93338	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	1.000
		Adult + J IIB	0.007*
		Senile IIIA	0.03*

SD: Standard deviation

J: pomegranates and grapes juice-treated group

This table showed that there was a statistically significant difference between the studied groups as regard Comparisons between the mean area percent of GFAP immune reactivity in all experimental groups (n:10) except Adolescent IA in Senile + IIIB.

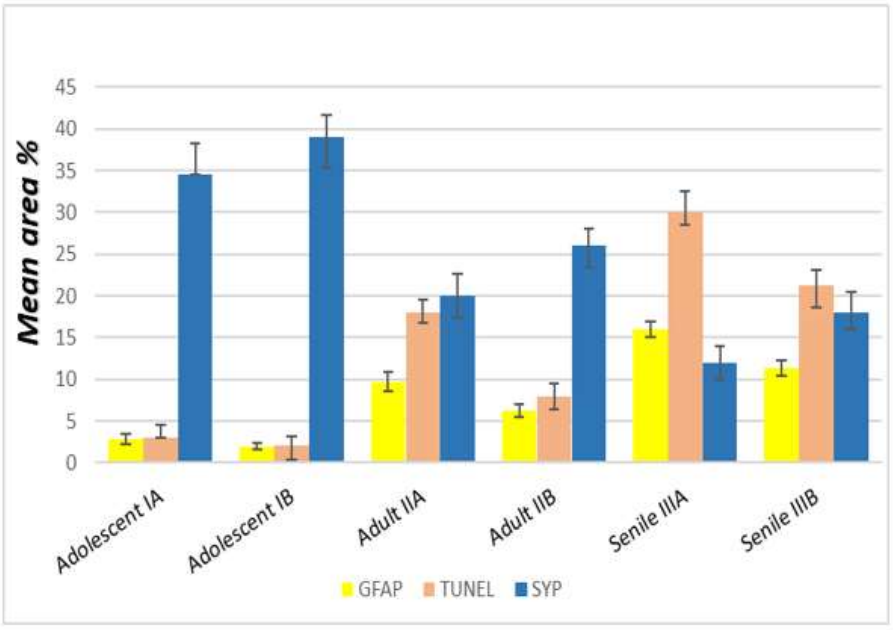

Figure 5: Bar chart showing the mean area % of GFAP, TUNEL & SYP immune stains of all experimental groups.

Table (3): Comparisons between the mean level of α-Synuclein gene expression in all experimental groups (n:10)

	Mean ± SD	Versus groups	P-value
Adolescent IA	1.0310 ± 0.02168	----------	----------
Adolescent + J IB	1.0100 ± 0.15811	Adolescent IA	0.891
Adult IIA	3.4800 ± 0.31937	Adolescent IA	0.043*
		Adolescent + J IB	0.031*
Adult + J IIB	3.1950 ± 0.16920	Adolescent IA	0.05*
		Adolescent + J IB	0.048*
		Adult IIA	0.57
Senile IIIA	5.3380 ± 0.48033	Adolescent IA	0.002*
		Adolescent + J IB	<0.001**
		Adult IIA	0.03*
		Adult + J IIB	0.01*
Senile + J IIIB	4.9680 ± 0.37772	Adolescent IA	0.007*
		Adolescent + J IB	0.005*
		Adult IIA	0.043*
		Adult + J IIB	0.05*
		Senile IIIA	0.49*

SD: Standard deviation

J: pomegranates and grapes juice-treated group

This table showed that there was a statistically significant difference between the studied groups as regard Comparisons between the mean level of α-Synuclein gene expression in all experimental groups (n:10) except in Adolescent IA in Adolescent + J IB and Adult IIA in Adult + J IIB.

Table (4): Comparisons between the mean level of BDNF gene expression in all experimental groups (n:10)

	Mean ± SD	Versus groups	P-value
Adolescent IA	4.9120 ± 0.48329	----------	---------
Adolescent + J IB	5.5340 ± 0.30908	Adolescent IA	0.063
Adult IIA	1.7600 ± 0.16985	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
Adult + J IIB	2.5380 ± 0.42903	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	0.009*
Senile IIIA	0.5780 ± 0.11256	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	<0.001**
		Adult + J IIB	<0.001**
Senile + J IIIB	0.9500 ± 0.15572	Adolescent IA	<0.001**
		Adolescent + J IB	<0.001**
		Adult IIA	0.002*
		Adult + J IIB	<0.001**
		Senile IIIA	0.046*

SD: Standard deviation

J: pomegranates and grapes juice-treated group

This table showed that there was a statistically significant difference between the studied groups as regard Comparisons between the mean level of BDNF gene expression in all experimental groups (n:10) except Adolescent + JIB in Adolescent + J IB.

DISCUSSION

In the present study, hippocampal-dependent ability to learn, acquire, store and retrieve spatial memory was investigated by applying the famous Morris water maze test (MWMT). The best performance during the acquisition phase in the current study was observed in the youngest age groups (IA & IB), as denoted by the least mean escape latency, with obvious progressive decrease in recorded time along the training days and the highest number of platform-site crossovers and the percentage of time spent in the target quadrant (proximity), in the probe test which reflected well-formed spatial memory and successful task learning.

The adult group IIA, however, exhibited significant better performance than the senile ones, regarding the training trials escape latency time and the probe test results, but it was still significantly worse than the adolescent rats.

The aforementioned results were in accordance with **Taridi et al. (8)** and **Hamezah et al. (9)**, who attributed the marked cognitive impairment exhibited by the aged rats in MWMT, and the difficulty in acquiring new tasks and remembering them later, to the reduction of hippocampal volume with age. **Shavitt et al. (10)**, supported this assumption in humans. They reported a significant positive correlation between the hippocampal volume in aged subjects and the higher performance in spatial memory tasks and intelligence quotient (IQ). Thus, in subjects having Alzheimer's dementia with severe amnesia, hippocampal atrophy is commonly encountered.

Regarding the hippocampal structural changes with the advance in age, the current work revealed mild degree of disorganization and neuronal degeneration in hippocampal CA1 & CA3 regions in the adult group IIA compared with the adolescent group IA. Likely, the DG showed loss of evenly arranged appearance of the granular cell layer, neuronal loss, numerous glial cells with obvious cytoplasmic vacuolations, in addition to deceased neuronal stem cells in the SGZ. The decrease in the count of pyramidal cells, in both CA1 & CA3, and granular neurons in the DG of the adult group IIA was statistically significant, in comparison to the cell count of the adolescent group IA.

Matching cytoarchitectural figures was observed by **Hwang et al. (11)** and **Gasparova et al. (12)** who reported the inception of neuronal loss with reduction in the pyramidal and granular cell count, since the middle age and become significantly evident in late age. Additionally, they assumed that, neuronal loss is presumably, reflected upon the compromised functional integrity of the hippocampus among the middle-aged rats.

On the contrary to the present histological findings, **Morterá & Herculano-Houzel (13)** speculated as a novel uncommon finding, that hippocampal neurons keep rising in number to reach the peak at the adolescent period then progressive age-concomitant

decline of neurons begins, as soon as, the end of adolescence.

In the present study, the DG of both the adult group IIA & senile group IIIA showed variable degrees of reduction in the neuronal stem cells in the SGZ, with marked reduction in the senile rats. This indicates decreased adult hippocampal neurogenesis since the adult age to become markedly explicit in senile one. This finding might be one of the factors explaining the progressive deterioration of performance in spatial memory test, observed in these two groups.

Supporting data was disclosed by **Baptista & Andrade (14); Ghallab et al. (15)** and **Abbott & Nigussie, (3)** who underlined the importance of the newly generated neurons in DG in maintained hippocampal-dependent mental functions, when they successfully integrate into the established hippocampal circuitry. The Authors presumed that adult neurogenesis is fundamental in the continued ability of learning, healthy brain aging and cognitive resilience till late ages.

In the current work, PGJ-treated adult group IIB displayed significant improvement of the histopathological features seen in the age-matched group IIA. The three hippocampal regions displayed apparent organized, evenly arranged, compact pyramidal and granular neurons, which appeared vesicular, with narrow neuropil in between and the degenerated neurons were distinctly reduced.

Similarly, the senile PGJ-recipient group IIIB exhibited variable degrees of recovery among hippocampal regions but in a milder form, as compared with the younger adult group IIB. Most of pyramidal and granular neurons regained its normal appearance and packed organization, signs of neuronal degeneration and tissue damage were less intense, areas of neuronal loss were restricted, with clear increase in cell number. Degenerated neurons were less numerous. In addition to decreased glial cells and amyloid deposits. The highly significant rise in the cell count of the three hippocampal regions; CA1, CA3 & DG of groups IIB & IIIB versus the age-matched groups IIA & IIIA, respectively, denoted evident improvement.

The current improved histological pictures and cell count results of the adult and senile PGJ-administered groups IIB & IIIB, respectively, complied with **Gadouche et al. (16); Ghorbanian et al. (17)** and **Harakeh et al. (18)** who described the cytoprotective role of antioxidants, specially, polyphenols, flavonoids and resveratrol, that are concentrated in pomegranates and grapes. These active compounds have the potential to restore the structural integrity of the aged hippocampus, as they can permeate the blood brain barrier to act directly on the hippocampus, which is harmed by the aging-concomitant oxidative stress.

In the present work, immunohistochemical study for GFAP in group IIA showed moderate increase in the intensity of positive GFAP immune reactive astrocytes; whose glial fibers were thick, dense and irregular. Meanwhile, senile group IIIA exhibited strong positive reaction; the GFAP immune reactive astrocytes appeared dense and widespread in the different hippocampal fields, their glial fibers were coarse, dense and highly-branched as compared with the younger adolescent and adult rats, with highly significant difference in the area percent of GFAP immune stain between each of the adult & senile rats (IIA & IIIA), respectively and the adolescent group IA.

In agreement with the present results **Teissier et al. (19)** reported that astrocytes undergo proliferation and activation paralleled with aging, or any insult affecting the astrocyte-nearby neurons**.**

Up to our knowledge, there is a notable paucity in publications discussing the anti-apoptotic influence of a juice mixture of pomegranates and grapes, on the hippocampus of aged rats, by applying TUNEL technique. Nevertheless, **Dulcich (20)** demonstrated the antiapoptotic power of PJ by ameliorating radiation-induced DNA damage due to enhanced apoptosis in hippocampal neurons, in addition to the pomegranates ability to stimulate cellular proliferation in the DG.

In the current work, the highest level of SYP immune reactivity was detected in the adolescent group IA, with a progressive decrement as the rats get older; the adult group IIA and the senile group IIIA demonstrated a highly significant diminution in the area percentage of SYP immune reactivity versus each of the adult and adolescent rats. This synaptic dysfunction with the advance in age might be one of the factors underlying learning and memory deterioration displayed by the adult and senile rats in MWMT.

These outcomes run in parallel with the results of **Bazzari & Parri (21)** who observed higher SYP expression in young age, which is critical for continued learning ability, encoding of new information and hippocampal circuitry development; the life period in which there is intense synaptic activity in response to bursts of neuronal stimuli. Additionally, the former authors deduced a positive correlation between SYP level and the degree of intelligence.

CONCLUSION

Regular intake of functional food such as pomegranates and grapes juice is necessary for the maintenance of normal cognitive functions required for healthy aging. They can mitigate or even reverse the early structural and functional adverse effects of aging on hippocampus, moreover, they can slow down or prevent further progression of cognitive decline and memory deterioration in neurodegenerative disorders, if present.

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