Anti-aging properties of whey against brain damage of senile Wistar rat

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Abstract

Aging of mammalian species results in impaired biological function and cognitive decline. The purpose of this study was to determine the capacity of whey supplementation to improve aging –related changes of cognitive impairment markers; tau and amyloid-B and α-amylase in the brain of old rats. These have been conducted in conjunction with histopathology, immunohistochemistry and flow cytometry of apoptosis. Twenty-four male Wistar albino rats (*Rattus novergicus*) ages 8 and 30-M (months) old were used. They were arranged into four main groups; adult (8-month old) and old rats (30 month old) with or without buffalo whey syrup supplementation. Oral whey supplementations was given daily twice doses of 2 mL^3 of whey syrup for two months. At the end of experiment, the rats were sacrificed by light anesthesia. The brain was examined for histological, immunohistochemical of synaptophysin and caspase 3 and biochemical and flow cytometric investigation.

Old rats presented with depletion of superoxide dismutase (SOD), adenosine triphosphatase (ATP), dopamine (DA) and serotonin (5-HT). The 30 M old rats also presented with increased lipid peroxidation MDA, inflammatory markers (tumor necrosis factor- α and 5-lpooxygenase), apoptic marker caspase 3, Annexin-v and aging marker tau-protein, amyloid-β and α-amylase. The combination of these findings in old rats predicts cognitive impairment. Among old rats, whey supplementations reduced inflammatory and oxidative stress markers. Whey supplementation also enhanced neurotransmitters and decreased tau-protein, amyloid-β, α- amylase cognitive impairment markers. Improved the histopathology and immunohistochemistry of cerebrum, cerebellum and hippocampus of old rats confirmed these effects of supplementation. The rates of apoptosis were decreased by assessment of Annexin v via flow cytometry. Whey supplementation to 8M old rats resulted in maintenance of the brain structure and function. The authors concluded that whey contains antioxidants and amino acids that decrease brain oxidative stress and restore normal cognitive function. These findings were evaluated by enhanced antioxidant defense and DA and 5-HT neurotransmitters which coincides with improved histology.

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Introduction

Aging, is characterized by abnormal autonomic dysregulation, arterial stiffness and damage to the blood-brain barriers. The processes have been linked with the production of cortical and subcortical microinfarcts, microblems and diffused disease of white matter. These injuries result in demyelination and axonal damage [1]. The development of cerebral micro-hemorrhages of venous origin, and ischemic damaging of the neuronal cells also facilitate the development of cognitive impairment and dementia [2]. In old age, gray and white matter were affected by these changes, resulting in loss of memory [3]. Aging also led to an increase in TDP-43 [4], which increased neuronal and glial cell inflammation. This inflammation leads to excessive accumulation of pro-inflammatory microRNA cytokines [5] targeting genes involved in neuronal apoptosis. Old age displayed irregular glycolytic enzyme activities which impede synaptic function and trigger neuronal cell loss [6] Also, advanced age can lead to heme degradation due to heme-oxygenase-1 regulation which causes damage to the mitochondrial membrane in neuronal cells [7]. The major diseases associated with aging are Alzheimer's disease, Parkinson's disease and glaucoma. Glucoma is the manifestation of oxidative stress assessed by mitochondrial and endoplasmic reticulum dysfunction and endothelial cell damage. Glaucomatous patients exhibit an abnormal aggregation of Tau- protein or the β-amyloid in the retinal ganglion cells [8].

Whey protein, is a byproduct of the cheese-making process. It is rich in amino acids plays a pivotal role in glucose homeostasis and treatment of type 2 diabetes [9]. Dietary supplementation of sheep/goat whey protein (1 g kg b.w/day) improved antioxidant capacity and decreased free radical and protein carbonyl [10]. Administration of whey proteins to rats fed a diet containing a high phytooestrogen for 10 weeks improved T4, estradiol levels and glucose homeostasis [11]. Female C57BL/6J mice supplemented with whey protein (100g WPI/L drinking water for 12 weeks) exhibited activated brain function with increased levels of cytochromes [12].

Secondary effects of diabetes mellitus can damage the brain through oxidative stress [13[. High whey-protein supplementation antagonized the development of diabetic disease via increased cerebral oxygen, cerebral blood flow and insulin secretion, and decreased blood sugar level [14]. The intake of fermented dairy products improved cognitive and improved symptoms of ‚Alzheimer's disease in a mice model. These improvements resulted were caused by Trp-Tyr (WY)-containing peptides that increased dopamine levels and inhibited monoamine oxidase-B activity in brain tissue [15]. The progress of oxidative stress resulted in degenerative neurons was reduced in CD1 mice supplemented double oral doses of Immunocal® (whey protein)for 28 days prior to receiving a moderate TBI. This reduction in negative effects resulted reduced axonal demyelination and brain-derived neurotrophic factor and improved both Iba1 (microglial marker) [16]. Patients with Parkinson's disease supplemented whey protein for 6 months revealed increased plasma glutathione levels, upregulation of branched and essential amino acids and reduction of plasma homocysteine [17]. A total of 130 sarcopenic elderly people (53 men and 77 women; mean age: 80.3 years) supplemented whey protein (22 g), essential amino acids (10.9 g, containing 4 g leucine), and vitamin D [2.5 μg (100 IU)] for 12 weeks showed increased muscle strength with a handgrip dynamometer, and improved health condition based on blood biochemical indices [18].

Whey protein supplementation has been shown to alleviate oxidative stress and improve the brain diseases [19]. Growing rats fed on a diet containing lactoferrin, milk fat globule and a polydextrose/galactooligosaccharide prebiotic led to a marked increase in total dendritic spine density in hippocampal dentate gyrus neurons [20].

Whey proteins, especially lactoferrin and bovine serum albumen, are major components of milk [21], These nutrients represent important components of the human diet [22] and are widely used in infant formula [23].

The present study aimed to assess the capacity of whey supplementation in improve these aging related changes via reduced accumulation of the cognitive impairment markers, tau and amyloid-B and α-amylase in the brain of old rats. We also examined histopathological, immunohistochemical and flow cytometry of brain markers.

Materials And Methods

1. Animal model and design of experiment:

Twenty-four adult Wistar albino rats (*Rattus novergicus*) (8-month-old, n = 12, weight 200 ± 12) and senile rat (30 month –old,n = 12, weight 400 ± 20) were obtained from Breeding laboratory farm of Ministry of Health, Egypt. The experiments were approved by the local Experimental Animal Ethical Committee of Faculty of Science, Mansoura University, Egypt (decisionl statement No. RZ19004). This study was carried out according to the guidelines from the National Institute of Health for the use of laboratory animals (NIH Publication No, 8523, updated 1996). Animals were kept in an aerated room with approximately 12 hour of light / dark cycle and light intensity exposure at an of 180–200 lx. Free access to a standard diet and water were allowed *ad libitum*.

2. Whey syrup supplementation:

Fresh bovine whey is the byproduct of coagulated milk and cheese production. It was daily obtained from the Dairy Product Lab, Faculty of Agriculture, Mansoura Univ., Egypt. Each rat was orally administered twice daily doses of 2 mL^3 for two months by interagastric tube Whey protein was determined as described by Parris and Baginskla [24]. The lactose content [25] and overall antioxidant potential [26] of the whey syrup were determined. The number of lactobacilli colonies in whey was also measured [27] (Table 1).

Table 1
Bovine whey contents of lactose, protein, antioxidants, lactobacilli and SNF and freezing point.

Item	Content
SNF (standard solid factor)	9.9%
Lactose (mg/mL)	5.5
Total protein(mg/mL)	3.75
Total antioxidant capacity (mg/L)	4.7
Freezing point	0.63
Lactobacilli cell count	60/mL

3. Animal grouping and investigations:

Twenty-four male Wistar albino rats (*Rattus novergicus*) at 8 and 30-M (months) old, Hellwan Breeding Farm, Ministry of Health, Cairo, Egypt, were used for experimentation. The animals were divided into two main groups; 8 month and 30 months old. Each group was subdivided into two, non- supplemented and whey supplement-treatments. For the whey supplemented group, each individual was supplemented 2 mL^3 twice daily for two months by interagastric tube. At the end of treatment, At the end of 8 weeks the rats were fasted overnight and euthanized for analysis by exposure to halothane (2- bromo-2-chloro-1,1,1-trifluoroethane), followed by cervical dislocation and dissected (Fig. 1).

4. Body and brain weight (g):

Both absolute body and brain weight as well as relative brain weights were measured for both the young and old age groups with or without whey supplementation.

5. Assessments of superoxide dismutase and lipid peroxidation (MDA):

Super oxide dismutase activity (SOD),was measured using 100 µL of the supernatant of brain tissue samples added to100 µL xantine oxidase, 100 µL xantine, 100 µL xantine oxidase, 100 µL nitroblue tetrazolium and 3100 µL phosphate buffer solution (PBS) and incubated for 30 minutes at 30°C.The developed colour .was measured spectrophotometrically at 500–600 nm 1996) [29]. Malondialdhyde eacts with thiobarbituric acid producing thiobarbituric acid reactive substance (TBARS), TBARS is a pink colour and was determined calorimetrically at 532 nm [30].

6. Determination brain dopamine and serotonin:

The assayed neurotransmitters were determine by Rat ELISA Kit of CUSABIO TECHNOLOGY LLC ,Houston, USA, following the manufacture's instruction. Dopamine (DA) was assayed by the ELISA Kit, Cat Nu. CSB-E08660r while

serotonin (5-HT) was measured by Kit Cat Nu. E-El-0033.

7.Assessments of neurodegenerative markers:

The rat ELISA Kit (CUSABIO TECHNOLOGY LLC,Houston, USA) was used for determination of brain α-amylase (CSB-EL001689RA), tau protein (Cat Nu. CSB-E13729r) amyloid B-peptide CSB-E-10786r),acetylcholinesterase (Ache) (CSB-E11304r), brain natriuretic peptide (CSB-E07972r) and nerve growth factor(CSB-E04685r), Adenosine triphosphate (ATP) was measured by ELISA Kit (My Bioscource Company, MBS723034). The method was based on the competitive inhibition reaction between labeled biotin and unlabeled tau-protein with the pre-coated antibody specific to either tau or AB1-42 or DA or 5-HT or TNF-α or casp-3. Avidin conjugated with horseradish peroxidase was added to the samples, The amount of bounded HRP was proportional to the amount of the assayed parameter and the absorbance was measured at 540 nm within 30 minutes to avoid fading. The standard curve was calculated using the assayed parameter. In case of brain creatine kinase (catalogue no. K777-100) and xanthine oxidase activity (catalogue no.K710-100)were determined by Biovision incorporated(,Milpitas boulevard, Milpitas, GA,95035USA).

8. Histopathological investigations:

Brain specimens were fixed in 10% phosphate buffered formalin (pH 7.4), dehydrated in ascending grades of ethyl alcohol, cleared in toluene, and mounted in molten pararplast 58 – 62°C. Serial 5 µm thick histological sections were cut and stained with hematoxylin and eosin (H&E) and examined under bright field light microscopy to visualize the changes in cerebrum, cerebellum and hippocampus.

9. Immunohistochemistry of caspase 3and synaptophysin:

Serial 5µm thick histological paraffin sections were cut and mounted onto super frost t plus glass slides (Fisher Thermo Scientific, Nepean, Ontario, Canada). The tissue sections were processed for antigen retrieval by digestion in 0.05 % trypsin (pH 7.8) for 15 min at 37°C and incubated against either caspase 3 or mouse anti-synaptophysin (Thermo Fisher Scientific, Fremont, CA, USA) overnight at 4°C. These were followed by treatment with a horseradish peroxidase streptavidin, then DAB plus Chromagen to detect the immunoactivity, and counterstained with Mayer haematoxylin. Negative control sections were incubated with 1% non-immune serum PBS. The brain regions were examined with a Leica BM5000 microscope (Leica Microsystems, Wetzlar, Germany) and photographed.

10. Flow cytometry assessments of annexin V:

Flow cytometric assessment of Annexin -v was carried out following Logue et al (2009) by using V-FITC/ PI double staining assay. The brain tissue was lysed with Tris–EDTA buffer (pH 7.4) and fixed in 70% ethanol. Cells were then washed with PBS, suspended at a concentration of $0.1–0.3 \times 10^6$/ml and stained with fluorescein isothiocyanate-conjugated annexin-v (annexin V-FITC). The specimens were allowed to incubate for 15 min at room temperature and were measured using Becton Dickinson Fac Scan Fluoescence Activated Cell Analyzer (Becton Dickinson, Sunnyvale, CA, USA).

11. Statistical analysis:

Data were presented as means ± standard deviations (SD). The statistical analysis was conducted using the software package SPSS (version 13) one way Anova post hoc analysis for windows ,comparing between the aged and adult group and/or whey supplementation. Significant at $p < 0.05$

Results

1.Whey nutrient contents:

The whey is rich in nutrient components. From table (1), total protein, lactose contents, total antioxidant, Lactobacilli contents and standard solid factor were illustrated.

2. Absolute and relative brain weights:

Whey supplementation caused a non-significant increase in absolute brain weight and a decrease in relative brain weight in 8-month old rat compared to non-supplemented group. Whey supplementation decreased absolute brain weight of old age and caused a non-significant increase of relative brain weight (Fig. 2A-C).

3. Brain superoxide oxide dismutase and lipid peroxidation (malondialdhyde):

Figure 3 (A) shows brain SOD of 8M old rats (G1) was15.46 ± 0.85 and coincides with the apparent depletion in 30 M old rats (G2) which was 9.81 ± 0.88. G1Y supplemented whey showed a non-significantly increase of the SOD activity compared to G1. However it increased significant in G2Y group compared to G2. On the other hand, there were no variations of brain malondialdhyde contents in either G1 & G1Y. However, a significant increase was detected at G2 (6.97 ± 0.15) compared to G2Y (4.66 ± 0.37). Whey supplementation decreased the malondialdhyde level (5.78 ± 0.16) in G2Y compared to G2 (6.97 ± 0.15) but was significantly higher when compared to G1 (Fig. 3B)..

4. Brain neurotransmitters dopamine and serotonin:

Dopamine (DA), serotonin(5-HT) and adenosine triphosphate (ATP) showed no variations in G1 and G1Y. However during aging, the brain levels of DA, 5-HT and ATP were markedly depleted in G2 reaching 6.78 ± 0.66, 103.57 ± 3.06 and 82.69 ± 2.55, respectively compared to 13.09 ± 0.45, 147.37 ± 1.68 and 117.65 ± 16.3 respectively. Whey administration improved the levels of the assayed neurotransmitters and ATP, but these compounds were still significantly decreased in G2 compared to G1 (Fig. 4A&B).

5. Brain biomarkers of cell death and inflammation:

Figure (5) shows that tumor necrosis-α, 5-lipooxygenase and caspase 3 markedly increased in G2 at 8.68 ± 0.57, 127.35 ± 5.68 and 100.64 ± 6.31 respectively compared to G1 6.21 ± 0.57, 67.47 ± 5.06 and 6.28 ± 0.87 respectively. Whey supplementation showed slight alterations in G1. G2Y exhibited moderate improvements in the brain levels of both cas-3 and TNF-α but 5-lipooxygenase were still significantly increased compared to G1.

6. Neurodegenerative markers:

Old group (G2) showed significant increase of both tau-protein, amyloid-β and α-amylase (218.83 ± 4.62, 11.23 ± 0.64 and 92.98 ± 6.47 respectively) compared to 162.71 ± 3.43, 6.83 ± 0.38 and 9.75 ± 1.54 respectively in G1. Whey administration moderately improved the assayed parameters but their level were still above the normal range in G1. ATP was markedly depleted in the aged group at 82.69 ± 2.55 compared to 117.65 ± 16.3 in G1.Whey administration improved the levels of the assayed neurotransmitters and ATP but they were still significantly decreased compared to G1 (Table,2).

Table 2
Neurodegenerative markers in serum and brain of senile male rats with or without whey supplementation.

	Serum					Brain			
	NGF (pg/mL)	XOR (mU/mL)	CK (mU/mL)	BNP (pg/mL)	AchE (pg/ml)	Tau-Protein (Pg/mgP)	Amyloid-β (Pg/mgP)	α-amylase (U/mg)	ATP (ng/mg)
G1	30.22 ± 1.34	5.02 ± 0.13	66.26 ± 0.79	318.88 ± 3.63	130.54 ± 1.27	162.71 ± 13.43	6.83 ± 0.538	9.75 ± 1.54	117.65 ± 16.3
G1Y	29.88 ± 0.31	4.76 ± 0.44	62.71 ± 1.46	321.61 ± 1.44	126.03 ± 4.45	150.92 ± 14.21	6.19 ± 0.71	8.76 ± 0.69	122.98 ± 6.51*
G2	19.4 ± 0.93*	7.38 ± 0.11*	86.76 ± 1.69*	252.89 ± 1.46*	169.43 ± 3.62*	218.83 ± 17.62*	11.23 ± 0.64	92.98 ± 6.47*	82.69 ± 2.55*
G2Y	22.19 ± 0.36*	6.02 ± 0.45	73.48 ± 0.84*	290.19 ± 1.83*	144.92 ± 3.25*	184.66 ± 9.61*	8.29 ± 0.71*	63.30 ± 5.78*	95.49 ± 3.98
F-test	50.26	25.42	77.88	111.73	52.55	25.93	56.15	112.12	18.59
P < 0.05	S	S	S	S	S	S	S	S	S

Data were presented as mean ± SD(n = 8), *Significant at p < .05 compared to G1. Abbreviations; AchE, acetyle cholinesterase; ATP, adenosine triphosphatase; BNP, brain natriuretic peptide; CK, creatine kinase; G1; 8month old; G1Y, 8 month old supplemented whey; G2, 30 month old; G2Y, 30 month old supplemented whey; NGF, nerve growth factor; XOR, xanthine oxido-reductase

In addition, old rats exhibited increased serum levels of xanthine oxido-reductase (XOR), creatine kinase (CK) and acetyle choline esterase, while decrease the levels of nerve growth factor (NGF) and brain natriuretic peptide (BNP). Whey supplementation improved the assayed serum levels of old rats. Also, whey administration maintained the assayed serum levels in adult rats (Table, 2).

7. Histopathological observations:

In control and supplemented whey groups (G1& G1Y), the cerebral external granular (OGL) exhibited a dense distribution of pyramidal and stellate cells with centrally located nuclei (Fig. 6A & A1 and B& B1). However in old rats (G2), many of the neuroglial cells showed either clumping nuclear chromatin (pyknosis) or chromatolysis (karyolysis). Angiogenesis of the blood vessels appeared wide spread throughout the cerebral tissue clarifying the sticky pathological feature. Glial cells appeared grouping manifesting inflammation of the brain tissues Numerous edematous, necrotic and spongiform degenerated zones were detected (Fig. 6C & C1). Whey supplementation improved these aged related changes (Fig. 6D & D1)

The cerebellar cortex of both 8 M old and whey supplemented groups (G1 & G1Y possessed normal pattern structures of the molecular (MCL), Purkinje (PCL), and the granular cell layer (GCL) (Fig. 6A2 & B2), In old age (G2), the Purkinje cell showed either pyknotic nuclei or karyolysed nuclei embedded in a wide necrotic spaces. The granular cells were reduced and infiltrated by wide glomerular spaces (Fig. 6C2). Whey supplementation to aged group comparatively improved the cerebellar structure especially in Purkinje cells (Fig. 6D 2).

Histological investigation of the hippocampus of 8 M-old rat with or without whey supplementation (G1&G1Y) revealed revealed a well-defined polymorphic, pyramidal, and molecular layer. The pyramidal layer is made up of small

tightly backed up pyramidal cells. Each cell appeared as a large polygonal with rounded nuclei, prominent nucleoli and scanty cytoplasm. The dentate gyrus is made up of small granule cells. The molecular layer had regular neuronal axons and dendrites distribution (Fig. 6A3 & B3). The old age group (G2) had either chromatolysis or grouping nuclear chromatin that manifested as apoptosis of pyramidal cells (Fig. 6C3). Whey-treatment improved the neuronal structure in old rats through some deformed blood vessels were still observed (Fig. 6D3).

8. Immunohistochemistry of caspase 3 and synaptophysin:

In cysteine-aspartic acid protease 3 (caspase-3), cerebral neurons, cerebellar Purkinje and granular cells and pyramidal hippocampus cells displayed overexpression of the immunohistochemical reaction in old rats (G2) with increased apoptic cells (Fig. 7A2,B2 & C2). Whey supplementation, decreased the immune- histochemical reaction in G2Y group but did not fully return to the rates to the state the young group with or without whey supplementation (Fig. 7A3,B3 &C3) compared to control an whey supplementation (Fig. 7A,B,C,A1,B1 & C1). Image analysis revealed the increased intensity of the caspase-3 imunohistochemical reaction in the aged group compared to that of the old rats supplemented whey or young rats with or without whey supplementation (Figs. 7B).

Imnunohistochemistry with synaptophysin showed a decrease in the G2 expression of the synaptic axon's cerebrum, cerebellum and hippocampus and increased in studied groups of supplemented whey but less than in younger rats (Fig. 8A, A3,B3andC3) compared to aging (Fig. 9A2,B2 &C2) and both young and whey supplemented group (Fig. 8A, A-C & A1-C1). Image analysis revealed the decreased intensity of the synaptophysin imunohistochemical reaction in the aged group compared to that supplemented whey as well as young with or without whey supplementation (Fig. 8A-B).

9. Flow cytometry of Annexin v:

Old brain exhibited by increased average of apoptotic cells. These cells reached reached 92.44% compared to 54.13% in experimental group supplemented whey. Adult rats with or without whey supplementation showed 14.35 and 17.36% respectively (Figs. 9A &B).

Discussion

Aging rats exhibited a marked increase of body weight, an increase of absolute brain weight and a decrease of relative of brain weight. The increase of body weight and decrease of relative brain weight reflected the decreased of metabolic rates of body organs [31] and the decrease of body fat oxidation [32, 33]. Whey is rich in amino acids [21] shows protective properties against oxidative stress [10]. Its higher antioxidant activity has promoted the cessation of oxidative chain reactions by removing free radical radical intermediates [33].This supplement also provides increased protein during aging [34] and improves brain function [19] .

Here aging rats showed substantial depletion of the antioxidant SOD and a marked increase in lipid peroxidation malondialdhyde. These changes facilitated the progress of neuronal cell damage in the cerebrum, cerebellar cortex, and hippocampus. Histopathological results showed increased vasculogenesis associated with cerebral cortex breakdown of neuronal cells. Degeneration of Purkinje cells and loss of many of the granular cells in cerebellum were also detected. In hippocampus, the pyramidal layer attained a considerable atrophy and was missing almost of the pyramidal neurons, which acquired pleomorphic forms. The damaged neurons were explained by increased brain caspase 3, UR + LR of Annexin V and immunohistochemistry of caspase 3.

The present findings support the work of Garg et al. [35] in d-galactose (150 or 250 mg/kg) induced alterations of hippocampus in experimental aging in rats for 8 months. Li et al. [36] reported significantly increased levels of β-

galactosidase and MDA coinciding with decreased SOD activity and decreased expression of erythropoietin, EPOR, p-JAK2, and HIF-2αin in aging rats.

Sousa et al. [37] recorded that aging resulted in a depletion of antioxidant including catalase activity and glutathione / oxidized glutathione ratio (GSH / GSSG) in the rat brain cortex. Sousa et al. also showed increased levels of malondialdehyde (MDA). Impairment of antioxidant enzymes]33] increased the release of active forms of oxygen [38–40] which induced cell death. Cell death can result in cognitive defects and increase the risk of disorders of the brain [41].Alzheimer's disease, and associated neurodegenerative disorders were found to increase oxidative stress and lipid peroxidation [42], involving the production of free radicals that directly damages cell membranes and secondary reproduce secondary byproducts leading to neurodegenerative disorders [43] .

These result showed a marked increase in brain TNF-α and 5- lipooxygenase coinciding with spreading angiogenesis, new formation of blood vessels,, tightly grouped glia cells in the cerebrum, damage to Purkinje cells and degeneration of granular cells in the cerebellum and damage to pyramidal cells and widespread pleomorphic cells in hippocampus necrotic sites.

These result align with the work of Garg et al. [35], who reported increased inflammatory markers (TNF-α, IL-1β, IL-6), lipid peroxidation, reactive oxygen species, advanced protein products for oxidation, and decreased activity of acetyl cholinesterase in aged rats.The susceptibility of the hippocampus to aging related damage caused by the reduction of astrocytes and neuronal stem cells in the dentate gyrus subgranular region, which affected the learning and memory function resulting in cognitive impairment [44] .

5-lipoxygenase (5-LO) mRNA was expressed in all the brain regions especially cerebral cortex, hippocampus, and cerebellum [45]. Highest expression of 5-LO has been reported in the old mouse hippocampus [46].

Uz et al. [47] and [46] reported damaged of the dendrites of pyramidal neurons in limbic structures, including the hippocampus, and V pyramidal cells of the frontoparietal cortex and their apical dendrites. These authors attributed the upregulation of the neuronal 5-Lipoxygenase (5-LO) to increased 5-LO gene expression. It is known that 5-LO is expressed in neurons and activated by neurodegeneration.5-LO also enhanced synthesis of leukotrienes and inflammatory eicosanoids and results in neurodegeneration [48].

The observed increase of lipid peroxidation and impair of the antioxidant system were reflected by depletion of dopamine and serotonin and consequent impaired cognition. Similar results of aging-related loss of serotonin secretion have been documented in old rats cerebral cortex, hippocampus, hypothalamus and pons-medulla [49].

Dopamine affects motor and limbic functions in reward processing [50]. The dopamine neurons transmit reward-related signals from the brain regions to the post-synaptic sites basal ganglia. Dopamine is involved in managing the various aspects of mental brain functions [51]. During pre- or post-synapse, serotonin serotonin (5-HT) also has a characteristic receptor. Aging has been shown to result in increased oxidative stress leading to loss of motor function. The mechanism of this loss of function is the decreasing dopaminergic neurons in substantia nigra pars compacta ,reduction of dopamine in the nigrostriatal pathways [52] and the reduction in sensorimotor function related to the depletion of GABA levels [53].

The dramatic change observed in the old brain mirrored the reported substantial depletion of the content of ATP in the old rat relative to the adult. Mitochondria are the brain's primary energy organelle to maintain its vital function.. Each mitochondria have copies of a double stranded, closed circular and maternally inherited DNA of 5–10 mtDNA.The human brain uptakes 20% of the body's oxygen. The brain neurons require about 4.7 billion ATP molecules per second

to satisfy the synapse's increased energy demands [54]. Depletion of ATP content reflects the loss of mitochondrial function and failure of neuronal cellular energy [55].

The activities of ATPases were significantly decreased during aging reflecting the reduction of energy requirements for brain function [56]. In cases of AD, the ATP-binding cassette transporter A1 produced a double triple rise in hippocampal neurons. These changes were associated with increased APOE and PUMA gene expression as a result of neuronal stress [57].

The extracellular ATP played a great role in glial neuron communications, especially in modulating synaptic plasticity. Aging led to depletion of the neuronal P2X receptor-associating less spontaneous currents which originated from the release of ATP from both synapses and astrocytes [58] .

Navarro and Boveris [59] reported impaired function of mitochondria in aged rat hippocampus and frontal cortex, in the human cortex in Parkinson's disease and dementia with Lewy bodies, and in substantia nigra in Parkinson's disease..

The observed findings influenced in injuring brain structure, reducing brain function and significantly upregulated brain tau-protein, amyloid-β and α-amylase.

Tau is a highly regulated microtubule-associated protein in neurons. In diseased conditions, abnormal aggregation of insoluble tau was associated with neuronal cell loss and synapses degeneration [60, 61]. Tau phosphorylation is known to be a secondary to amyloid-beta (Aβ) accumulation. It is occurred at serine or threonine residues and associated with aging [62]. These were found to be linked to neuronal loss and synaptic damage, in the brains of both male wild-type (Wt) and male P301L transgenic mice (a mouse model of human tauopathy. Activation or inhibition of cyclin-dependent kinase 5 and glycogen synthase kinase-3β were markedly altered Aβ/$p75^{NTR}$-mediated p-tau levels in neurons [63]. Dysfunction of lysosomes and autophagosomes were the main cause of neurodegeneration. The neurons facilitated the accumulation of undegraded cellular material by the secretion of extracellular vesicles [64]. Extracellular β –amyloid plaques and intracellular neurofibrillary tangles of tau phosphorylated protein [65, 66] were associated with the development of cognitive impairments and memory loss. In a macaque model of AD that amyloid β oligomers were found to infiltrate the brain tissues and deposited in brain regions. These depositions lead to astrocyte and microglial activation, synapse loss, abnormal tau phosphorylation and neurofibrillary tangle formation and ultimately impaired brain function [67]. Aging was found to deplete brain cellular glutathione content or impairment its biosynthetic enzyme glutamate cysteine ligase. Soluble amyloid-β (Aβ) oligomers were found to induce oxidative stress, synaptic dysfunction and memory deficits [68]. Also, Lénárt et al. [69] found that an elevated ApoB-100 level (cholesterol- and triglyceride-rich LDL and VLDL lipoproteins) and the hypertriglyceridemia can lead to impaired neuronal function and neurodegeneration, especially via hyperphosphorylation of tau protein.

These may illustrate the neuroinflammation and clumping of the glial cells which possessed the brain resident immunity and predicted deficits in cognition associated age-related neurodegenerative diseases [70].

It is known that the hippocampus is involved in memory formations [71]. The observed decreased expression of synaptophysin in neuronal axons in old rats reflected impairment of memory capacity, neurodegeneration and development of cognitive failure [72].

Also, the present findings show the increased level of alpha amylase in old brain compared to the young rat. These data supported the findings of Byman et al.[73] who indicated the importance of synapse activity and plasticity due to its requirement for 84% of energy for postsynaptic actions [67].

Following amylase investigation or omission of lead citrate staining strongly recommended that the electron-dense granular structures incorporated with Ab immunoreactivity are glycogen. Some of it had gliofilaments and immunolabeling with glial fibrillary acidic protein confirmed that they were astrocytic [68].

Alpha (a)-amylase, is involved in degradation of glycogen in the gastrointestinal tract. It is also, expressed in the hippocampal CA1/subiculum and the expression is altered in old patients. The authors added the presence of the α-amylase isotypes AMY1A and AMY2A and HB- inclusions in neuronal dendritic spines, pericytes and astrocytes as well as in CA1 of AD patients [73].

Although, AD patients showed reduced gene expression of a-amylase, it was overexpressed expressed and increased levels of a-amylase were present. Periodic acid-Schiff showed positive polyglucosan bodies in the brain of AD patients, correlated with the increased a-amylase activity [74]. Glycogen is composed of several glucose units linked together with alpha (a)(1–4) glyosidic bonds and branched f by a(1–6) glyosidic bonds [75]. It is manufactured by the brain glycogen synthethase [76] and present in the cytosol of astrocytes, endothelial cells, pericytes and neurons [77] .

Also, glial cells were found to express both glycogen synthethase and glycogen degrading enzymes [75] .

In addition, Old rats exhibited increased serum levels of xanthine oxido-reductase (XOR), creatine kinase (CK) and acetylcholine esterase, while decrease the levels of nerve growth factor(NGF) and brain natriuretic peptide (BNP).

The finding of increased serum acetylcholinesterase in old brain reflected the neurodegenerative disorder as mentioned by García-Ayllón et al. [78] in Alzheimer patients. Also, XOR-generated ROS leading to for the catabolism of purine bases [79] via liberating electrons to oxygen and intern generate O_2^- and H_2O_2 within the cell [80] .resulting in imbalance in the redox state of the cell, by either overproduction of free radicals, or by impairment of the antioxidant systems. Also, increased serum creatine kinase reflected damage of mitochondria as a result of oxidative stress in old brain [81]. Depletion of serum nerve growth factor [82] and natriuretic peptides [83] .

From the present finding aging rats orally supplemented whey exhibited a moderate depletion of lipid peroxidation, caspase 3, inflammatory markers (TNF-α and **5- LOX**) and increased Tau-protein and amyloid—β and α-Amylase which coincided with increase SOD activity and ATP contents of the brain. These changes facilitated improvements of brain function and improved histological and immunohistochemistry results in the cerebral, hippocampus and cerebellum. These improvements were attributed to increased antioxidant activity of whey due to its contents of phenolics, flavonoids and tannins.

The current results support previous studies of whey protein and aging. Whey protein has been found to improve aging-related galactosaemia rat disease associated with SOD depletion and MDA[84]. Young (4 months) and old (24 months) male Wistar rats supplemented whey protein (300 mg/kg body weight) for 28 days down-regulated the inflammatory markers (tumor necrosis factor alpha, interleukin (IL)-1β, IL-6) associated oxidative stress in aged rats [35]. Also, whey administration to diabetic rats decreased lipid peroxidation and improved brain co-ordination [85, 86], increased mitochondrial activity [12] and managed brain structure and function [87,88]. There was a detected increase of the dendritic spine density in the hippocampal dentate gyrus neurons of weanling rats supplemented diet containing milk fat globule membrane, lactoferrin and a polydextrose/galactooligosaccharide prebiotic [89].

Finally, whey supplementation improved the histopathological changes in cerebrum, cerebellum and hippocampus of aged rats while also improving antioxidant defense and inflammation. There was a detected reduction of brain tau and amyloid –β illustrating the antioxidant and nutritive properties of whey supplementation.

Declarations

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Author Agreement Statement:

We declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs Signed by all authors.

Author Declaration and Conflict of Interest

The authors confirm that there is no conflict of interest associated with this publication and there is no significant financial support of the work that could have influenced its outcome. The corresponding author declares that he is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs Signed by all authors.

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Figures

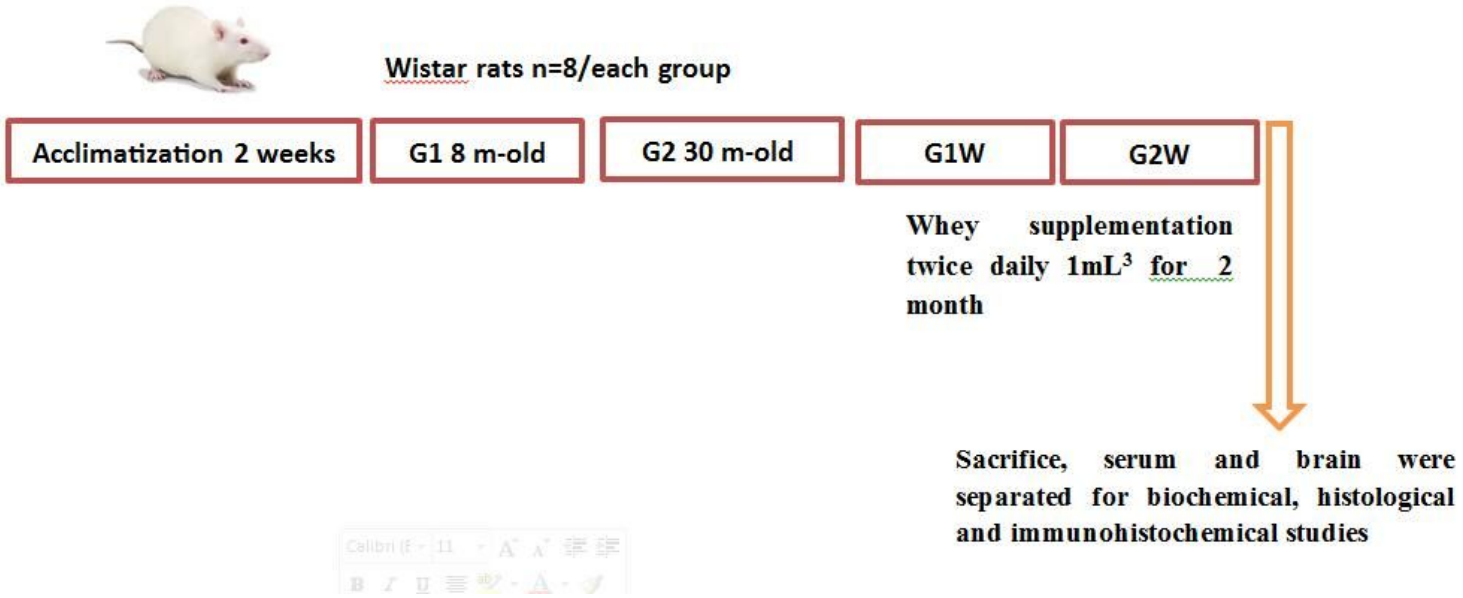

Figure 1

Chart illustrating experimental design.

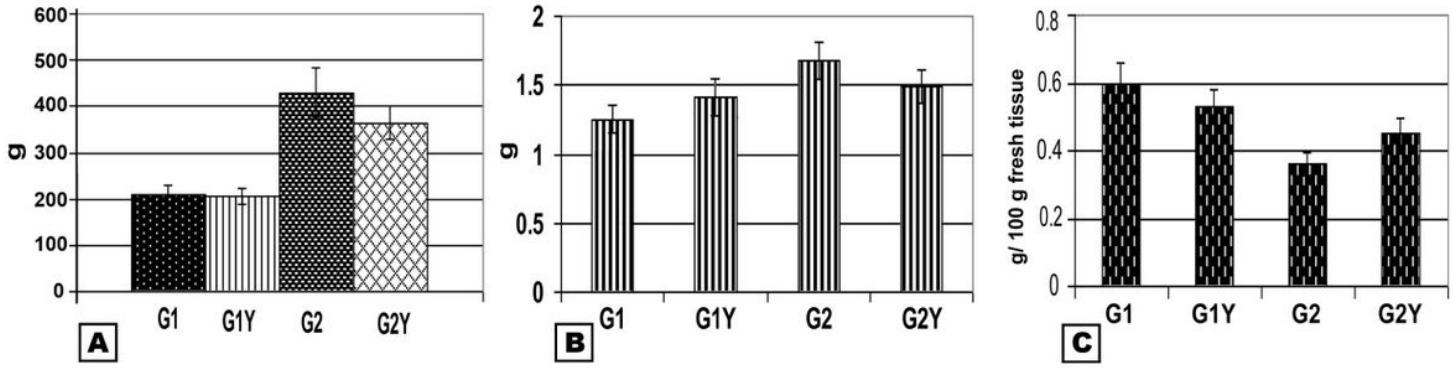

Figure 2

Mean body weight (A) and absolute (B) and relative brain weight (C) of aging rat with or without whey supplementation. Data represent the mean ± SD (n = 5). *Significant at $p < 0.05$.

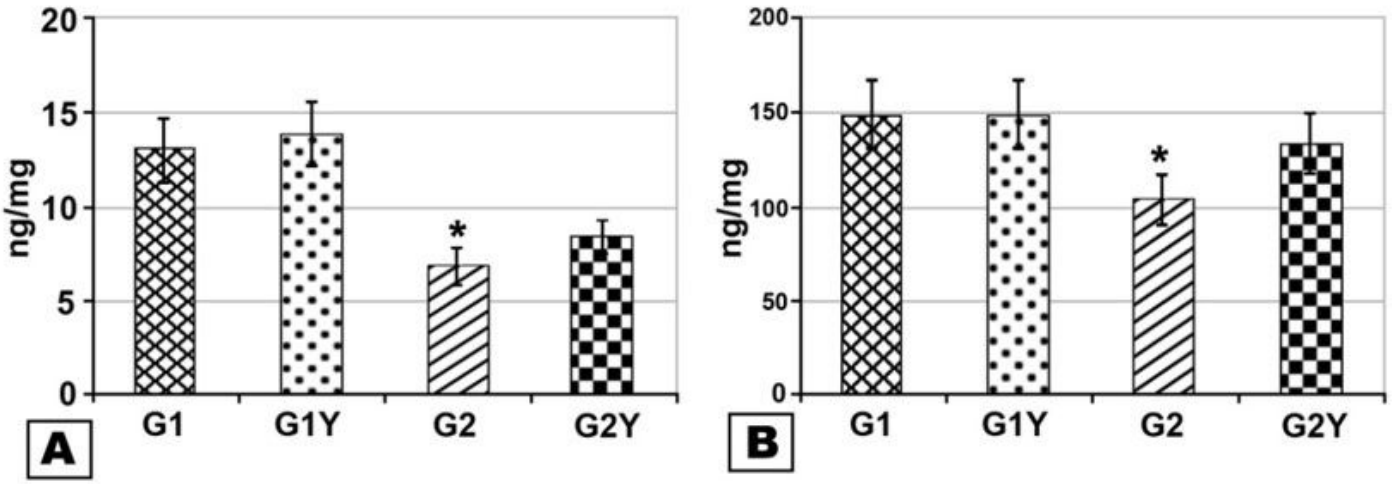

Figure 3

Superoxide dismutase (SOD, A) activity and malondialdhyde (MDA, B) content of aging brain compared to young age with or without whey supplementation. Data represent the mean ± SD (n = 5); *Significant at $p < 0.05$.

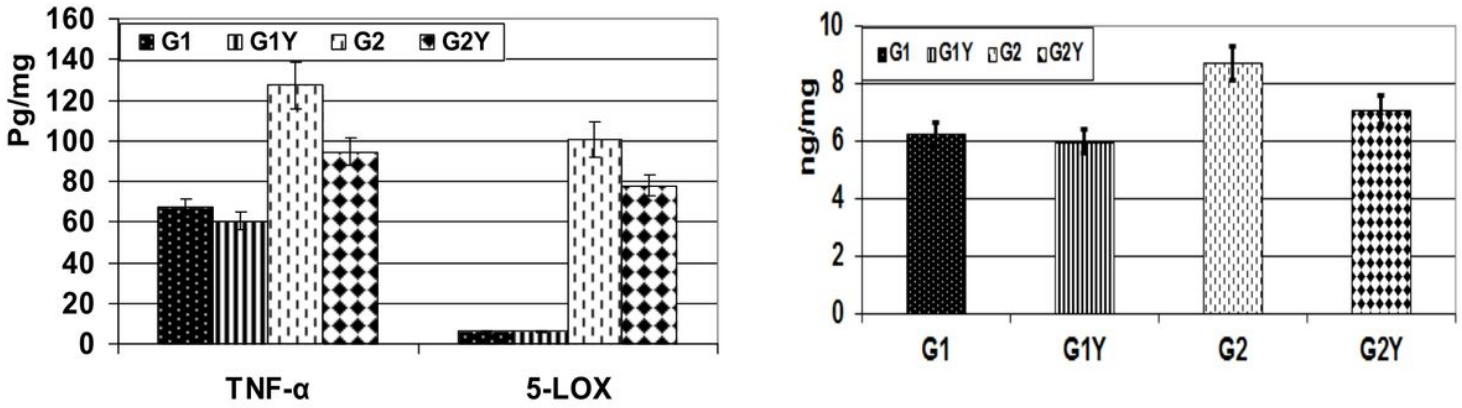

Figure 4

Brain dopamine (A) and serotonin (B) levels in aging rat compared to young age with or without whey supplementation. Data represent the mean ± SD (n = 5)); *Significant at $p < 0.05$.

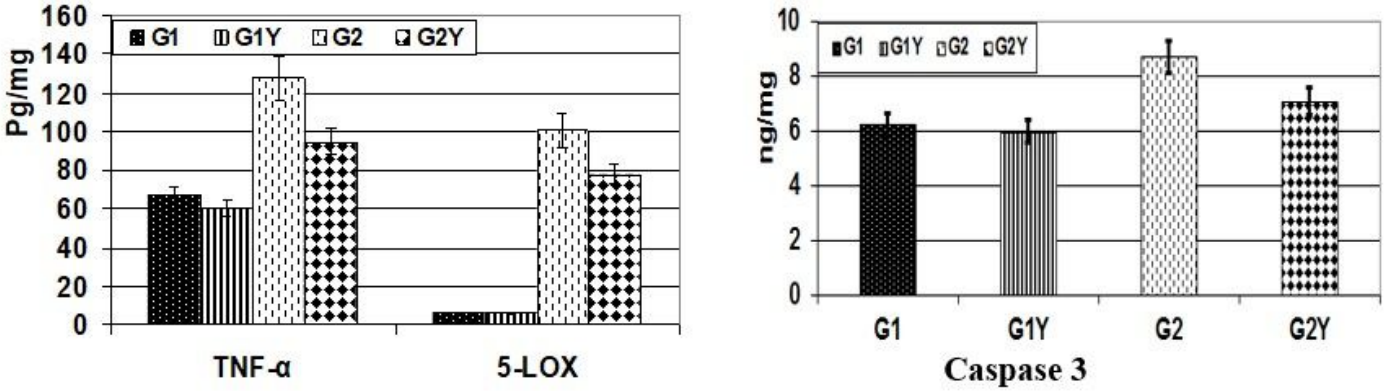

Figure 5

Biomarkers of TNF-α, 5-LOX and caspase 3 of brain of senile male rats with or without whey supplementation. Data were presented as mean ± SD (n = 8), *Significant at $p < .05$ compared to G1. Abbreviations; Cas-3, caspase 3; 8month old; G1Y, 8 month-old supplemented whey; G2, 30 month old; G2Y, 30 month old supplemented Whey; 5-LOX, 5-lpooxygenase; TNF-α, tumor necrosis factor—α.

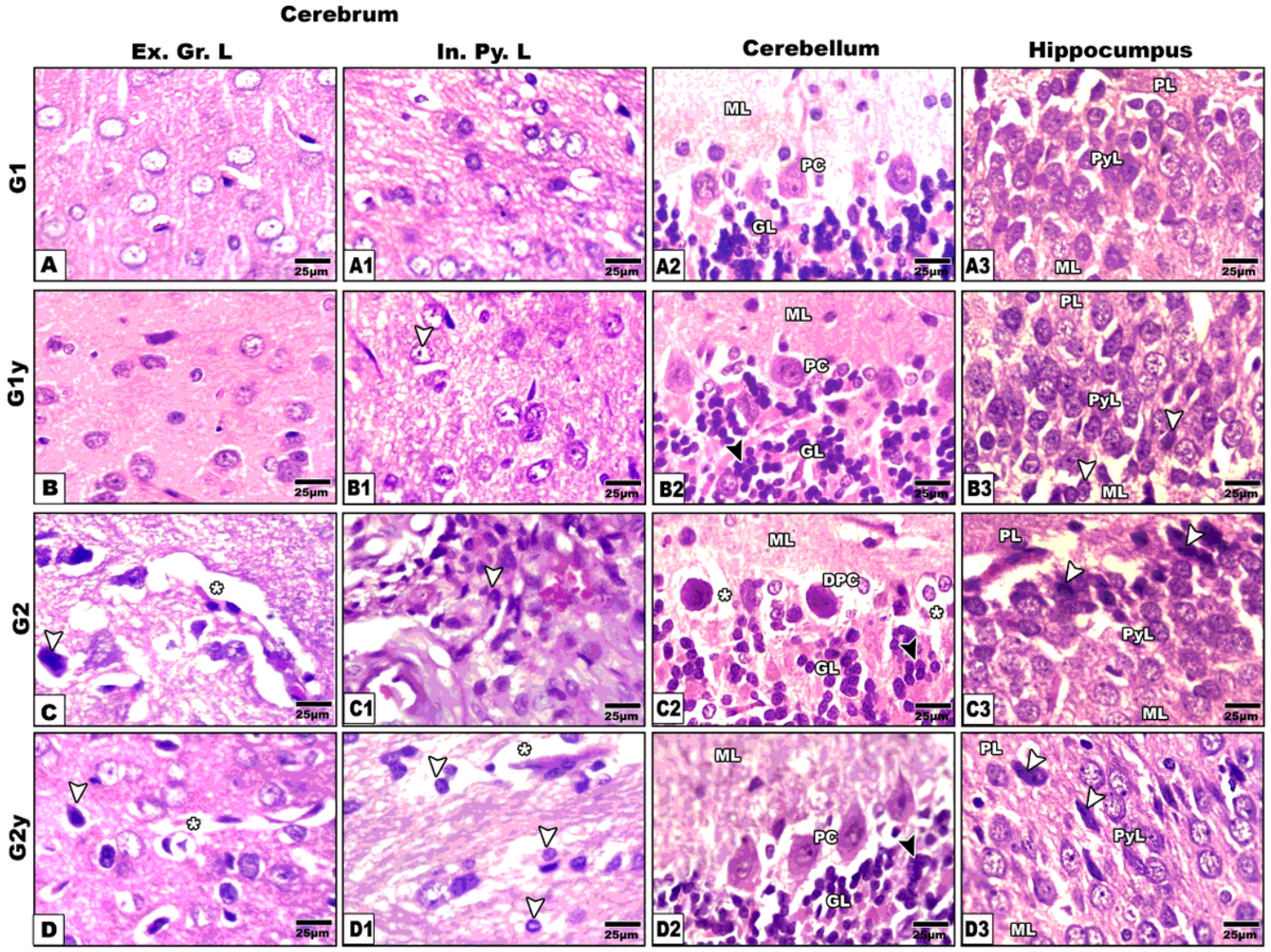

Figure 6

Photomicrographs of sagittal histological section of cerebrum (A- D & A1-D1), cerebellum (A2-D2) and hippocampus (A3-D3). A&A1. 8-month old cerebrum. B& B1. Whey supplemented 8-month old. Note normal cerebral neurons. C&C1. Cerebrum of old rat (G3) showing edematous lesions and fragile white matter (star) and dense aggregation of glial cells (arrow head) infiltrated in between degenerated neuron. D&D1. Whey supplemented old group showing less damaged neurons with vesicular nuclei. A2. Cerebellum of adult rats. B2. Whey supplemented cerebellum of adult rat. Note normal molecular, Purkinje and granular cells.C2. Cerebellum of old rat showing pyknotic of Purkinje cells with pyknotic nuclei and reduction of granular cells. D2.Cerebellum of old rats supplemented whey showing partial improvement of Purkinje cells and regenerated granular cells. A3. Hippocampus of adult rat. B3. Hippocampus of adult rat supplemented whey. Note normal pattern of pyramidal cells ((PyL) and outer polymorphic layer (PL). C3. Hippocampus of old rat showing vacuolar degeneration of pyramidal layer((PyL). D3. Old rats supplemented whey showing moderate improvement in pyramidal cells.

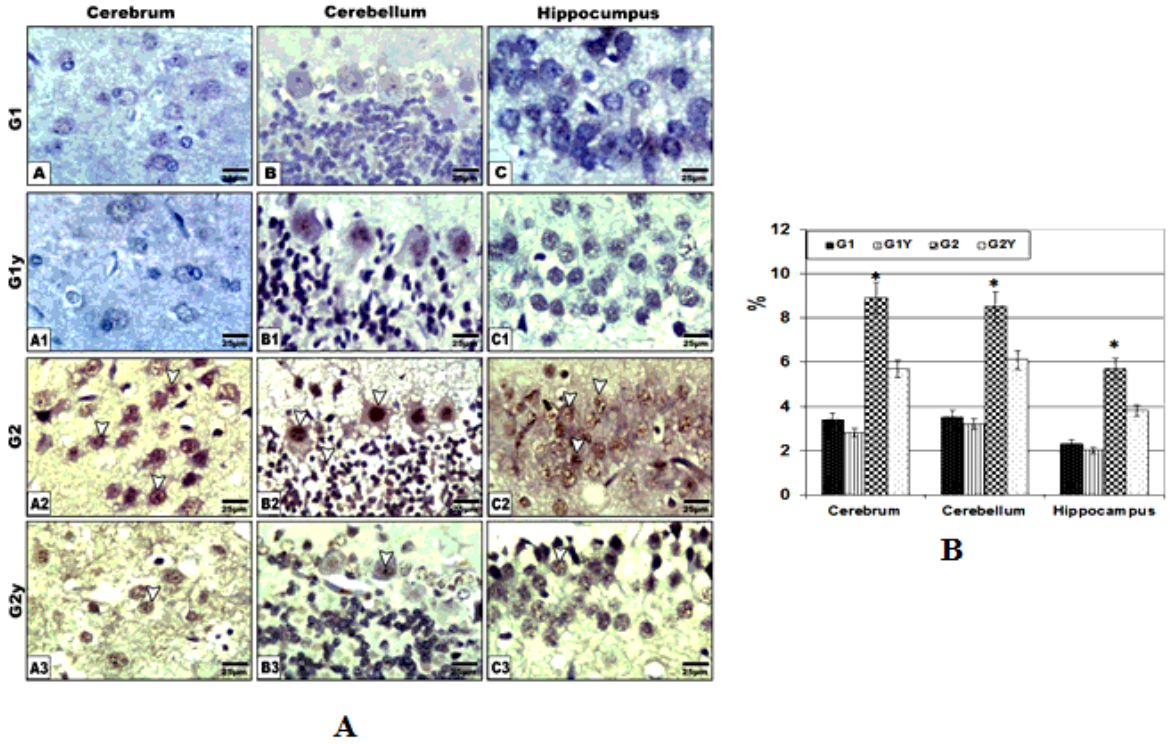

Figure 7

A. Photomicrographs of sagittal histological sections of formalin fixed immunostained with caspase 3 of cerebrum (A-A3), cerebellum (B-B3) and hippocampus (C-C3). A. Adult rat cerebrum. A1. Whey supplemented adult rat. Note negative immune reaction.A3. Old rat showing increased immune reaction in neuronal cells. A3.Whey supplemented old rat showing decreased immune reaction. B& B1. Cerebellum of adult and whey supplemented group showing negative immune reaction. B2. Cerebellum old rats showing dense immune reaction. B3. Old cerebellum supplemented whey showing decreased immune reaction. C. & C1. Adult hippocampus and whey supplemented group. C2. Hippocampus of old rat showing dense immune reaction. C3. Hippocampus of old rat supplemented whey showing decreased immune reaction.. B. Chart illustrating Image analysis of caspase 4 showing overexpression of caspase 3 in aging group compared to adult and aging group supplemented whey syrup. Each result represent the mean ± SD (n = 5); *Significant at $p < 0.05$. Abbreviations; G1, adult group; G1Y, adult group supplemented whey; G2, old rat; G2Y, old group supplemented whey.

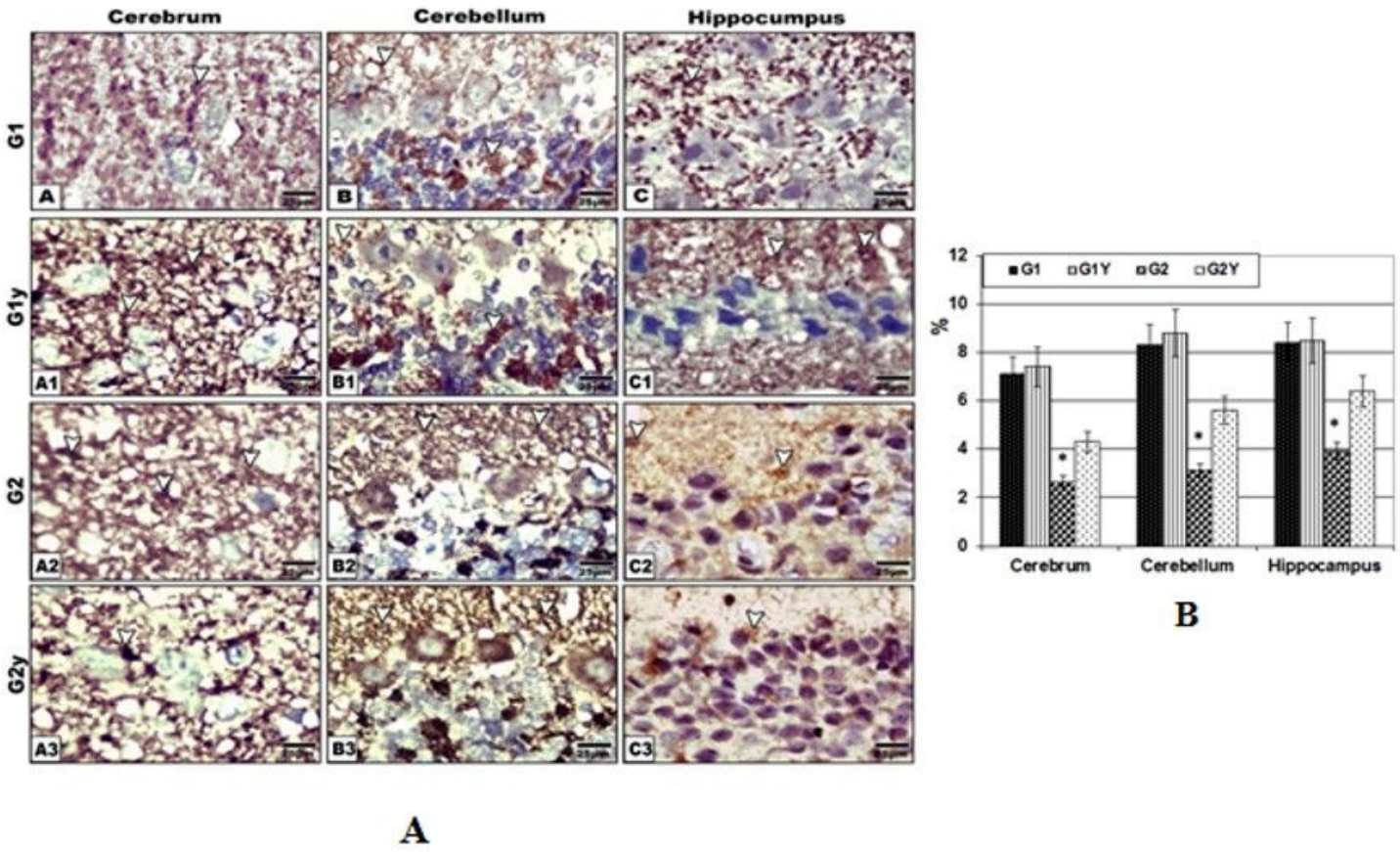

Figure 8

A. Photomicrographs of sagittal histological sections of formalin fixed immunostained with synaptophysin of cerebrum (A-A3), cerebellum (B-B3) and hippocampus (C-C3) of different ages of rats with or without whey supplementation. A &A1. Cerebrum of adult and whey supplemented group.A3. old cerebrum showing decreased immune reaction. B &B1. Adult and whey supplemented cerebellum. C2. Old cerebellum showing decreased immune reaction. C.&C1. Adult and whey supplemented hippocampus. C2. Old hippocampus showing decreased immune reaction. C3. Old hippocampus supplemented whey showing improvement. B. Chart illustrating Image analysis of synaptophysin showing decreased expression in aging group compared to adult and aging group supplemented whey syrup. Each result represent the mean ± SD (n = 5); *Significant at $p < 0.05$. Abbreviations; G1, adult group; G1Y, adult group supplemented whey; G2, old rat; G2Y, old group supplemented whey

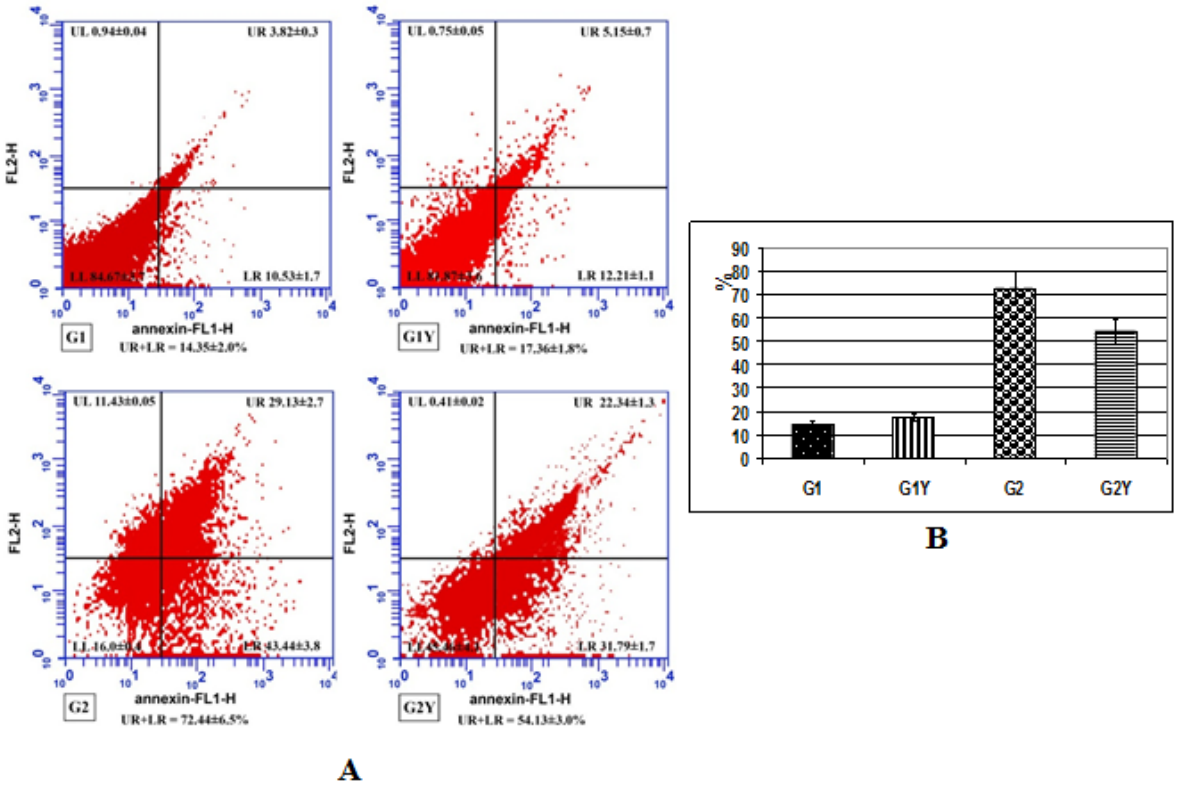

Figure 9

A. Flow cytometry analysis of annexin-v of brain cells of aging rats with or without whey supplementation. From the chart, UL & LL showing negative annexin-v and negative propidium iodide (PI), indicating viable cells. UR showing positive annexin-v and positive propidium iodide (PI), indicating early apoptosis. LR showing negative annexin-v and positive propidium iodide (PI), indicating necrotic cells. UR plus LR, illustrating apoptosis. B. Chart illustrating the apoptotic rate in brain of old rat and percent of improvement post whey supplementation. Abbreviations: G1, young rat; G1Y, young rat supplemented whey; G2, old rat; G2Y, old rat supplemented whey. Data represent as the mean ± SD (n = 5); UR + LR in G2 is Significant at $p < 0.05$.

Supplementary Files

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- GraphicAbstractFinal.pdf