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Anti-Aging Effect of Apigenin Through Modulating Mitochondrial Dysfunction, Cellular Senescence, and miR-34a in D-Galactose Induced Brain Aging in Mice

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ABSTRACT

Background: Brain aging is gradual deterioration of brain functions, including cognitive impairment. Apigenin is a natural anti-oxidant. **Objective:** This study aims to evaluate the anti-aging impact of apigenin on D-galactose-induced brain aging via modulating mitochondrial dysfunction, cellular senescence, redox status and miR-34a.

Materials and methods: Thirty mice were randomly allocated into control group (0.9% saline + dimethyl sulfoxide orally), D-galactose group, D-galactose/apigenin group. D-galactose was subcutaneously injected (150 mg/kg/day). Apigenin was given orally (20 mg/kg/day). All chemicals were administrated for eight weeks. Object location recognition (OLR) test was conducted. Senescence associated β-galactosidase (SA-β-gal) enzyme activity, gene expression, and immunohistochemical expression levels of P16, the super oxide dismutase (SOD) activity, reactive oxygen species (ROS), and malondialdehyde (MDA) levels, gene expression levels of miR-34a, and mitochondrial dynamics markers mitofusin -2 (MFN-2) and fission 1 (Fis-1) were assayed.

Results: The OLR test showed impaired memory in D-galactose group, which was ameliorated by apigenin. D-galactose group showed decreased Fis-1 levels, and SOD activity, and increased activity of SA-β-gal, levels of MFN-2, ROS, MDA, and gene expression of miR-34a and p16. Apigenin administration helped in maintaining mitochondrial homeostasis, increased SOD activities, reduced MDA, ROS levels, SA-β-gal activities and expression levels of miR-34a and p16 in D-galactose/apigenin group. **Conclusion:** Collectively, apigenin might have anti-aging effect through modulating mitochondrial dysfunction, cellular senescence, redox status, and miR-34a expression.

Keywords: Brain aging, Apigenin, Senescence, Mitochondrial dysfunction, miR-34a.

INTRODUCTION

Aging is a progressive decline of body functions over time, which can be coupled with cognitive impairment. Aging predisposes to various age-related diseases [1]. Cellular senescence, which is a process of cell cycle arrest to prevent damaged cells proliferation [2], plays key role in driving aging [1], as senescent cells release pathogenically active molecules, which spread chronic inflammation in nearby tissue, and promote age-related diseases. This state is called inflammaging [2].

One promising biomarker for cellular senescence is the rise in the activity of senescence associated β-galactosidase (SA-β-gal), which catalyzes the hydrolysis of terminal β-linked galactose residues in glycolipids, glycoproteins, and proteoglycans [3] . P16 is a cell cycle inhibitor, whose upregulation induces cellular senescence [2] . Cellular senescence is categorized into replicative senescence linked to progressive shortening of telomere and stress-induced senescence, which can be provoked by mitochondrial dysfunction and oxidative stress [4] .

Mitofusin-2 (MFN-2) is an enzyme, localized at the outer mitochondrial membrane, and mediates the mitochondrial fusion process [5] . Fission 1 (Fis-1) is an anchored protein in the outer mitochondrial membrane, that causes mitochondrial fragmentation via inhibiting MFN1/2 [6]. Abnormal mitochondrial dynamics are involved in neurodegenerative diseases [4].

The free radical theory assumes that increased age leads to increase in free radicals, which causes cellular functional impairment [7]. Malondialdehyde (MDA) is a breakdown metabolite of peroxidation of polyunsaturated fatty acids [8]. Superoxide dismutase (SOD) detoxifies the superoxide radicals into hydrogen peroxide and oxygen; hence it plays anti-oxidant defense mechanism [9] .

MiR-34a is involved in senescence and inflammaging, all of which are strongly linked to a number of aging-related disorders. Thus, in the treatment of aging-related diseases, miRNA-34a may represent interesting therapeutic target for pharmacological interventions[10] .

Apigenin is a natural flavonoid, present in onions, and parsley and olives, with potent antioxidant, and anti-inflammatory properties. However, despite the fact that apigenin has been demonstrated to ameliorate aging associated cognitive impairment[11], the underlying mechanisms, including the impact of apigenin on

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important factors that contribute to brain aging, have not been thoroughly explored. The current study intends to assess the anti-aging effect of apigenin on brain aging caused by D-galactose (D-gal) by modifying cellular senescence, mitochondrial dysfunction, redox state, and emphasizing its impact on miR-34a as innovative therapeutic target for brain aging.

MATERIALS AND METHODS

Study design:

Thirty male mice, of 8 weeks old and matched weight, were kept in well-ventilated cages at 23 ± 2 °C, and a 12 h dark and light cycle. Mice with preexisting disease were excluded. Free access to water was allowed as well as standard laboratory rodent chow contained approximately 21% crude protein, 60% carbohydrates, 5% crude fat, 3% crude fiber, and 1% vitamins and minerals, purchased from El-Nasr Pharmaceutical Chemicals Co. (New Maadi, Cairo, Egypt).

Following acclimatization for one week, mice were randomly allocated into three groups (each group has 10 mice): control group, D-gal group, and D-gal/apigenin group as follows: The control group received an oral gavage of 0.9% saline (containing 0.5% dimethyl sulfoxide (DMSO)) once a day for 8 weeks. The other two groups received subcutaneous injections of D - Galactose (CAT# G0750, from Sigma, St. Louis, MO, USA), in the back, at the dose 150 mg/kg/day, after being dissolved in normal saline, for 8 weeks [12]. In the meantime, D-gal/apigenin group received apigenin (CAT #10798, Sigma, St. Louis, MO, USA) after being dissolved in DMSO at a dose of 20 mg/kg orally once daily, for 8 weeks [11].

Object location recognition test (OLT):

This test evaluates spatial learning, and was carried out as described in literature at the end of drug administration period [13]. The test includes habituation, training, and test phases. In habituation session, mice explored empty box (50 X 40 X 50 cm) for 10 min, along three consecutive days, for acclimatization.

Followed by the training phase, then the test phase with an interval of 30 min in between. At training and test phases, mice explored two similar small, plastic objects, placed in the box. The two objects differ only in color. In the test phase, an object was maintained in the same place (familiar). However, the other one was displaced (novel).

70% ethanol was used to clean objects and box, to avoid odor clue, between each trial. The exploratory behavior of mice was recorded by video camera, and it was only considered if the mice were touching or sniffing the objects. Discrimination index (DI)= TN − TF/TN + TF, in which time for novel object exploration was referred to as (TN), and the time for familiar object exploration was referred to as (TF).

Blood sampling and tissue homogenate preparation:

Mice were anaesthetized and euthanized. Blood samples were collected and left to clot, then centrifuged at 3000 rpm for 15 min. Subsequently, the sera were collected and frozen at -20 °C for subsequent analysis.

Brain tissue and hippocampus were isolated and cut into pieces, for histopathological examination (after being fixed in 10% formalin), tissue homogenization, mitochondrial isolation and gene expression (after being frozen at -80 °C).

For tissue homogenization, the tissue pieces were weighed then homogenized in ice chilled phosphate buffer saline (PH 7.2-7.4) [7] . This was followed by centrifugation at 4°C, for 20 min (2000-3000 rpm). Resulting supernatant was kept frozen for further biochemical analysis. Total protein content of tissue homogenate was detected by Lowry method [14] .

For mitochondrial isolation, portions of brain tissues were homogenized in a mitochondrial buffer (0.8% NaCl, 0.01 M Tris-HCl, 0.01 M sucrose, 0.0001 M EDTA-2Na) with a pH of 7.4 [15]. 2 mL of the homogenizing buffer were added to 100 mg of brain tissue. Then, for 10 min, the mixture was centrifuged at 1500 rpm, followed by centrifugation at 10,000 rpm for 15 min [16]. Lowry method was utilized to estimate the protein content in mitochondrial pellet [14].

Biochemical analysis:

Colorimetric evaluation of SA-β-galactosidase activity in hippocampus tissue:

An aliquot of hippocampal tissue homogenate (20 µl) was added to ortho-nitrophenyl- β-galactoside (ONPG) buffer (150 µl) (CAT # 34055). The ONPG buffer was made by dissolving 1 mM $MgCl_2$, 40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 10 mM KCl, 4 mg/ml ONPG, and 0.4% β-mercaptoethanol (pH 6) (Acros Organics, New Jersey, USA). This was followed by incubation at 37°C till the appearance of a pale-yellow color. 60 µl of 1 M Na_2CO_3 were added as stop solution, followed by determining the optical density (OD) at 420 nm. SA-β-galactosidase activity was measured in units per milligram protein at 37 °C [17]. Calculation was as follows [18]:

1) nmoles / milliliter= OD_{420}/0.0045
2) To get the total concentration in nmoles, the total assay volume (ONPG buffer, lysate, and stop solution) was multiplied by the concentration in nmoles/milliliter
3) Total concentration in nmoles/incubation time = nmoles/min (units)
4) Units/ lysate used in the assay (µl)/concentration of protein in mg of 1 µl lysate = Units/mg protein.

Assay of Liver and kidney function Markers:

For assessment of the liver functions, enzyme activities of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined at 340 nm in the serum of all experimental groups using kits from Diamond diagnostics company,Cairo, Egypt, in accordance with the manufacturer's instructions.

For renal functions assay, serum creatinine (Cr) and, blood urea nitrogen (BUN) levels were measured using commercial kits (MyBiosource, San Diego, CA, USA, Cat# MBS3807501 and MBS751125 respectively).

Assay of redox status parameters in brain tissue:

Malondialdehyde (MDA) levels were evaluated by the **Ohkawa *et al.*** method as nmol/mg protein [19] . Additionally, SOD activity and reactive oxygen species (ROS) were estimated using commercial kits (BioDiagnostic, Egypt, CAT# SD 2521 and MyBiosource, San Diego, CA, USA, Cat# MBS9718216 respectively). SOD activity was expressed in U/mg protein and ROS were expressed as nmol/mg protein.

Assay of inflammatory Biomarkers:

The serum levels of interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) were determined by kits from (MyBiosource, San Diego, CA, USA, Cat# MBS730957 and MBS825075 respectively) following the manufacturer's instructions.

Quantitative real time PCR (qRT)-PCR of miR-34a in brain tissue and P16 in hippocampal tissue:

Extraction of total cellular RNA from brain and hippocampal tissue samples was conducted using TRIZOL (CAT#15596026, Invitrogen Inc., USA). NanoDrop Spectrophotometer (Implen, USA) was used to assess the concentration and purity of extracted RNA.The purity was qualified if the OD_{260}/OD_{280} was between 1.8 and 2.0.

cDNA synthesis was carried out using Revert Aid H Minus Reverse Transcriptase kit from Thermo Scientific, Fermentas, CAT# EP0451. According to the manufacturer instructions, the qRT-PCR was carried out by real-time thermal cycler using specific primers for the gene of the isolated cDNA and 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific, USA, CAT# K0221).

The relative expression of miR-34a was analyzed with normalization against U6 small nuclear RNA. However, relative expression of P16 was analyzed with normalization against GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as a control using the $2^{-\Delta\Delta Ct}$ method [20] . The used primer sequences are listed in table 1.

Table 1: Primer pairs for RT-qPCR

Gene	Nucleotide accession No.	Primers	Amplicon size
MIR-34a ID: 262206221	NR_029610.1	Forward: 5′- TGGCAGTGTCTTAGCTGGTT-3′ Reverse: 5′ - GCAGCACTTCTAGGGCAGTA-3′	75
U6 ID: 612339293	NM_020810.3	Forward: 5′- CTCGCTTCGGCAGCACA-3′ Reverse: 5′- AACGCTTCACGAATTTGCGT-3′	96
P16 ID: 89355818	DQ406745.1	Forward: 5′- GCCAGTCCTCAAAGCGGATA-3′ Reverse: 5′- AGGGAATATGAGTGCCCCCT-3′	140
GAPDH ID: 1519316078	NM_002046.7	Forward: 5′- TTTTGCGTCGCCAGCC-3′ Reverse:5′ Reverse: 5′- ATGGAATTTGCCATGGGTGGA-3′	208

Immunoassay of mitochondrial dynamics parameters:

The obtained mitochondrial fraction was used for estimation of (MFN-2), and (Fis-1) (CAT# MBS9717649, MBS8820358 respectively) levels using the kits (MyBiosource, Inc., San Diego, USA), with high compliance to the instructions of the manufacturers. At the optical density (450 nm), the color density was estimated by the microplate reader (Stat Fax 2100, New York, USA).

Histological and immunohistochemical assessments:

Alcohol and xylene were used to dry the brain tissue to form paraffin blocks. Five-millimeter-thick slices were made in series using a rotary microtome. The pieces were then hydrated, deparaffinized, immersed in hematoxylin and counterstained with eosin. These slides were prepared for examination with light microscope using hematoxylin and eosin stains (H&E) [7] .

For P16 reactivity assay, paraffin-embedded sections of brain tissue were immunostained . The paraffin blocks of brain tissues were heated in a 60 °C for 30 min, deparaffinized, and hydrated using a rabbit monoclonal antibody that is specific for P16 in a dilution: 1:150; pH 7.8 is optimal. Morphometric study was performed by measuring the thickness of the pyramidal cell layer in cornu ammonis 1 and 3 (CA1, CA3) and the granular cell layer in the dentate gyrus by using an image analysis tool. Five non-overlapping random fields from each selected area were taken, photographed, and the means obtained. The sections immunohistochemically labelled for P16 were used to determine the mean area percentage in regions of the hippocampus (400). Image J software (Media Cybernetics) was used for quantification of the morphometric study.

Ethical approval: The current study was performed, according to the recommendations of the Ethical Committee of Medical Research at Tanta Faculty of Medicine with approval code 36264PR240/6/23. The protocols established by the National Institutes of Health (NIH) of the United States in regard to the care and laboratory animals' utilization, were followed.

Analysis of statistical data:

The obtained findings were demonstrated as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey's post hoc test were used for data analysis, using SPSS, version 23.0 (Armonk, NY, USA). P value was deemed significant, if it was less than 0.05.

RESULTS

Effect of apigenin on object location recognition test (OLT): The current study evaluated spatial learning, through object location recognition test and revealed that D-galactose resulted in a significant decrement of the discrimination index (DI) in the D-gal treated group, whereas, apigenin treatment significantly reversed DI ,as presented in table (2).

Effect of apigenin on SA- β- galactosidase activity:

D-galactose administration resulted in a significant increase in the SA-β-gal activity in the D-gal group. However, apigenin administration significantly restored the SA-β-gal activity, as presented in table (2).

Effect of apigenin treatment on brain redox status:

D-gal administration for 8 weeks caused significant accumulation of MDA levels and concomitant significant decrease of SOD activity, relative to control group. On the other hand, apigenin effectively ameliorated the redox imbalance through restoring SOD activity and reducing the MDA levels, thus documenting potential antioxidant role of apigenin in brain aging. This redox imbalance was associated with significant increase in ROS production which was significantly improved after apigenin administration, as presented in table (2).

Effect of apigenin on mitochondrial dysfunction markers:

The current study assessed the role of apigenin in ameliorating the aberrant mitochondrial dysfunction induced by D-gal administration. Both mitochondrial fusion proteins represented in MFN-2, paralleled with mitochondrial fission protein Fis-1 were measured. As expected, D-galactose treated group revealed a significant increase of MFN-2 levels, paralleled with significant reduction of Fis-1 levels. Meanwhile, apigenin treatment for 8 weeks significantly improved mitochondrial dysfunction, illustrating the effective role of apigenin in modulating mitochondrial dysfunction associated with brain aging in mice. These data are presented in table (2).

Effect of apigenin on inflammatory markers:

When compared to the control group, the D-gal-treated group showed significantly elevated serum IL-6, and TNFα. However, administration of D-gal with apigenin resulted in their significant reduction. This demonstrated the potential anti-inflammatory role of apigenin on D-gal-induced brain aging, as presented in table (2).

Effect of apigenin treatment on liver and kidney functions:

D-galactose administration for 8 weeks resulted in significantly elevated serum ALT, AST, Cr, BUN which

suggested hepatorenal dysfunction. Conversely, co-treatment with apigenin resulted in their significant reduction, demonstrating the protective hepatorenal effects of apigenin as shown in table (2).

Table 2: Comparison of object location recognition test results, cellular senescence, redox status, inflammatory markers and hepatorenal functions among studied groups

	Control group N=10	D-galactose group N=10	D-galactose/apigenin group N=10	P
Object location recognition test (DI)	0.45 ± 0.1	-0.34[a] ± 0.08	0.32 [ab]± 0.08	< 0.05*
SA-β - galactosidase activity (U/mg protein)	0.0059 ± 0.001	0.027[a] ± 0.001	0.018 [ab]± 0.001	< 0.001*
SOD levels (U/mg protein)	198.81 ± 13.81	150.40 [a]± 28.43	176.7[ab]± 9.73	< 0.05*
MDA levels (nmol/ mg protein)	6.89 ± 0.147	10.92[a] ± 0.4	8.64[ab] ± 0.51	< 0.001*
ROS levels (nmol/mg protein)	5.247 ±0.77	10.6[a] ±2.35	7.79[ab] ±1.1	< 0.001*
MFN-2 level (ng/ mg protein)	424 ± 67.58	791.6 [a]± 85.35	546.7[ab] ± 69.25	< 0.05*
Fis-1 level (ng/mg protein)	506.4 ± 53.7	232.7 [a]± 41.13	401.2[ab] ± 57.96	< 0.05*
ALT(U/L)	43.40 ± 0.69	124.9[a] ± 1.82	49.65[ab] ± 0.84	<0.001*
AST(U/L)	93.60 ± 0.73	374.1[a] ± 1.52	106.3[ab] ± 1.69	<0.001*
Cr (mg/dl)	0.64 ± 0.05	2.15[a] ± 0.16	0.92[ab] ± 0.09	<0.001*
BUN(mg/dl)	23.28 ± 0.88	60.09[a] ± 1.40	27.19[ab] ± 1.27	<0.001*
TNFα (pg/ml)	38.77 ± 0.60	86.70[a] ± 0.95	40.98[ab] ± 1.28	<0.001*
IL-6(pg/ml)	29.40 ± 0.11	91.26[a] ± 1.17	34.86[ab] ± 1.22	<0.001*

Data were expressed as Mean ± SD, a: Significant with Control, b: Significant with D-gal, P <0.05* is considered significant, p < 0.001* is highly significant. SA-β - galactosidase activity: Senescence associated β-galactosidase, SOD: super oxide dismutase, MDA: malondialdehyde, ROS: reactive oxygen species, MFN-2: Mitofusin-2 , Fis-1: fission 1, ALT: alanine aminotransferase, AST: aspartate aminotransferase, Cr: creatinine, BUN: blood urea nitrogen , TNFα: tumor necrosis factor alpha, IL-6: interleukin-6.

Effect of apigenin on relative gene expression of hippocampal P16 mRNA and miR-34a in brain tissue:

D-galactose administration for 8 weeks induced significant increment in both P16 and miR-34a genes expression in D-gal group. On the contrary, apigenin treatment significantly decreased their levels of expression compared to the D-gal group. Concomitantly, the lowest levels of expression were shown in the control group, as illustrated in figure (1).

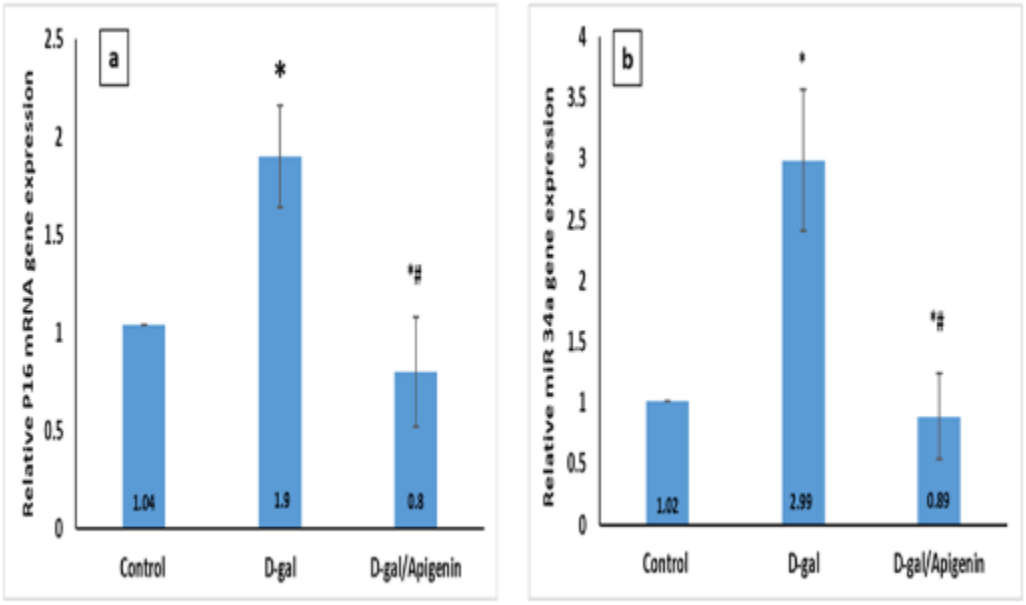

Figure (1): The apigenin effect on the relative gene expression of (a) P16 in hippocampal tissue and (b) miR-34a in brain tissue. Results are presented as mean ± SD. P was considered significant at < 0.05. *Significant changes versus control group, # Significant changes versus D-gal group. Concerning P16 and mir-34a, p was < 0.001.

Effect of apigenin on hippocampal histopathological changes:

Upon examination of the control group's hippocampus CA1 and CA3, three distinct layers were identified: the molecular layer (ML), the pyramidal layer (PL), and the polymorphic layer (POML). The pyramidal layer was composed of densely packed pyramidal cells, which are grouped in 4-5 rows for CA3 or 3-4 rows for CA1. These cells were characterized by large nucleoli and vesicular nuclei, and there was minimal basophilic cytoplasm in general. The POML and ML contained many glial cells and astrocytes, along with typical blood capillaries.

There are three distinct layers of the dentate gyrus (DG): molecular layer (ML), granule layer (GL), and polymorphic layer (POL). The granule layer (GL) was made up of circular to oval granule cell bodies. In the sub-granular zone (SGZ), there were tiny and intensely pigmented juvenile neuron nuclei. Glial cells and astrocytes with normal blood vessels could be detected. Pyramidal cells (P) and granule cells (G) of dentate gyrus appear with basophilic cytoplasm. In D-galactose group, the hippocampus showed hypocellularity in the dentate, CA1, and CA3 areas. The cells had a haphazard appearance and were loosely arranged, with several pyknotic cells and numerous red neurons. Furthermore, compared to the control group, the D-gal/apigenin group showed some restoration of normal structure in the hippocampus slices, with certain red neurons and pyknotic nuclei being maintained, as shown in figure (2).

The granular cell layer in the dentate gyrus and the pyramidal cell layer in CA1, and CA3 of D-galactose group exhibited a significant thickness decline in the earlier data, when compared to control group. Whereas, apigenin administration showed significant increase in thickness of the granular cell layer in the dentate gyrus and the pyramidal cell layer in CA1, and CA3 relative to D-galactose group and non-significant difference with control group, as illustrated in figure (3).

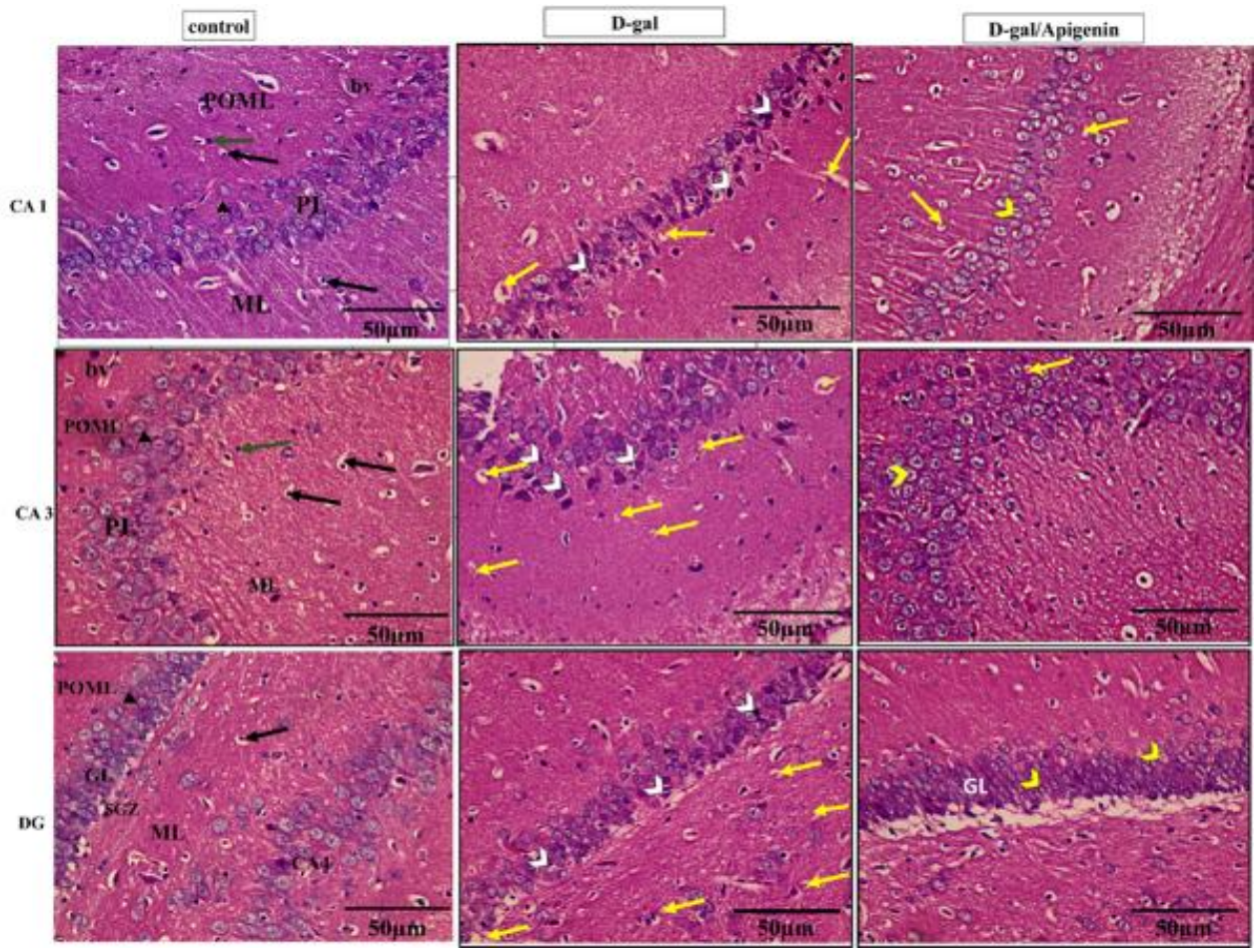

Figure (2): H&E-stained sections of hippocampus: control Group shows, cornu aminos (CA) CA1 and CA3, which exhibit three well-defined layers: polymorphic layer (POML), pyramidal layer (PL), and molecular layer (ML). The PL region is composed of tightly packed pyramidal cells (black arrow heads), that are arranged in either 4-5 rows in CA3 or 3-4 rows in CA1. These cells are easily identified by their prominent nucleoli, vesicular nuclei, and small amount of cytoplasm. In contrast, the POL and ML contain many glial cells (black arrows) and astrocytes (green arrows), as well as normal blood vessels (bv). The dentate gyrus (DG) is made up of three distinct layers: ML, granule layer (GL), and POL. The GL is characterized by the presence of circular to oval granule cell bodies. The sub-granular zone (SGZ), which is located below the GL, contains tiny and intensely pigmented juvenile neuron nuclei. Glial cells (black arrow) with normal blood vessels (bv) can be detected. granule cells (G) of the dentate gyrus appear with basophilic cytoplasm (arrow head). D-gal group's CA1, CA3, and dentate regions are hypocellular, with disorganized, loosely arranged dark stained cells (arrow heads) with pyknotic nuclei and multiple red neurons (yellow arrows) with pericellular halo.CA1, CA3, and dentate regions of D-gal/Apigenin group shows the same features as control group with scanty red neurons (yellow arrow) (H&E X400; scale bar = 50 µm).

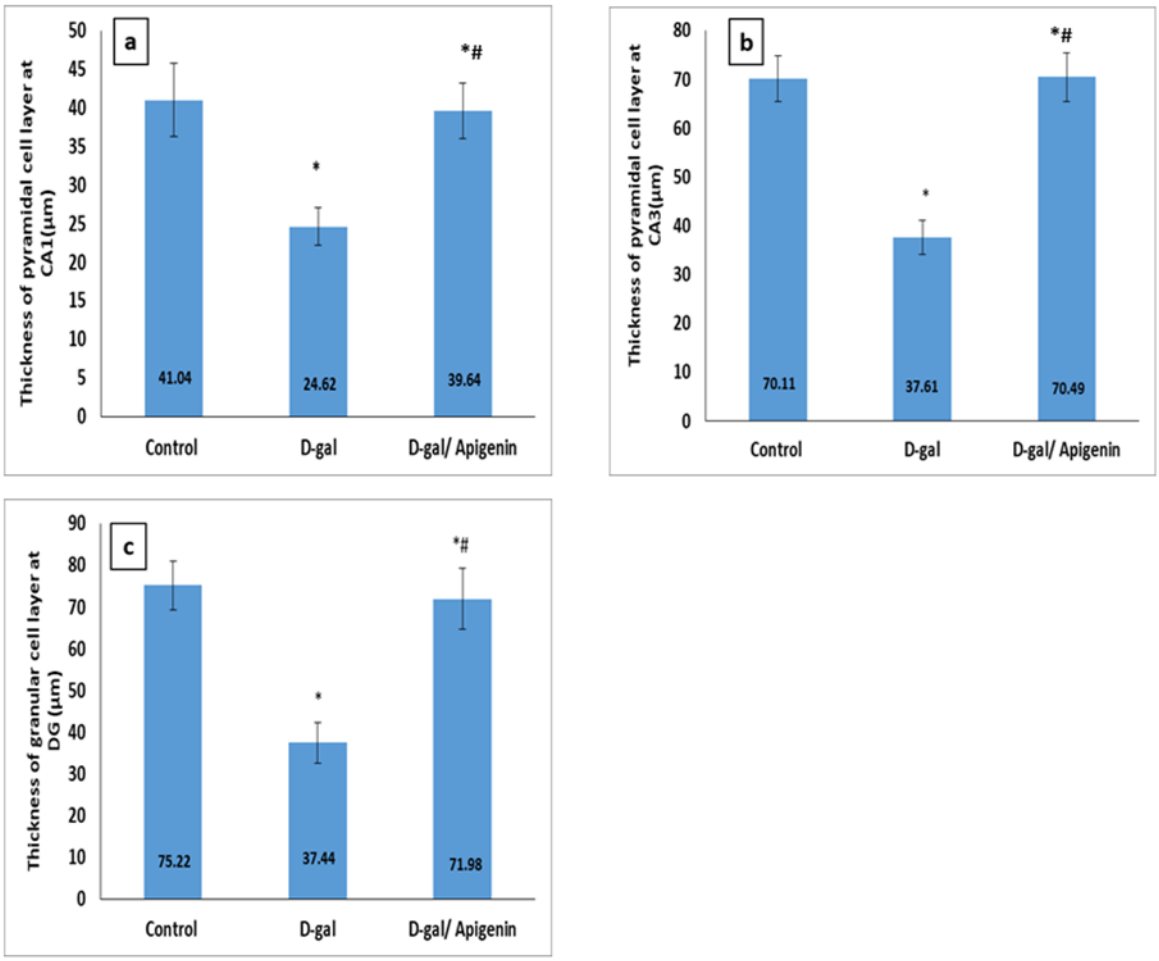

Figure (3): Graphical presentation of (a) Thickness of pyramidal cell layer at CA1, (b) Thickness of pyramidal cell layer at CA3, and (c)Thickness of granular cell layer at DG (µm): Results are represented as mean ± SD. P was considered significant at < 0.05. *Significant change vs. control group, #Significant change vs D-gal group. CA:Cornu Ammonis, DG: dentate gyrus.

Effect of apigenin treatment on hippocampal immunohistochemical expression of P16:

Examination of P16 immuno-stained sections from the hippocampal CA1, CA2, and dentate gyrus revealed a significant increment in the area percentage levels of P16 expression in D-gal group compared to the control group, whereas apigenin administration significantly decreased P16 level of expression, in D-gal/apigenin group relative to the D-gal group. Concomitantly, the lowest levels of area percentage expression were shown in the control group, as illustrated in figure (4).

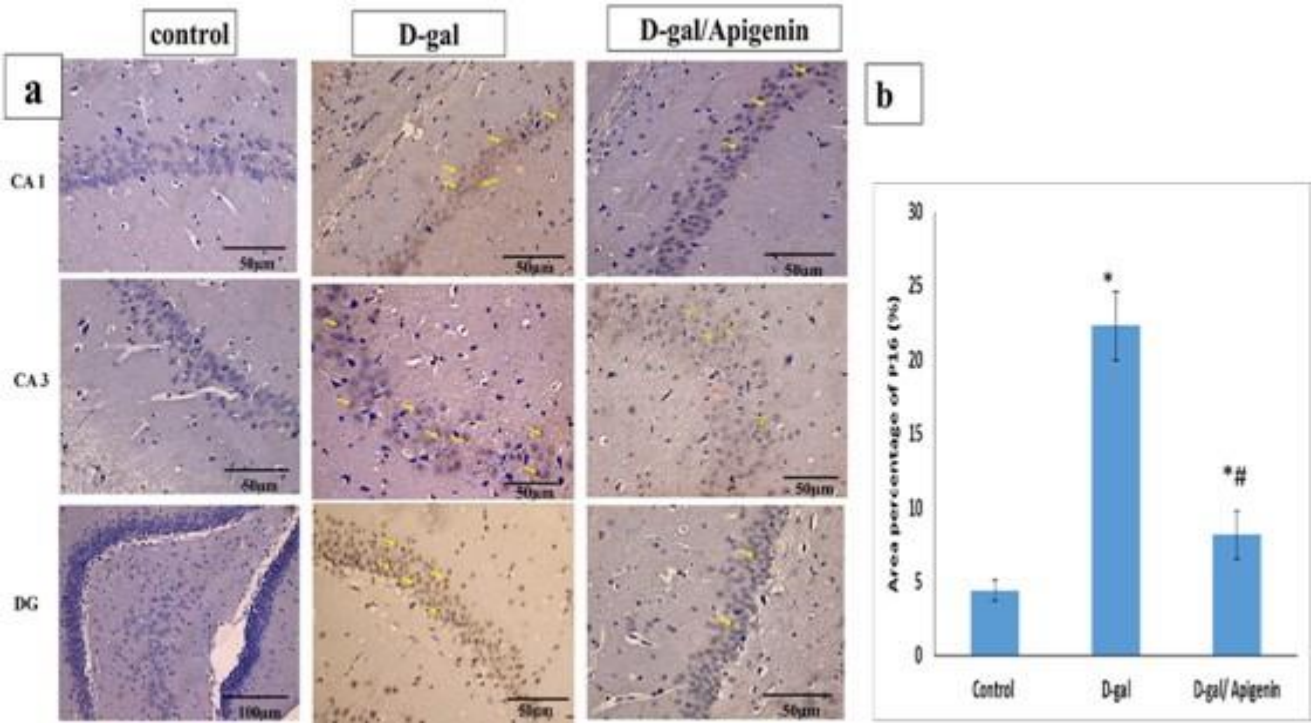

Figure (4): (a) A representative photomicrograph of hippocampus: CA1, CA3, and dentate regions stained with P16 immunostaining of the different groups. Control group shows negative P16 immunoreactions, while the pyramidal cells of CA1, CA3, and the dentate areas (marked with yellow arrows) in D-gal group shows brownish coloring in their cytoplasm and nuclei due to a positive P16 immunoreaction. In D-gal/apigenin group, there is a significant decline in the number of positive cells (yellow arrows). (P16-immunostaining ×200, scale bar = 100 μm and P16-immunostaining × 400, scale bar = 50 μm). (b) Graphical presentation of area percentage of P16 (%): Results are represented as mean ± standard deviation. P was considered significant at < 0.05. *Significant change versus. control group, # Significant change versus D-gal group.

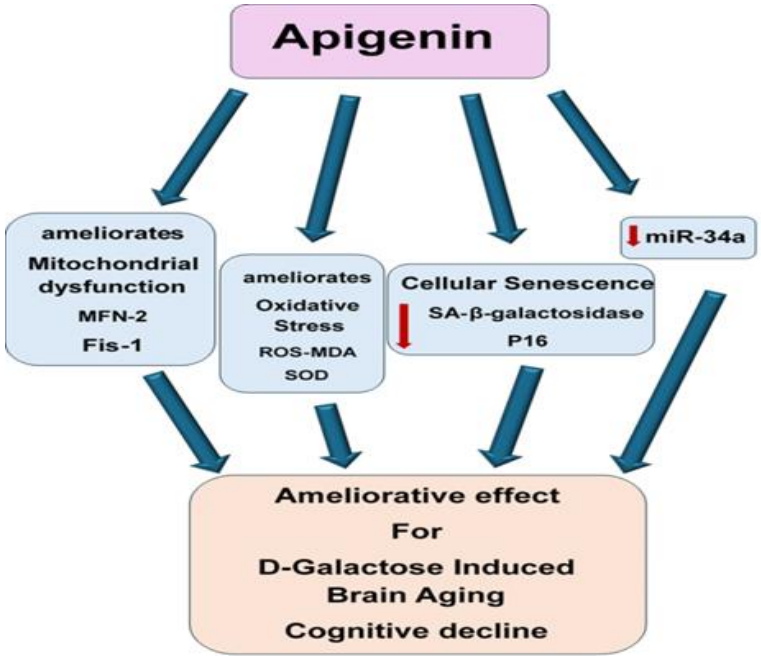

Figure (5): Proposed ameliorative role of apigenin.

DISCUSSION

Aging is a natural progressive deterioration of physiological functions of human body [1]. MicroRNA-34a mediates inflammaging [10]. Apigenin is a natural anti-oxidant compound [11]. The aim of the present study is to evaluate the anti-aging impact of apigenin on D-galactose-induced brain aging via modulating mitochondrial dysfunction, cellular senescence, redox status and miR-34a.

According to the current study, D-galactose administration for eight weeks, effectively induced aging, as the histopathological study of the hippocampus displayed structural alteration, as the granular cell layer in the dentate gyrus and the pyramidal cell layer in CA1, and CA3 of D-galactose group exhibited a significant thickness decline in D-galactose treated groups, when compared to control group. Apigenin significantly improved these histopathological alterations. This aligns with the findings of **Chesworth *et al.*** [21].

Moreover, galactose administration induced memory and learning deficit, which was significantly alleviated by apigenin, in the current study, as indicated by the object location recognition test results. This can be supported by the results of **Zhou *et al*.** and **Wang *et al.*** [22,23].

D-galactose in high concentrations, is converted by galactose oxidase into hydrogen peroxide and aldose, which triggers oxidative stress, mitochondrial dysfunction, and cognitive impairment [7]. High level of methylglyoxal in D-galactose induced aging model might be responsible for cellular senescence [24]. The D-gal group showed aggravated cellular senescence, as it exhibited a notable increase in SA-β-gal activities, as well as, marked rise in P16 gene expression and immunohistochemical expression levels. This is in harmony with **Hou *et al.*** and **Li *et al.*** findings [25,26].

The current research detected mitochondrial dysfunction in D-gal group, represented by significant decline in the level of mitochondrial fission-related protein Fis-1, and significant rise in the level of mitochondrial fusion protein MFN-2. These results can be supported by **Jeong *et al.*** findings [27].

Added to the mitochondrial dysfunction, the present study showed dysregulation of the redox status indicated by significant decrement of SOD activities and accumulation of MDA and ROS levels in D-gal treated group. This aligns with the results of **Sang *et al.*** [12]. The interplay between aging, cellular senescence, mitochondrial dysfunction, and oxidative stress can be explained by the theory of mitochondrial free radicals in senescence as it postulates that aging is accompanied by mitochondrial dysfunction, which results in increased ROS production, leading to further cellular and mitochondrial damage [7].

The current study revealed that the miR-34a expression showed significant upregulation in D-gal group. In the same context **Sarkar *et al.*** declared that upregulation of miR-34a promotes cognitive impairment [28]. **Li *et al.*** supposed that miR-34a provokes cellular senescence by shortening telomere length, repressing telomerase activity, and inducing DNA damage [29]. According to **Thounaojam *et al.***, miR-34a exacerbates mitochondrial dysfunction, cellular senescence, and decreases SOD activity in retina[30].

Apigenin is a natural flavonoid [11], which induced reduction in SA-β-gal activities and P16 levels, in the current research. According to **Li *et al***, apigenin modulates the SIRT1- NAD^+-cluster of differentiation 38 (CD38) axis, which in turn decreases SA-β-gal activity and P16 levels to ameliorate cellular senescence [31]. In addition, **Sang *et al.*** declared that apigenin affects the Nrf2 pathway to ameliorate cellular senescence [12].

Additionally, mitochondrial homeostasis was restored by apigenin administration, in the current study. Likewise, **Ahmedy *et al.*** outlined that apigenin has neuroprotective potential through maintaining mitochondrial homeostasis, activating sirtuin-3 and preserving NAD^+/ reduced nicotinamide adenine dinucleotide (NADH) ratio [32].

Notably, apigenin reversed the findings of SOD, ROS and MAD. This can reinforce the results of **Sang *et al.*** [12]. Apigenin has antioxidant impact through inhibiting oxidant enzymes, modulating redox signaling pathways as Nrf2, enhancing free radical scavenging, and reinforcing enzymatic and non-enzymatic antioxidants [11]. Apigenin pretreatment downregulates miR-34a expression in the current study. This could be corroborated by the findings of **Zhang *et al.*** in mouse peritoneal fibrosis [33]. Concerning the interplay between miR-34-a, senescence, and oxidative stress. MiR-34a acts as a regulator for cellular senescence. This was postulated by the studies of **Wan *et al.*** [34]. MiR-34a has triggering effect for oxidative stress in necrotizing enterocolitis, according to **Zhu *et al.*** [35].

Apigenin has anti-inflammatory effect with no organ toxicity at low dose. The present study displayed elevated levels of TNF-α and IL-6 in D-galactose group, which was diminuted with apigenin administration. According to **Li *et al.***, D-galactose activated inflammatory cells with subsequent release of TNF-α and IL-6, leading to renal injury[26]. According to **Allemailem *et al.***, apigenin attenuates serum IL-6 and TNF- α [11]. In the current study, D-galactose administration in large dose, results in significant renal and hepatic toxicity by increasing serum Cr, BUN, ALT and AST levels. This is in harmony with **Li *et al***. and **Ma *et al.*** findings [26,36]. Low dose administration of apigenin exerted protective effect. According to **Sahindokuyucu-Kocasari *et al.***, apigenin reduces renal and hepatic

toxicity by reducing oxidative stress, and inflammation[37].

CONCLUSION

In conclusion, mitochondrial dysfunction, cellular senescence, and oxidative stress are highly interconnected, and contribute in aging pathogenesis. Apigenin could exert ameliorative role through attenuating oxidative damage, mitochondrial dysfunction, cellular senescence and miR-34a expression. These results imply that apigenin might be a viable option for additional preclinical research on its impact on aging.

Strengths, limitations and recommendations:

This study demonstrates the interplay between senescence, mitochondrial dysfunction, oxidative stress, and miR-34a in brain aging. It evaluated the anti-aging impact of apigenin via targeting cellular senescence, mitochondrial dysfunction, redox status, and miR-34a. P16 gene expression was evaluated in hippocampal tissue, while miR-34a gene expression was assayed in brain tissue. Nonetheless, consistency and interpretability may be enhanced by examining both markers in the same area of the brain (such as the hippocampus). DMSO, which is used as solvent for apigenin, has anti-oxidant properties. Therefore, DMSO+saline control group at matching concentrations was included in the study to exclude solvent-induced effects. It is recommended to evaluate the effect of apigenin in further studies, while encapsulating it in nanoparticle systems, as this could enhance its bioavailability. Further research is recommended to assess how SIRT1, FOXO, or GSK-3β/Nrf2 as important aging factors could interact with senescence, oxidative stress, mitochondrial dysfunction, and mir-34a, which provides mechanistic insights.

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