Microvesicles Therapy with Melatonin in Targeting Mitochondrial Dysfunction in Alzheimer's Disease Model of Female Albino Rats: Histological and Biochemical Study

Original Article

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ABSTRACT

Background and Objectives: Alzheimer's disease (AD) is a progressive neurodegenerative disease that causes cognitive impairment.

The Aim of this Study: Was to evaluate microvesicles (MVs) and melatonin effects on mitochondrial biogenesis and apoptosis in an experimental model of Alzheimer's disease in rats

Methods: Forty-five female rats were divided randomly (9 rats in each group) intoGpI (control) and experimental groups, which included: GpII (AD), GpIII (AD+MVs), GpIV (AD+melatonin), GpV (AD+MVs+melatonin). Induction of AD was done by a single intraperitoneali.p injection of lipopolysaccharide (LPS) of 0.8mg/kg. One week after the AD induction, the treated groups received a single i.p injection of MVs at a dose of 0.2mg/kg or melatonin by i.p injection of 10mg/kg once daily for three weeks or combined therapy of both. When the experiment was over, Y-maze & open field tests were used to assess the cognitive functions. The hippocampus was subjected to histological and immunohistochemical studies for assessment of the morphological changes. Besides, biochemical investigations were performed for AD pathogenesis, mitochondrial biogenesis, inflammatory mediators, and apoptosis.

Results: The AD modeling group demonstrated impaired cognitive function, a significant increase in AD markers; Aβ plaques deposition in Congo-red stained sections and the levels of Aβ-42 and p-tau protein, increased apoptotic markers; Casp-3 positive immunostaining and Cyt-c level, a significant reduction in mitochondrial biogenesis markers; gene expressions of PGC-1α, Nrf-2, TFAM and Sirt-1, affection of the brain energy metabolism represented by the AMPK level. Besides, the IL-6 level increased significantly. The therapy with MVs, melatonin, or combined therapy had a curative role against AD with the best results in the combined therapy group.

Conclusion: Combined therapy with MVs and melatonin was superior to single therapy for AD.

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Key Words: Aβ- plaques; alzheimer's disease; casp-3; melatonin; microvesicles.

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INTRODUCTION

The most frequent neurodegenerative disease is Alzheimer's disease (AD). It is characterized by cortical and hippocampal shrinkage, enlargement of ventricles, and abundant evidence of intracellular tau neurofibrillary tangles andextracellular-amyloid (Aβ) plaques in the gross histopathology[1]. In Alzheimer's disease, tau protein is phosphorylated at several locations, causing tau to be removed from the microtubule and the microtubule to collapse, affecting cellular processes such as protein trafficking and overall cellular morphology[2]. Another study has demonstrated a crosslink between mitochondrial oxidative stress and the hyper-phosphorylation of tau protein.The efficient internal antioxidant defense system prevents tau hyper-phosphorylation and protects against AD neuropathology[3].

The degree of cognitive impairment in AD has also been linked to the extent of the mitochondrial accumulation of Aβ[4]. Aβ was found to impair mitochondrial functions, including the electron transport chain, reactive oxygen species (ROS) production, mitochondrial dynamics, and mitochondrial motility[5].

The mitochondrion is engaged in the pathogenesis of many neurodegenerative diseases due to its important role

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in regulating cell energy and metabolism. Mitochondrial defects, such as a lack of oxidative phosphorylation (OXPHOS), reactive oxygen species (ROS) increase, a decline in ATP output, and an imbalance in mitochondrial fission and fusion, are early events in AD patients' and animal models brain[6]. On the other hand, proteins implicated in Alzheimer's disease pathogenesis, such as amyloid precursor protein (APP) and Aβ, have been discovered to invade mitochondria and interrupt their activity[7].

Melatonin is a neurohormone secreted from the pineal gland and has a regulatory and neuroprotective role. Melatonin is a potent antioxidant and free radical scavenger that activates several antioxidative enzymes and increases mitochondrial energy metabolism. It has been reported that melatonin level is disturbed in AD, indicating its involvement in the pathophysiology of AD[8]. Melatonin has a remarkable role in governing the molecular events that occur during the development of AD disease and regulating stem cells that can repair brain damage and may help develop novel strategies for neurodegenerative disease therapy[9].

Melatonin is a known inducer of Silent Information Regulator Type 1 (SIRT-1)[10], a NAD+-dependent protein deacetylase that regulates the key targets via deacetylation and is a key player in several biological mechanisms such as inflammation, necrobiosis, and metabolism[11]. In neurodegenerative diseases, SIRT-1 activation plays a significant neuroprotective role,according to previous studies[12,13]. During transcription,SIRT-1 deacetylates histone and non-histone proteins as PGC-1α (peroxisome proliferator initiated receptor gamma-coactivator 1 alpha), which is thought to be a core regulator of mitochondrial biogenesis[14]. According to some studies,the pathway of SIRT-1/PGC-1αhas been shown to mediate the protective effects of SIRT-1[15].

Mitochondrial biogenesis is important for mitochondrial homeostasis to be maintainedto satisfy the physiological needs of eukaryotic cells. Mitochondrial biogenesis is regulated by multiple elements,such asnuclear respiratory factor-2 (Nrf-2), which controlsthe nuclear genes that code for mitochondrial proteins as well asmitochondrial transcription factor A (TFAM), which inducesmtDNAtranscription and replication[16]. PGC-1α regulates the expression of Nrf-2 and TFAM; thus,the affection of these markers contributes to reduced mitochondrial biogenesis observed in AD[17].

Microvesicles are small cell-derived membranous particles, and their content and surface markers reflect the cells they are originating from and the underlying pathology. Therefore, circulating endothelial microvesicles (EMVs) in differentbody fluids, such as blood, cerebrospinal fluid, and urine,function as accuratepathophysiological processes,such as neurodegenerative diseases, biomarkers[18]. Growing data have shown that mesenchymal stem cells, dendritic cells, and regulatory T cell-derived exosomes possess anti-inflammatory, anti-apoptotic, immunomodulatory, and pro-angiogenic properties[19]. They can cross the blood-brain barrier and deliver drugs and genetic elements to treat neurological disorders.They can act as potent Aβ scavengers suggesting their role in Aβ clearance in the central nervous system[20].

Regulating the status and state of MVs may be a 'Trojan- horse' approach to deliver drugs into the brain and treat neurodegenerative and other disorders[21].

The aim of this work was to study how MVs and melatonin, either separately or in combination, affect mitochondrial biogenesis and apoptosis in the AD rat model.

MATERIALS AND METHODS

A) The experimental design

In this study, 45 female albino Wistar rats of similar age and weight were used (6 months-one year &150-200 gm). Animals were inbred at Cairo University, Faculty of Medicine, Animal House. They had unrestricted access to water and ate a regular laboratory diet while being exposed to a 12:12 hour daylight/dark cycle. The experiment received ethical approval with the approval number (CU/III/F/5/18)from Cairo University Institutional Animal Care and Use Committee (IACUC).

Rats were randomly divided into control and experimental groupsas the following:

The control group (GpI): included nine rats that were equally subdivided into three subgroups (3 rats in each):

- Subgroup Ia: each rat received a single i.p. injection of 0.5ml phosphate buffer saline (PBS), the solvent of lipopolysaccharide (LPS) and MVs.
- Subgroup Ib: each rat received dailyi.p. injection of 0.5ml dimethyl sulfoxide, the solvent of melatonin, for three weeks.
- Subgroup Ic: each rat received a single i.p. injection of 0.5ml PBS and a daily i.p. injection of 0.5ml dimethyl sulfoxide for three weeks.

Experimental groups

Using a single i.p. injection of 0.8mg/kg LPS in 36 rats, the AD was induced[22].

The rats were separated into the following groups after one week (n=9):

GpII (AD-induced): the rats received no further treatment.

GpIII (MVs-treated): each rat received MVs as 0.2 mg/kg single MSC-MVs i.p injection[23].

GpIV (Melatonin-treated): each rat received melatonin by daily i.p injection of 10 mg/kg for 3 weeks[24].

GpV (MVs+Melatonin-treated): each rat received MVs and melatonin (with the same route and the same dose of each drug in GpIII and GpIV).

After four weeks from the start of the experiment, all rats were euthanized.

B) Isolation and identification of bone marrow-mesenchymal stem cells (BM-MSCs)

BM cells were flushed from the tibia of white albino rats with PBS.Nucleated cells were isolated with a density gradient Ficoll-Paque (Gibco-In*vitro*gen, Grand Island, NY) and suspended in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 0.5% penicillin and streptomycin, then incubated with 5% CO2 at 37°C till reaching 80-90% confluence within 7days. Using morphology and Florescent Analysis of Cell Sorting (FACS), cultured BM-MSCs were characterized by assessingthe positivity and negativity of CD90and CD45, respectively, which are specific to BM-MSCs.

C) MVs derived from BM-MSCs: preparation and identification

Microvesicles were extracted from BM-MSC supernatants cultured in RPMIovernight without Fetal Calf Serum (FCS). After centrifugationto remove debrisfor 20 minutes at 10,000 g, cell-free supernatants were centrifuged at 100,000 g (Beckman Coulter Optima L-90K ultracentrifuge) at 4° C for one hour, washed in serum-free medium 199 containing N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) 25 mM (Sigma) and, under the same conditions, applied to the second ultracentrifugation[25]. The MVs were detected using transmission electron microscopy (TEM) on purified MVs[26] (Figure 1) as well as a polymerase chain reaction (PCR) study of integrin gene expression.For the integrin gene, we used the following oligonucleotide primers: forward, 5'-AATGTTTCAGTGCAGAGC-3'; reverse, 5'-TTGGGATGATGTCGGGAC-3' (accession to gene bank number: NM017022.2).

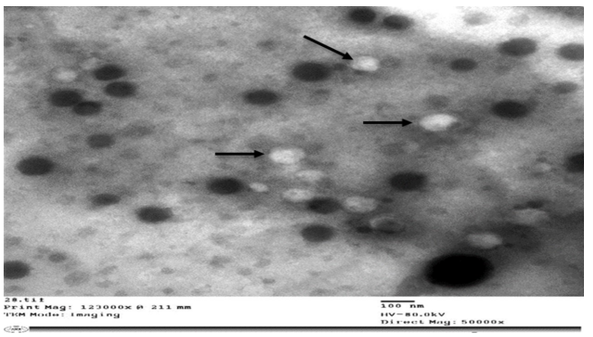

Fig. 1: TEM Ultra-magnification (50000x) for purified MVs showing their spheroid morphology (arrows)and confirming their size.

D) Assessment of the rats' cognitive functions

Open Field Test

It is the easiest and most cost-effective approach for evaluating rodent locomotion, exploration, and anxiety. Usually, the number of line crosses and the frequency of rearing are reported. A lower degree of anxiety and increased locomotion and exploration were correlated with a high prevalence of these behaviors[27].

Y-Maze Test

The natural inclination of rats to select alternate arms during a Y-maze is known as spontaneous alternation in rats. It is considered a fast and relatively easy test of spatial working memory[28].

E) Biological sample collection

At the experiment end, rats were euthanized by anesthesia overdose using 90 mg/kg ketamine/15mg/kg xylazine i.p injection[29]. The scalp was reflected, and the sagittal suture was traced with dissecting blade to obtain the brain tissue.The harvested right brain hemispheres were subjected to histological assessment, and the left ones were subjected to biochemical investigations.

F) Histological Studies

I. Hippocampus preparation

For histological assessment, the harvested brain hemispheres were placed in Bouin'ssolution for 2 hours, then removed and placed in a new Bouin's solution for 24 hours. The brain was sectioned coronally to expose the hippocampus and processed into paraffin blocks. The specimens were dehydrated in ascending grade of alcohol (70%, 90%, and 100%), cleared in xylene to be embedded in paraffin.

II. Hematoxylin and Eosin (H&E) Staining

Using a microtome (Leica RM 2025, Germany), Serial sections (5-7 m thick) were placed on glass slides and rehydrated in descending alcohol grades before being stained with H&Eand examined at Histology Department, Faculty of Medicine, Cairo University[30].

III. Congo Red Staining

The sections were de-paraffinized, rehydrated in descending grades of alcohol then stained by Congo Red solution (0.2%) for 20 minutes. The sections were rinsed in distilled water, dehydrated, cleared in xylene, mounted, and covered with a glass slide. The Aβ plaques were observed and illustrated as darkly stained cytoplasmic red plaques[31].

IV. Immunohistochemical Staining

The paraffin-embedded hippocampal sections were dewaxed and rehydrated. Endogenous peroxidase was blocked for 15 minutes, followed by washing in PBS. Retrieval was done by microwave-heating for the tissue sectionsfor 20 min in 10mM citrate buffer (Neomarkers', Cat. # AP-9003), pH 6, followed by cooling for 20 min at room temperature. The sectionswith the primary antibodies were then incubated in the humified chamberduring the night at room temperature. Anti-cleaved caspase-3 (Casp-3) rabbit polyclonal antibody (NEO markers, Thermo Scientific Laboratories-USA, Cat. # RB-1197-R7) was used as in a dilution of (10µg/ml) as an apoptotic marker

indicator. One step mouse/rabbit poly-detector DAB HRP brown (Bio SB-USA, Cat. # BSB 0201) was used to visualize the antigen antibody-positive reaction. Mayer's hematoxylin was used for counterstaining of the nuclei. The Casp-3 positive cells showed brown cytoplasmic deposits[32].

G) Biochemical Investigations

I. Detection of gene expression of PGC-1α, Nrf-2, TFAM, SIRT-1 and cytochromec by real-time PCR

RNA extraction from brain tissue: to isolate total RNA from brain tissue homogenate, the SV Total RNA Isolation System was used (Promega, Madison, WI, USA). Using a UV spectrophotometer, the concentration of RNA was measured.

c-DNA synthesis:using high capacity c-DNA reverse transcription kit (Cat. #K1621, Fermentas, USA), the total RNA (1µg) was used for c-DNA conversion.Random primers (3µl)were appliedto the 10 µl of RNA, denaturedin the thermal cyclerat 65°C for 5 minutes. The temperature of the RNA primer mixture was lowered to 4°Cand reverse transcription mixture containing 5 µlfirst-strand buffer, 2 µl of 10 mM of deoxyribonucleotide triphosphate (dNTP), 1 µl RNase inhibitor (40 U/µl), 1 µl of moloney murine leukemia virus reverse transcriptase (MMLV-RT enzyme -50 U/µl), and10 µl DEPC-treated water. For each sample,the master mix total volume was 19 µl. This was added to the 13 µl RNA-primer mixture. The last mix was incubated at 37°C for 1 hour in the programmed thermal cycler, followed byenzyme inactivationfor 10 minutes at 95°C before its temperature was lowered at 4°C. RNA was then converted toc-DNA. Minus 20°C was used to store the transformed c-DNA.

Real-time qPCR using SYBR Green I: An Applied Biosystem with software version 3.1 (StepOneTM, USA) was used to perform real-time qPCR amplification and analysis. At the annealing temperature, the qPCR assay with the primer sets (Table1) was optimized. All c-DNA including previously prepared samples (for cytochromec, PGC-1α, TFAM, Nrf-2, and SIRT-1gene expression), internal control (for β-actin gene expression as housekeeping gene), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture), were in duplicate.PCR reactions consisting of 50ºC for 2 min (1 cycle), 95 ºC for 15 s, 60ºC for 1 min, and 72ºC for 1min (40cycles), were performed on step one plus Real-Time PCR system (Applied Biosystems). ABI Prism 7500 sequence detection system software was used for the data analysis. Data quantification was done using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Using the method of the comparative threshold cycle, the relative expression of the studied genes was computed.All values were normalized to the beta-actin genes. All these steps were described by[33].

Table 1: Primers' Sequences

Gene	Primer sequence
Cyt-c	Forward primer:5′-TTTGGATCCAATGGGTGATGTTGAG-3′ Reverse primer:5′TTTGAATTCCTCATTAGTAGCTTTTTTGAG-3′
PGC-1α	Forward primer:5'-CATTGAGGTGTATTTCACGG -3 Reverse primer:5'-GGCAAGTGGCCATTGTGTTC -3
TFAM	Forward primer: 5′AAAAATCTGTCTCATGATGAAAAGCAG-3′. Reverse primer: 3′ CTTCATTTCATTGTCATAACGAATTCTAT-3′
Nrf-2	Forward primer: 5′-CTTTTGGCGCAGACATTCC-3′. Reverse primer:5′-AAGACTGGGCTCTCGATGTG-3′.
SIRT-1	Forward 5'-TCG TGG AGA CAT TTT TAA TCA GG -3', Reverse 5'-GCT TCA TGA TGG CAA GTG G-3'
Beta actin	Forward primer :5'-AGGTCGGAGTCAACG GATTTGGT-3 Reverse primer:5′- CATGTGGGC CATGAG GTC CACCAC-3

II. Enzyme-Linked ImmunoSorbent Assay (ELISA) in estimation of the levels of IL-6, Aβ-42, and p-tau

Following the instructions of the manufacturer, estimation of IL-6 was done by ELISA kit supplied by R&D system-USA; estimation of Aβ-42 was done by rat amyloid beta-peptide 1-42 ELISA kit (My Biosource-USA). Rat phospho tau protein (p-tau) ELISA kit (MyBioSource-USA) was used for estimation of p-tau

Estimation of AMPK Protein by Western Blot Technique

In 400l RIBA lysis buffer PL005, brain tissue (50 mg) was homogenized using a Bio Basicpolytron homogenizer (Markham Ontario L3R 8T4 Canada).The supernatant was collected after centrifuging the homogenate at 4ºC for 30 minutes. At -80°C, the samples were held before they were used. After boiling for 5 minutes at 95º C, samples (20 mg/lane) were applied toa SDS-PAGE gel (7%) then transferred to nitrocellulose membranes (Bio-Rad-USA). The membrane was blocked for 1 hour at room temperature in tris-buffered saline with Tween 20 (TEST) buffer and 3 % bovine serum albumin (BSA), then incubated overnight at 4ºC with primary antibodies specific for AMPK (Cat.#07-350) at dilution factor 1:2000 (Thermo Fisher, USA). The membranes were washed for10 minutes3 times with TBS-T before being incubated for 1 hour at room temperature with a secondary horseradish peroxidase-conjugated (Goat antirabbit IgG- HRP-lmg Goat mab -Novus Biologicals) Enhanced chemiluminescence (ClarityTM Western ECL substrate - Bio-Rad-USA, Cat. #170-5060) was used to visualize proteins. With a CCD camera-based imager, the chemiluminescent signals were recorded. On the Chemi Doc MP imager, software for image analysis was used to compare the target proteins band strength to the control sample after beta-actin normalization.

H. Morphometric and Statistical Analysis

The "Leica Qwin 500 C" image analysis computer system Ltd (Cambridge, UK) wasused. For each group, five slides of five different specimens were examined with five non-overlapping fields at a magnification of x100.The

following parameters were assessed:

1. The area % of Aβ plaques in Congo red-stained sections.
2. The area % of Casp-3 +ve immuno-stained cells in Casp-3 immuno-stained sections.

Data analysis was done using SPSS software version 22. Numerical data werepresentedusing means and standard deviations. ANOVA testwas used to compare groups when comparing more than twogroups. Multiple comparisons were done and adjusted using post hoc tests[34]. Pearson correlation coefficient was used for correlation analysis[35].

RESULTS

No variations were observed between subgroups Ia, Ib and Ic, so their findings will be discussed as group I (control group).

Improvement of the cognitive functions in melatonin and MVs treated rats

In AD-induced group, the cognitive functions were signigicantly decreased as compared to control group (group I). All treated groups displayed a significant increase in the cognitive functions as compared to AD group but still significantly decreased as compared to the control group. Interestingly, a significant increase was observed in the cognitive functions of group V as compared to groups III&IV (Figure 2).

Melatonin and MVs therapy protected against neurodegenerative changes in AD

In the control rats, light microscopic inspection of H&E-stained hippocampus sections (GpI) revealed C-shaped hippocampus proper formed of the cornuammonis, which consisted of four regions as CA1, CA2, CA3, the hilus regions, and the dentate gyrus having upper and lower limbs (Figure 3A). Three layers were observed; polymorphic, pyra¬midal and,molecular layers. The polymorphic and molecular layers showed glial cells within the eosinophilic neuropil, while the pyramidal layer was the most characteristic layer located between the previous two layers. This pyramidal layer illustrated the pyramidal cells with prominent nucleoli and vesicular nuclei (Figure 3B).

Sections of the hippocampus (CA1 and CA3 regions) from the AD group (GpII) demonstrated multiple shrunken degenerated pyramidal cells having dark eosinophilic cytoplasm with pyknotic nuclei and surrounded by pericellular spaces (Figure 4A).

Animals that received a single treatment with MVs therapy (GpIII) or melatonin therapy (GpIV) revealed mild to moderate neurodegenerative changes in the pyramidal layer. Some of the pyramidal neurons displayed vesicular nuclei and prominent nucleoli.

In contrast, others were still shrunken and degenerated (Figures 4B,5A). Combined treatment administration in MVs and Melatonin group (GpV) showed substantial protection and normal hippocampus structure as the pyramidal layer showed multiple pyramidal cells with prominent nucleoli and vesicular nuclei. Yet, few degenerated cells were observed (Figure 5B).

Melatonin and MVs therapy markedly decreased β-amyloid plaques deposition in AD

Light microscopic examination of Congo red staining of the control rat hippocampus revealed no obvious red Aβ plaques deposition in the pyramidal cells of the pyramidal layer (Figure 6A). Group II showed a marked deposition of red Aβ plaques in all layers of the hippocampus (Figure 6B). Aβ plaques mean area percent (%) was significantly increased in Group II as compared to the control group (Figure 10A). Groups III & IV revealed red Aβ plaques in some cells of the hippocampus (Figures 7A,B). The mean area % of Aβ plaques in groups III & IV was significantly increased as compared to control group, and significantly decreased as compared to AD-induced group (Figure 10A). Meanwhile, groupV appeared normal with no red Aβ plaques deposition in the hippocampus cells (Figure 7C) in most of fields. The mean area % of Aβ plaques in groupV was significantly decreased as compared to groups III & IV (Figure 10A).

Melatonin and MVs therapy markedly reduced Casp-3 immunostaining in AD

Light microscopic examination of Casp-3 immuno-staining (IS) the control rat hippocampus (group I) revealed negative IS in pyramidal layer cells (Figure 8A). Group II revealed multiple positive IS in the pyramidal layer cells (Figure 8B). The mean area % of Casp-3 immuno-staining showed a significant increase as compared to the control group (Figure 10B). Groups III & IV showed some positive IS in the pyramidal layer cells (Figures 9A,B). The mean area % of Casp-3 immuno-staining was significantly increased in groups III & IV as compared to the control group and significantly decreased as compared to AD-induced group (Figure 10B). Meanwhile, groupV exhibited negative IS in the cells of the pyramidal layer in most of the fields (Figure 9C). Group V revealed a significant decrease as compared to groups III & IV (Figure 10B).

Therapy with melatonin and MVs decreased the pathological markers of AD (Aβ-42 and P-tau)

In terms of Aβ-42, AD group revealed a significant increase in its level as compared to control group. All treated groups showed a significant decrease in its level compared to the AD group. Its level was significantly decreased in GpV (MVs+Melatonin) compared to Gp IV. (Figure 11A).

P-tau level, AD group revealed a significant increase in its level as compared to control group. Its level was significantly decreased in all treated groups compared to the AD group. However, in comparison to the control group, it was still significantly higher in Gp III and Gp IV. (Figure 11B).

Melatonin and MVs therapy improved mitochondrial biogenesis

The expression of mitochondrial biogenesis markers (PGC-1α, Nrf-2, TFAM, SIRT-1) was significantly lower in the AD group than in the control. At the same time, it was increased significantly in all treated groups in comparison to the AD group, especially the GpV compared to Gps III & IV. Compared to GpIV, sirt-1 expression significantly was increased in GpV (Figure 11C).

Melatonin and MVs therapy improved cellular energy

Compared to the control, AD group showed a significant decline in AMPK expression. All treated groups showed a significant increase in AMPK expression as compared to AD group,yet they were decreased significantly in comparison to the control group (Figures 11D,E).

Melatonin and MVs therapy improved the cellular apoptosis

In comparison to the control group and all the treated groups, Cyt-c gene expression showed a significant increase in the AD group. Compared to Gps III&IV, GpV showed a significant reduction in Cyt-c gene expression (Figure 11F).

Melatonin and MVs therapy improved the neuro-inflammation

In comparison to control group and all the treated groups, AD group had a significant rise in IL-6 level. In contrast to to Gps III&IV, GpV showed a significant reduction in IL-6 level (Figure 11G).

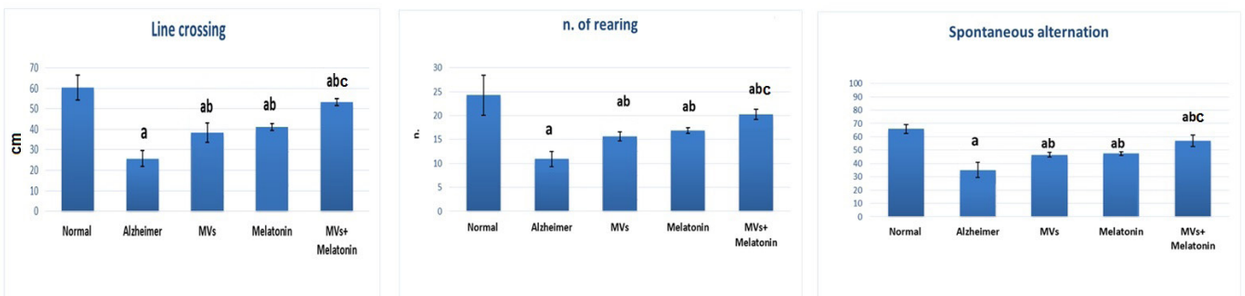

Fig. 2: Cognitive functions assessment for number of line crossing, number of rearing and spontaneous alternation. Data were expressed as Mean ± SD, (a) Significant (*p value* <0.05) difference versus the control group; (b) Significant difference versus AD group; (c) Significant difference versus MVs-treated and Melatonin-treated groups.

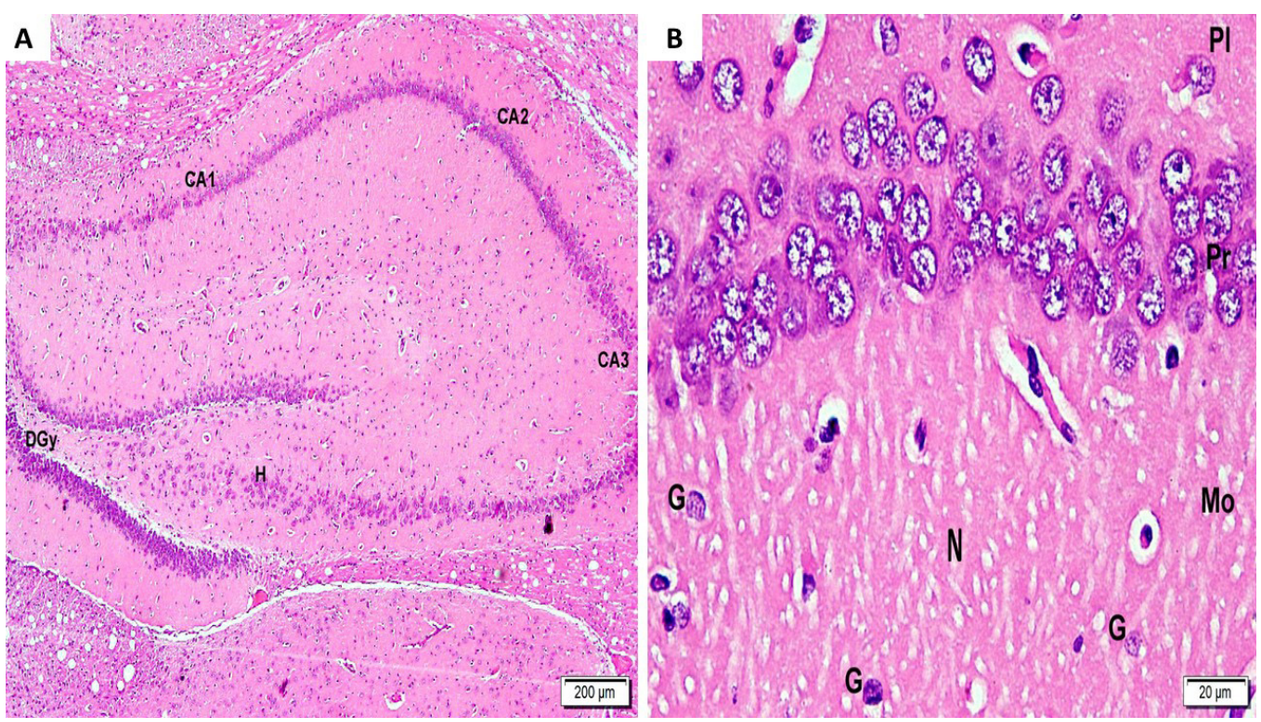

Fig. 3: Photomicrographs of H&E-stained hippocampus of the control group. (A): the hippocampus proper is formed of the cornuammonis as CA1, CA2, CA3 the hilus (H) regions and the dentate gyrus (DGy) having upper and lower limbs (40x). (B): A higher magnification shows the three layers of the hippocampus; polymorphic layer (Pl), pyramidal layer (Pr) and molecular layer (Mo). The pyramidal layer illustrates pyramidal cells with vesicular nuclei and prominent nucleoli. The molecular layer displays glial cells (G) among eosinophilic neuropil (N). (400x)

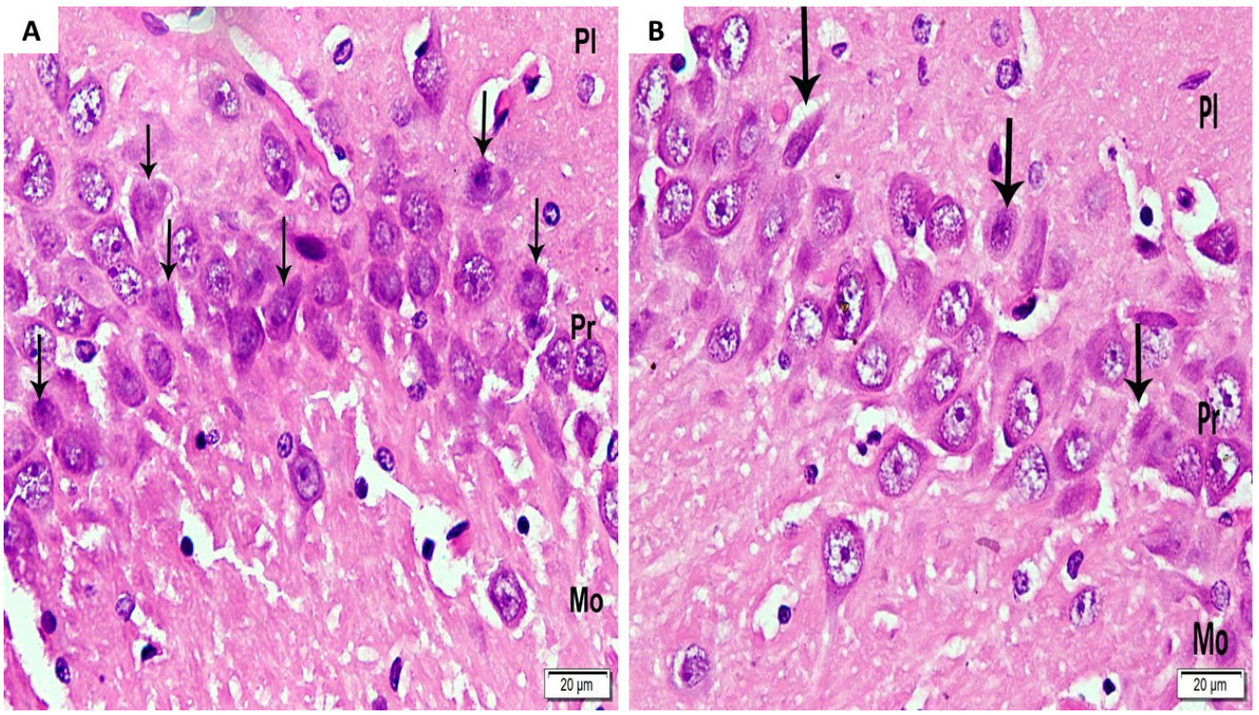

Fig. 4: Photomicrographs of H&E-stained hippocampus (400x) show: (A)GpII: the pyramidal layer (Pr) with multiple shrunken pyramidal cells which have dark eosinophilc cytoplasm and pyknotic nuclei surrounded by pericellular space (arrows). (B) GpIII: some shrunken pyramidal cells with dark eosinophilic cytoplasm, pyknotic nuclei and surrounded by pericellular space (arrows).

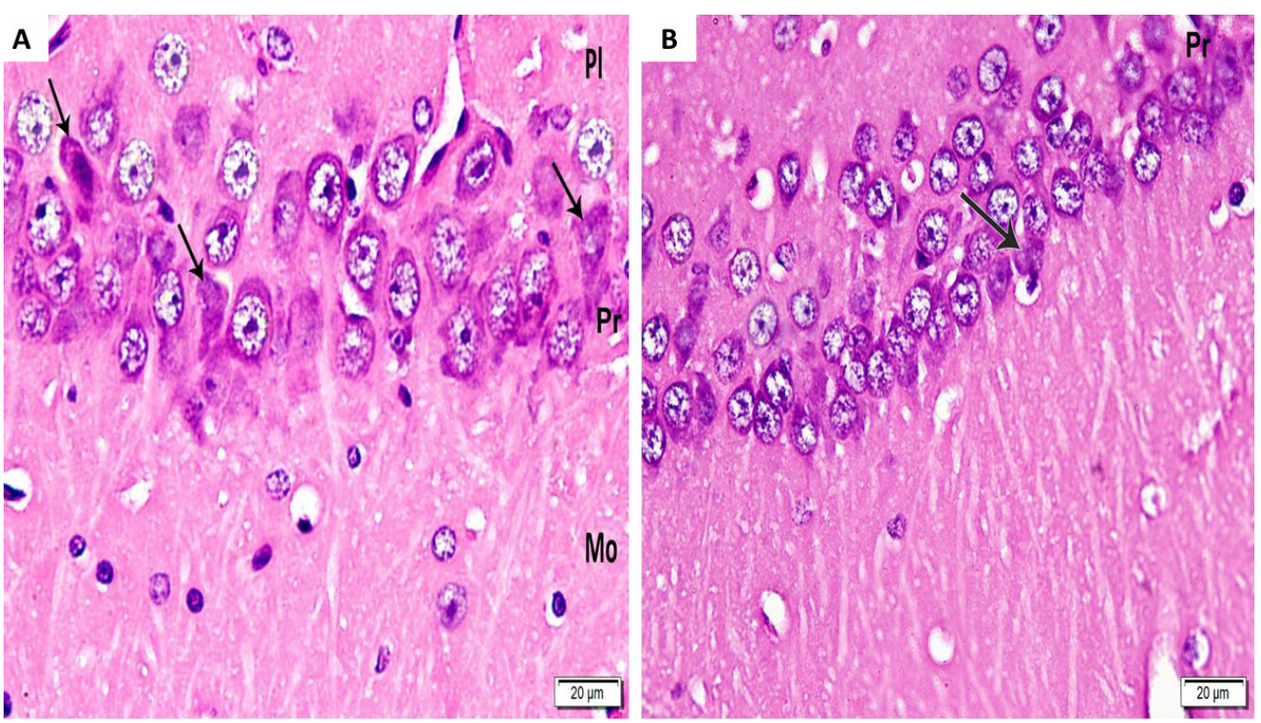

Fig. 5: Photomicrographs of H&E-stained hippocampus(400x) show: (A) GpIV: many preserved pyramidal cells with vesicular nuclei and prominent nucleoli. Some degenerated pyramidal cells (arrows) are noted. (B) GpV: the pyramidal layer (Pr) illustrates most of the pyramidal cells with vesicular nuclei and prominent nucleoli. Few degenerated pyramidal cells (arrow) are observed.

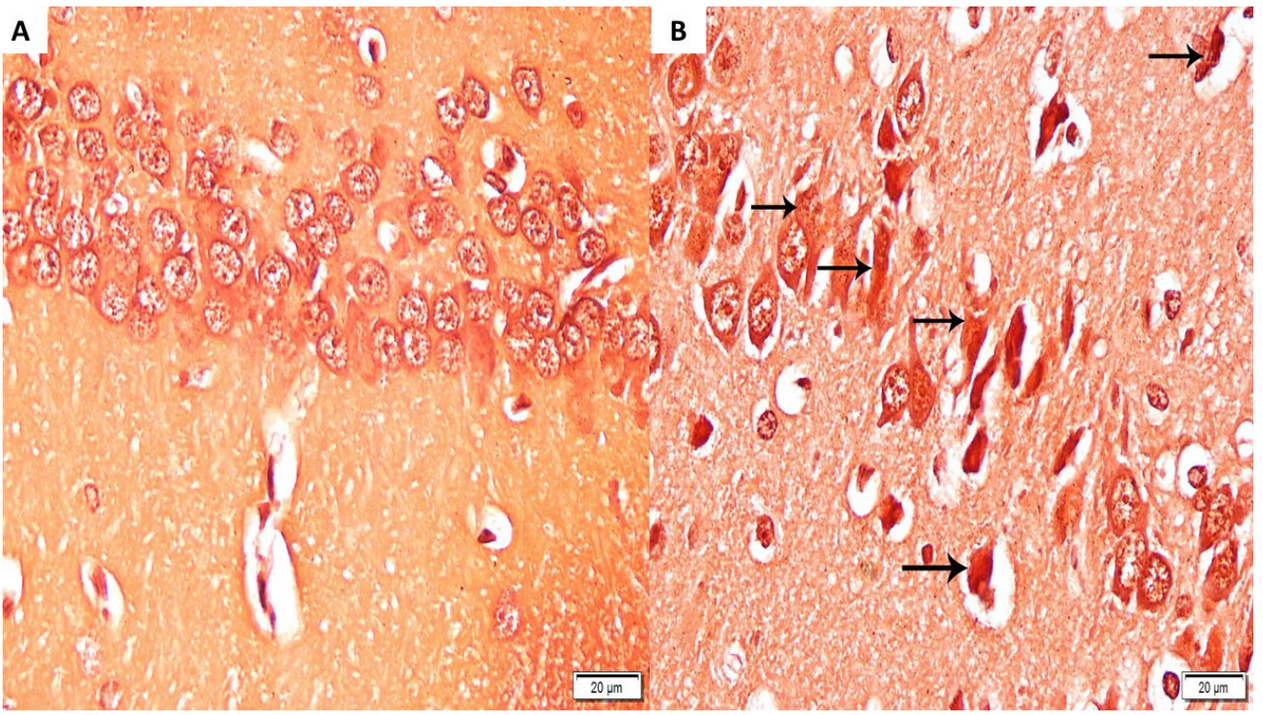

Fig. 6: Photomicrographs of Congo red stained rat hippocampus (400x): (A) Control Gp: showing no red Aβ plaques deposition in the hippocampus layers. (B): GpII: exhibiting extensive positively Congo red stained cytoplasmic Aβ plaques (arrows) accumulated in all layers of hippocampus.

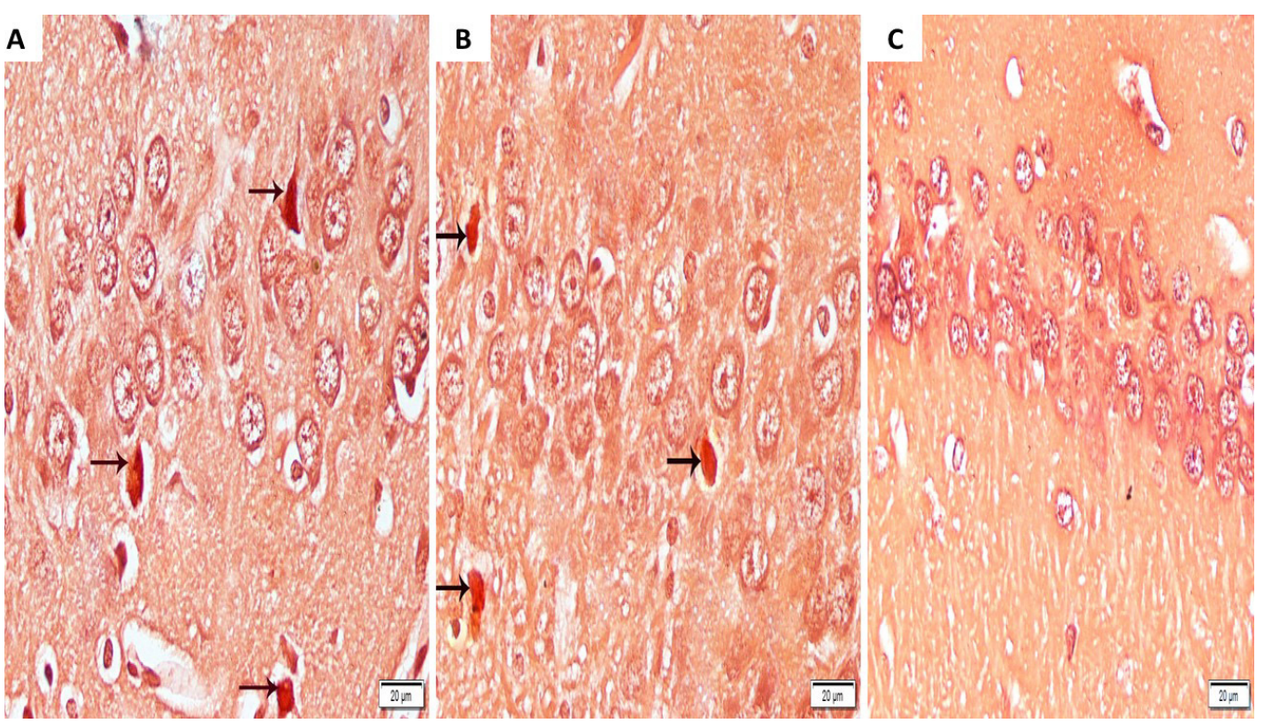

Fig. 7: Photomicrographs of Congo red stained rat hippocampus (400x): (A) GpIII& (B): GpIV: reveal some positively Congo red stained Aβ plaques (arrows) accumulated in all layers of hippocampus. (C): group V: showing no clear red Aβ plaques deposition in hippocampus layers.

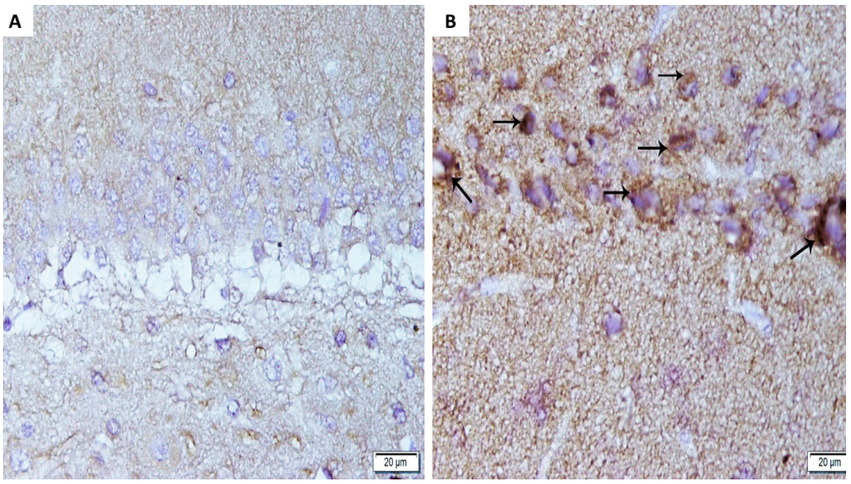

Fig. 8: Photomicrographs of rat hippocampus immunohistochemically stained with anti-Casp-3 antibody (400x): (A) Control group: shows negative IS in the pyramidal cells. (B): GroupII: reveals multiple pyramidal cells with Casp-3 +veIS (arrows).

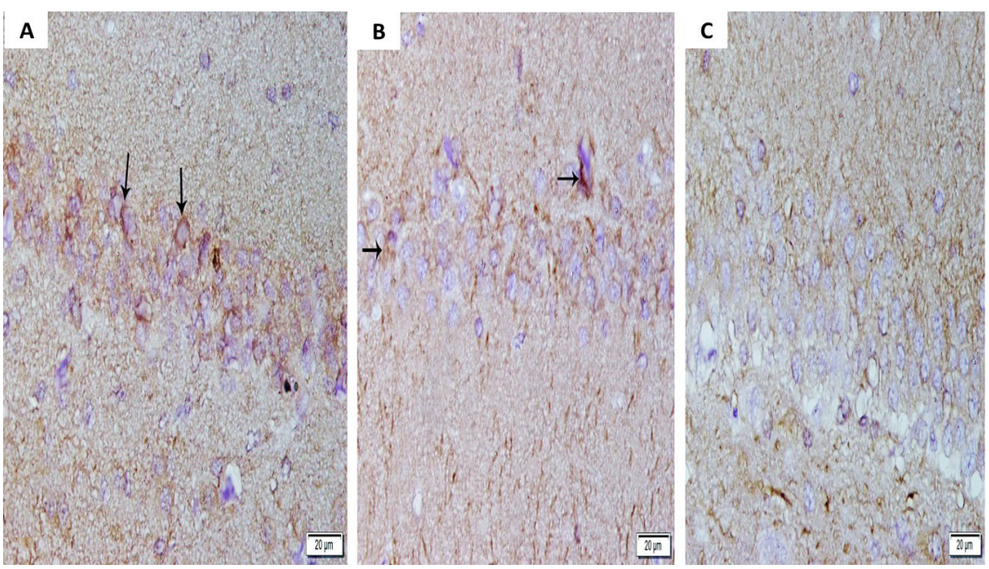

Fig. 9: Photomicrographs of rat hippocampus immunohistochemically stained with anti-Casp-3 antibody (400x): (A)&(B) Gps III &IV: shows some positive IS in the pyramidal cells(arrows). (C): GpV: reveals negative Casp-3 IS in the pyramidal cells.

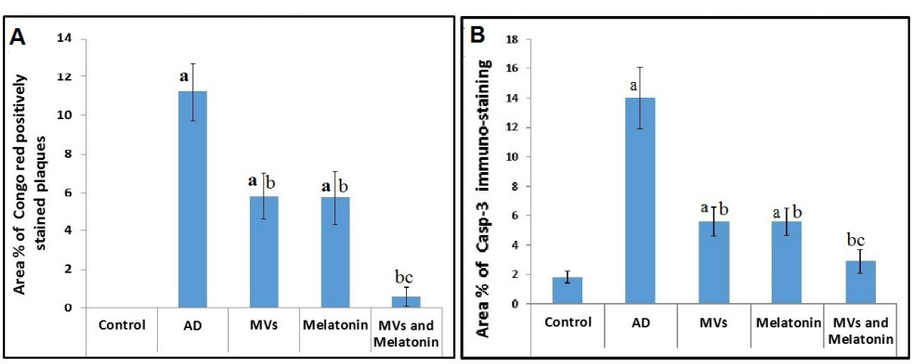

Fig. 10: Histograms illustrating the mean area % of of β-amyloid plaques &Casp-3 immuno-stained cells AD. Data were expressed as Mean ± SD, (a) Significant (p value <0.05) difference versus the control group; (b) Significant difference versus AD group; (c) Significant difference versus MVs-treated and Melatonin-treated groups.

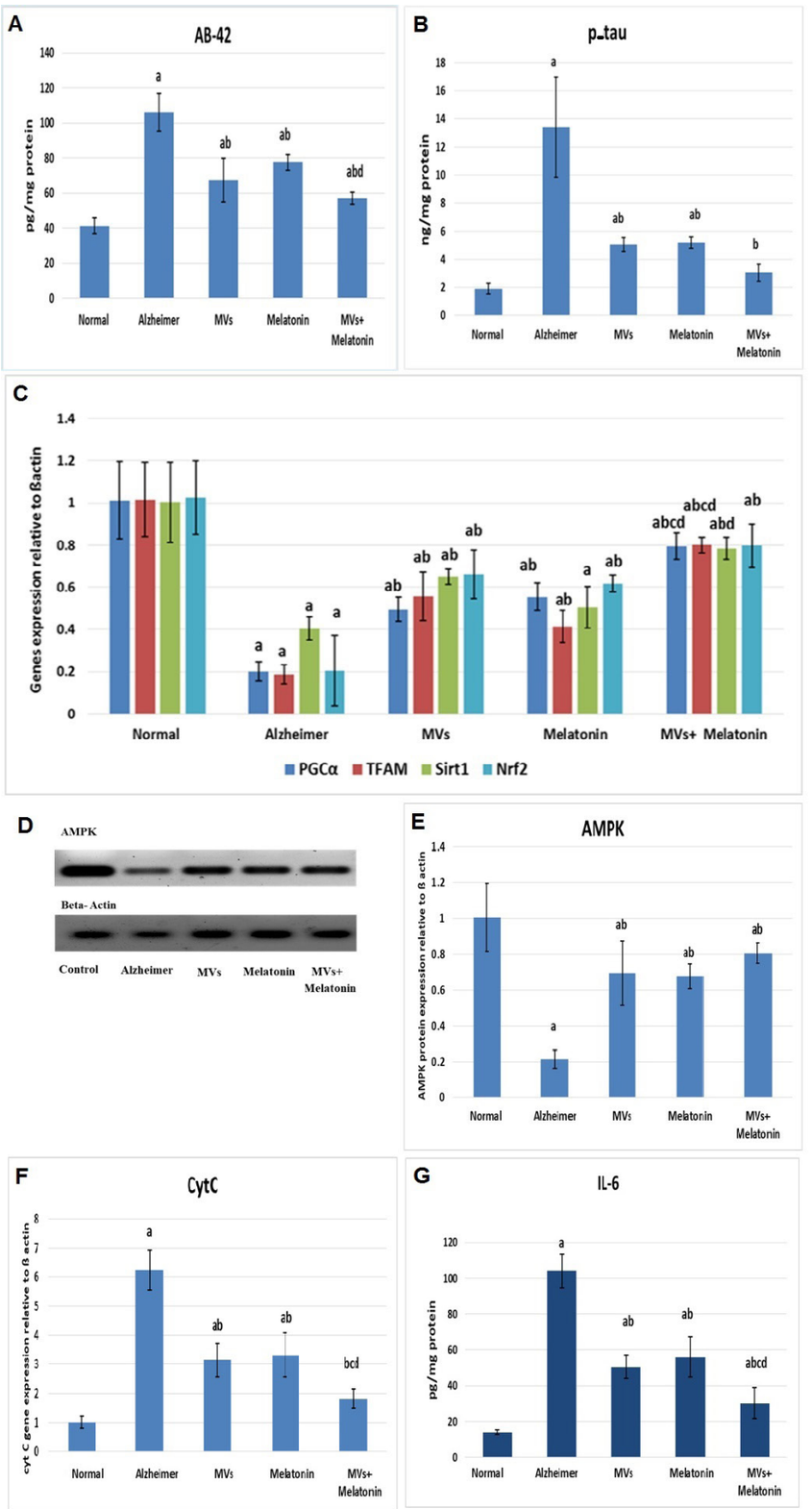

Fig. 11: A) The level of AB-42; B) The level of P-tau; C) The level of mitochondrial biogenesis markers expression (PGC-α, Nrf-2, TFAM, Sirt-1); D) Protein expression of AMPK by western blot analysis; E) Quantification of the AMPK expression; F) The level of Cyt-c gene expression; G) The level of IL-6. Data were expressed as Mean ± SD. (a) Significant (p value <0.001) difference versus the control group; (b) Significant difference versus AD group; (c) Significant difference versus MVs-treated group. (d) Significant difference versus Melatonin-treated group.

DISCUSSION

The most frequent neurodegenerativeage-related disease is Alzheimer's disease (AD). It is marked by neuronal cell loss and progressive brain atrophy, which results in cognitive decline[36]. This work aimed to study the effect of the BM-MSCs microvesicles and melatonin separately or as a combined therapy on mitochondrial biogenesis and apoptosis in the rat model of AD.

In this work, a single i.p. injection of 0.8mg/kg lipopolysaccharide (LPS) successfully induced AD. In comparison toall treated groups and the control group, the AD group (GpII) showed a significant decline in cognitive functions. Examination of the H&E-stained sections showed multiple shrunken degenerated pyramidal cellsexhibiting dark eosinophilic cytoplasm with pyknotic nuclei and surrounded by pericellular spaces in CA1 and CA3 regions of the hippocampus. Mean area % of Aβ plaques in Congo red-stained sections showed a significant increase. Besides, significant increases in p-tau and Aβ-42 levels were observed. It was documented that the cognitive impairment in AD is related to the accumulation of p-tau aggregates and fibrils in the degenerating neurons[37]. Our findings agreed with other authors[38,39]. Another study showed that survived neurons number was significantly decreased or disappeared in the transgenic AD model, and serious damage to the neurons was observed, including loss of integrity[40]. It was documented that the histopathological stigma of AD is the neurodegeneration of the brain with the deposition of neurofibrillary hyperphosphorylated tau protein and the Aβ peptides[41]. Hyperphosphorylated tau protein is a key component of neurofibrillary tangles, which may be used as an early indicator of Alzheimer's disease[42]. Another study also showed that AD exhibited an increase in Aβ plaques and a decrease in the neuronal population of the hippocampus compared to cortex and white matter by Congo red staining[43]. These Aβ plaques inhibit the antioxidant enzymes activity,trigger reactive oxygen species (ROS) generation, and disrupt mitochondrial function, resulting in the neuronal oxidative and non-resolving inflammatory response[44].

Senile plaques in AD patients' brains are associated with an increase in inflammatory cytokines as IL-6[45]. In the current analysis, the AD group had a significantly higher level of IL-6 than the control and all of the treated groups. This was in line with other studies that observed increased IL-6 cytokine production in AD patients, indicating abnormal cellular immunity in these patients[46]. This also agreed with another study that reported that patients with AD had higher IL-6 levels than the healthy controls[47].

Mitochondrial dysfunction in AD increased ROS production, which initiated lipid peroxidation of the cell membrane by promoting p-tau aggregation[48] and enhancing the neurodegenerative changes[49].This mitochondrial dysfunction in AD was proved by measuring different markers for mitochondrial biogenesis as silent information regulator Type 1 (SIRT-1), nuclear respiratory factor-2 (Nrf-2), peroxisome proliferation activator receptor gamma-coactivator 1α (PGC-1α), and mitochondrial transcriptional factor A (TFAM). SIRT-1 has been the most widely investigated sirtuin, especially in models of aging and neurodegenerative disorders as AD[50]. Sirtuins are involved in cell cycle regulation, DNA repair, cell survival, and apoptosis.PGC-1α is a transcriptional co-activator that promotes mitochondrial biogenesis and respiration, as well as adaptive thermogenesis and gluconeogenesis[51]. Nrf-2 is a transcription factor that controls antioxidant gene expression. In the presence of oxidative stress, Nrf-2 is released and then bound to antioxidant response elements that activate the synthesis and release of antioxidant molecules[52]. The TFAM controls mitochondrial transcription and retains mitochondrial DNA, which helps to preserve mitochondrial homeostasis. TFAM binds mitochondrial DNA and protects it from ROS damage[53]. In the AD group, compared toall the treated groups and the control group,our study showed a significant decrease in SIRT-1, PGC-1α, Nrf-2, and TFAM gene expression. Nrf-2 deficient mice had higher insoluble p-tau and Aβ56 levels and increased signs of oxidative stress and neuroinflammation. These findings suggest that the lack of Nrf-2 replicates a prodromal condition of human AD that is aggravated by the loss of Nrf-2-related homeostasis[54].

AMP-activated protein kinase (AMPK) is a vital intracellular energy sensor associated with energy metabolism[55]. AMPK activation suppresses ATP-consuming and protects against stress-mediated cellular pathophysiology[56]. Activation of AMPK facilitates autophagy and promoteslysosomal degradation of Aβ plaques[57]. In the present study,in comparison toall the treated groups and the control group, the AMPK level in the AD group was significantly lower. This could be explained by the oxidative stress involved in AD.

Regarding the apoptotic changes observed in this study, Casp-3 immunostaining and Cyt-c results confirmed these changes. In comparison to all the treated groups and the control group, the mean area % of casp-3 immunopositive cells and Cyt-c gene expression significantly increased in the AD group. Cytochrome-c acts as a key initiator of apoptosis by facilitating the assembly of apoptosomes that activates caspase cascade as Casp-3 leading to cellular death[58]. Other authors confirmed our findings, reporting that Aβ oligomers trigger Cyt-c release from neuronal cell mitochondria, which is an important phase in apoptosis. It induces a cascade of caspase activation, which results in cell death in Alzheimer's disease[59]. Moreover, it was demonstrated that increased oxidative damage of ROS in AD targets DNA proteins and lipids, leading to cell apoptosis[60].

All treated groups in this study showed improvement in cognitive functions. This agreed with other authors who reported that melatonin could protect against dementia and improve the cognitive performance in AD transgenic mice by decreasingthe p-tau burden in the hippocampus[61]. Moreover, intravenous administration of

adipose-derived stem cells also showed beneficial effects in dementia of induced AD[62]. In this study, single therapy in GpIII or GpIVrevealed mild to moderated protection. Some pyramidal neurons displayed vesicular nuclei with prominent nucleoli. Others were degenerated with reduction of Aβ plaques depositionin Congo-red stained sections.

In contrast, the combined administration of MVs and melatonin displayed synergetic improvement in the hippocampus structure against the degenerative AD changes with a significant decrease in Aβ plaques mean area % in Congo-red stained sections and p-tau and Aβ-42 levels as compared to single therapy groups (GpIII & GpIV).The MVs have immunomodulatory, anti-inflammatory, and neuroprotective effects capable of stimulating tissue regeneration[63]. Moreover, other authorsdemonstrated that intracerebral infusion of neuronal MVs into the brains of amyloid protein precursor (APP) transgenic micedecreased amyloid depositions, suggesting neuronal MVs key role in clearing Aβ in the brain[64]. The protective effect of melatonin is explained by other authors who stated that melatonin is an antioxidant that may help reduce proinflammatory cytokines expression, mitochondrial dysfunction, and neuronal cell death in Alzheimer's disease[65]. Also, melatonin exerts anti-Aβ aggregation effects and prevents neurotoxicity.

Regarding neuroinflammation in this study, we observed improvement in all the treated groups with a significant IL-6 declinein the combined therapy group (GpV) than single therapy groups (GpIII & GpIV). This is consistent with other authors who reported that melatonin inhibited LPS-induced production of inflammatory cytokines TNF-α, IL-1β, and IL-6 in mouse mammary cells[66]. Another study also showed that melatonin could reduce IL-6 levels in a concentration-dependent manner[67]. Other authors reported that transplantation of MVs derived-MSCs could significantly reduce the elevated serum levels of IL-6 in concanavalin A-induced hepatitis in mice[68].

Another study observed that genes regulating mitochondrial biogenesis (PGC-1α, TFAM, and Nrf-2) had been downregulated in AD patients' postmortem brains compared with age-matched control subjects indicating that these genes have an inverse relationship to AD pathogenesis[69]. All the treated groups showed improvement in mitochondrial biogenesis with a significant increase in SIRT-1, PGC-1α, Nrf-2, and TFAM expression in the combined therapy group than the single therapy groups. This was in line with other authors who showed an inverse association between melatonin treatment and aging with a simultaneous increase in SIRT-1 level in the dentate gyrus of rats[70]. In contrast,other authors reported that melatonin's anti-inflammatory effects occur due to up-regulation of SIRT-1 as observed in the ischemic brain injury model[71]. Melatonin also could inhibit oxidative stress and apoptosis by activating the PGC-1α/Nrf2 signaling pathway[72]. This is consistent with other authors who observed decreased hippocampal TFAM in Aβ-injected rats and when treated with melatonin[73]. Adipose-derived stem cells MVs treatment showed normalization of PGC-1α expression level[74]. AMPK levelsignificantly increased in GpVcompared tothe single therapy groups. These results agreed with other authors who indicated that melatonin increased AMPK phosphorylation and, as a result, expression levels in a dose-dependent manner[75].

The present study showed neurogenesis in all the treated groups with paid attention to the combined therapy group, in which the mean area % of Casp-3 positive immunostained cellsand Cyt-c levelsignificantly decreased compared to the single therapy groups. This was followingother authorswho showed that treatment with melatonin protects against apoptosis and cellular dysfunction by decreasing cyst-c expression in diabetic mice[76]. Another study documented that MVs can reduce neuronal loss by blocking apoptosis and increasing neurogenesis[77]. Besides, MVs can transfer several functional molecules like proteins, lipids, and regulatory RNA, modifying cell metabolism and promoting the recruitment, proliferation, and differentiation of other cells like neural stem cells. Thus, MVs and melatonin proved to have a synergetic protective effect against neurodegeneration in induced AD.

CONCLUSION

Our results revealed different therapeutic regimens for AD. With the morphological examination of the hippocampus structure and all biochemical parameters measured, we could conclude that a combined therapy (melatonin and MVs) could be superior to one therapeutic agent.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Snow AD, Castillo GM, Nguyen BP, Choi PY, Cummings JA, Cam J, Hu Q, Lake T, Pan W, Kastin AJ, Kirschner DA, Wood SG, Rockenstein E, Masliah E, Lorimer S, Tanzi RE and Larsen L.: The Amazon rain forest plant Uncaria tomentosa (cat's claw) and its specific proanthocyanidin constituents are potent inhibitors and reducers of both brain plaques and tangles. in Sci Rep (2019); 9 (561);1-28.

2. Guo T, Noble W, and Hanger D P: Roles of tau protein in health and disease. in Acta Neuropathol (2017): 133(5);665-704.

3. Su B, Wang X, Lee HG, Tabaton M, Perry G, Smith MA and Zhu X: Chronic oxidative stress causes increased tau phosphorylation in M17 neuroblastoma cells. in Neurosci Lett. (2010): 468(3):267-271.

4. Swerdlow R.H: Mitochondria and Mitochondrial Cascades in Alzheimer's Disease. in J Alzheimer's Dis (2018): 62(3):1403-1416.

5. Manczak M and Reddy PH: Abnormal interaction between the mitochondrial fission protein Drp1 and hyperphosphorylated tau in Alzheimer's disease neurons: Implications for mitochondrial dysfunction and neuronal damage. in Hum Mol Genet (2012): 21(11);2538–2547.
6. Mastroeni D, Khdour OM, Delvaux E, Nolz J, Olsen G, Berchtold N, Cotman C, Hecht SM and Coleman PD: Nuclear but not mitochondrial-encoded oxidative phosphorylation genes are altered in aging, mild cognitive impairment, and Alzheimer's disease. in Alzheimers. Dement (2017): 13(5):510-519.
7. Spuch C, Ortolano S and Navarro C: New insights in the amyloid-Beta interaction with mitochondria. in J Aging. Res ;(2012):2012;1-9.
8. Alghamdi B: The neuroprotective role of melatonin in neurological disorders. in J Neurosci. Res (2018): 96(7);1136–1149.
9. Shukla M, Sotthibundhu A and Govitrapong P: Role of melatonin in regulating neurogenesis: Implications for the neurodegenerative pathology and analogous therapeutics for Alzheimer's disease. In Melatonin Research (2020): 3(2);216-242.
10. Carloni S, Albertini MC, Galluzzi L, Buonocore G, Proietti F, Balduini W. Melatonin reduces endoplasmic reticulum stress and preserves sirtuin 1 expression in neuronal cells of newborn rats after hypoxia-ischemia. In J Pineal Res. (2014) :57(2);192-199.
11. Imai S, Armstrong C M, Kaeberlein M, and Guarente L: Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. In Nature (2000): 403 (6771); 795–800.
12. Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, Delalle I, Baur JA, Sui G, Armour SM, Puigserver P, Sinclair DA and Tsai LH. SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. in EMBO J. (2007):26(13);3169-3179.
13. Mudo G, Makela J, Di Liberto V and Tselykh T V: Transgenic expression and activation of PGC-1alpha protect dopaminergic neurons in the MPTP mouse model of Parkinson's disease. inCell.Mol. Life Sci (2012): 69 (7);1153–1165.
14. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P andAuwerx J: Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. in Cell (2006):127(6);1109-1122.
15. Zhou Y, Wang S, Li Y, Yu S and Zhao Y: SIRT1/PGC-1α Signaling Promotes Mitochondrial Functional Recovery and Reduces Apoptosis after Intracerebral Hemorrhage in Rats. in Front Mol Neurosci (2018):10(443);1-14.
16. Onyango IG, Lu J, Rodova M, Lezi E, Crafter AB andSwerdlow RH. Regulation of neuron mitochondrial biogenesis and relevance to brain health. in BiochimBiophys Acta. (2010):1802(1);228-234.
17. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC and Spiegelman BM: Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. in Cell. (1999): 98(1);115-124.
18. Muraoka S, Jedrychowski MP, Yanamandra K, Ikezu S, Gygi SP andIkezu T: Proteomic Profiling of Extracellular Vesicles Derived from Cerebrospinal Fluid of Alzheimer's Disease Patients: A Pilot Study. in Cells. (2020):9(9) ;1-17.
19. Zhang J, Guan J, Niu X, Hu G, Guo S, Li Q, Xie Z, Zhang C and Wang Y: Exosomes released from human induced pluripotent stem cells-derived MSCs facilitate cutaneous wound healing by promoting collagen synthesis and angiogenesis. in J Transl Med. (2015):13(49);1-14.
20. Yuyama K, Sun H, Sakai S, Mitsutake S, Okada M, Tahara H, Furukawa J, Fujitani N, Shinohara Y and Igarashi Y. Decreased amyloid-β pathologies by intracerebral loading of glycosphingolipid-enriched exosomes in Alzheimer model mice. in J Biol Chem. (2014):289(35);24488-24498
21. Cai ZY, Xiao M, Quazi SH and Ke ZY:Exosomes: a novel therapeutic target for Alzheimer's disease? in Neural Regen Res. (2018);13(5):930-935.
22. El Sayed NS, Kassem LA andHeikal OA:Promising therapy for Alzheimer's disease targeting angiotensinconverting enzyme and the cyclooxygense-2 isoform. in Drug Discov Ther. (2009): 3 (6);307-315.
23. Lee JY, Kim E, Choi SM, Kim DW, Kim KP, Lee I and Kim HS: Microvesicles from brain-extract-treated mesenchymal stem cells improve neurological functions in a rat model of ischemic stroke. in Sci Rep (2016) :6 (33038) ;1-14.
24. Kahya M, Nazıroğlu M and Övey I: Modulation of Diabetes-Induced Oxidative Stress, Apoptosis, and Ca2+ Entry Through TRPM2 and TRPV1 Channels in Dorsal Root Ganglion and Hippocampus of Diabetic Rats by Melatonin and Selenium. in Mol. Neurobiol (2017): 54(3);2345-2360
25. Bruno S, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F, Morando L, Busca A, Falda M, Bussolati B, Tetta C andCamussi G: Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. in J Am Soc Nephrol. (2009):20 (5);1053-1067.

26. Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C and Camussi G: Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. in Nephrol Dial Transplant. (2011) :26(5);1474-1483.
27. Ohl F: Testing for anxiety. In Clinical Neuroscience Research (2003): 3(4-5);233-238.
28. Arai K, Matsuki N, Ikegaya Y and Nishiyama N: Deterioration of spatial learning performances in lipopolysaccharide-treated mice. Jpn J Pharmacol (2001): 87(3);195-201.
29. Xu Q, Ming Z, Dart AM, Du XJ: Optimizing dosage of ketamine and xylazine in murine echocardiography. In Clin Exp Pharmacol Physiol (2007): 34(5-6);499-507.
30. Kiernan JK (2001): Histological and Histochemical Methods. In Theory and practice. 3rd edition, Arnold Publisher, London, New York, and New Delhy: 111-162.
31. Xiaoguang W, Jianjun C, Qinying C, Hui Z, Lukun Y andYazhen S: Establishment of a Valuable Mimic of Alzheimer's Disease in Rat Animal Model by Intracerebroventricular Injection of Composited Amyloid Beta Protein. in J Vis Exp. (2018) :137(56157);1-10.
32. Ismail ZM, Morcos MA, Mohammad MD andAboulkhair AG: Enhancement of neural stem cells after induction of depression in male albino rats (a histological & immunohistochemical study). In Int J Stem Cells. 2014: 7(2);70-78.
33. Kutyavin I, Lukhtanov E, Gamper H and Meyer R: Oligonucleotides with conjugated dihydropyrroloindole tripeptides: base composition and backbone effects on hybridization. in Nucleic Acids Res (1997):25(18);3718-3723.
34. Chan YH: Biostatistics102: Quantitative Data – Parametric & Non-parametric Tests. In Singapore Med J(2003a): 44 (8);391-396.
35. Chan YH: Biostatistics 104: Correlational Analysis. In Singapore Med J (2003b): 44 (12); 614-619.
36. Alzheimer's Association: 2016 Alzheimer's disease facts and figures. in Alzheimers Dement. (2016) ;12(4):459-509.
37. Gong C, Grundke-Iqbal I and Iqbal K: Targeting Tau Protein in Alzheimer's Disease. in Drugs & Aging (2010): 27 (5); 351-365.
38. Ghoneim FM, Khalaf HA, Elsamanoudy AZ, Abo El-Khair SM, Helaly AM, Mahmoud el-HM and Elshafey SH: Protective effect of chronic caffeine intake on gene expression of brain derived neurotrophic factor signaling and the immunoreactivity of glial fibrillary acidic protein and Ki-67 in Alzheimer's disease. in Int J Clin Exp Pathol (2015): 8(7);7710-7728.
39. Sun P, Yin JB, Liu LH, Guo J, Wang SH, Qu CH and Wang CX: Protective role of Dihydromyricetin in Alzheimer's disease rat model associated with activating AMPK/SIRT1 signaling pathway. in Biosci Rep. (2019):39(1);1-10.
40. Cui Y, Ma S, Zhang C, Cao W, Liu M, Li D, Lv P, Xing Q, Qu R, Yao N, Yang B and Guan F: Human umbilical cord mesenchymal stem cells transplantation improves cognitive function in Alzheimer's disease mice by decreasing oxidative stress and promoting hippocampal neurogenesis. in Behav Brain Res. (2017): 320;291-301.
41. DeTure MA and Dickson DW: The neuropathological diagnosis of Alzheimer's disease.in Molecular neurodegeneration (2019):14(1);1-8.
42. Kopeikina KJ, Carlson GA, Pitstick R, Ludvigson AE, Peters A, Luebke JI, Koffie RM, Frosch MP, Hyman BT and Spires-Jones TL: Tau accumulation causes mitochondrial distribution deficits in neurons in a mouse model of tauopathy and in human Alzheimer's disease brain. in Am J Pathol. (2011):179(4);2071-2082.
43. Nobakht M, Hoseini SM, Mortazavi P, Sohrabi I, Esmailzade B, Rahbar Rooshandel N andOmidzahir S. Neuropathological changes in brain cortex and hippocampus in a rat model of Alzheimer's disease. in Iran Biomed J. (2011);15(1-2);51-58.
44. More J, Galusso N, Veloso P, Montecinos L, Finkelstein JP, Sanchez G, Bull R, Valdés JL, Hidalgo C and Paula-Lima A: N-Acetylcysteine Prevents the Spatial Memory Deficits and the Redox-Dependent RyR2 Decrease Displayed by an Alzheimer's Disease Rat Model. in Front Aging Neurosci (2018):10 (399); 1-19.
45. Cacquevel M, Lebeurrier N, Cheenne S and Vivien D: Cytokines in neuroinflammation and Alzheimer's disease. in Curr Drug Targets (2004):5 (6); 529–534.
46. Cojocaru I, Cojocaru M, Miu G and Sapira V: Study of interleukin-6 production in Alzheimer's disease. Rom. in J. Intern. Med (2011): 49(1);55-58.
47. Kim YS, Lee KJ and Kim H: Serum tumour necrosis factor-α and interleukin-6 levels in Alzheimer's disease and mild cognitive impairment. in Psychogeriatrics. (2017):17(4);224-230.
48. M,Gong C, Grundke-Iqbal I and Iqbal K: Targeting Tau Protein in Alzheimer's Disease. in Drugs & Aging (2010):27(5);351-365.
49. El-Sokkary GH, I. Alghriany ASA andAtia MM. Protective effects of green tea and garlic on the morphological and oxidative stress changes of hippocampus in aged male and female albino rats. in J HistolHistopathol. (2018): 5(8); 1-9.

50. Donmez G: The neurobiology of sirtuins and their role in neurodegeneration. In Trends. Pharmacol. Sci (2012): 33(9); 494–501.

51. Handschin C and Spiegelman B: Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. In Endocr Rev (2006): 27(7);728-735.

52. Lim JL, Wilhelmus MM, de Vries HE, Drukarch B, Hoozemans JJ and van Horssen J: Antioxidative defense mechanisms controlled by Nrf2: state-of-the-art and clinical perspectives in neurodegenerative diseases. In Arch Toxicol. (2014): 88(10);1773-1786.

53. Kang D and Hamasaki N: Mitochondrial transcription factor A in the maintenance of mitochondrial DNA: overview of its multiple roles. In Ann N Y Acad Sci (2005):1042; 101–108.

54. Rojo AI, Pajares M, Rada P, Nuñez A, Nevado-Holgado AJ, Killik R, Van Leuven F, Ribe E, Lovestone S, Yamamoto M and Cuadrado A: NRF2 deficiency replicates transcriptomic changes in Alzheimer's patients and worsens APP and TAU pathology. in Redox Biol. (2017):13;444-451.

55. Liou CJ, Lee YK, Ting NC, Chen YL, Shen SC, Wu SJ and Huang WC: Protective Effects of LicochalconeA Ameliorates Obesity and Non-Alcoholic Fatty Liver Disease Via Promotion of the Sirt-1/AMPK Pathway in Mice Fed a High-Fat Diet. in Cells. (2019) :8(5);1-20.

56. Park JS, Leem YH, Park JE, Kim DY and Kim HS: Neuroprotective Effect of β-Lapachone in MPTP-Induced Parkinson's Disease Mouse Model: Involvement of Astroglial p-AMPK/Nrf2/HO-1 Signaling Pathways. In BiomolTher (Seoul). (2019) :27(2);178-184.

57. Mueed Z, Tandon P, Maurya SK, Deval R, Kamal MA and Poddar NK. Tau and mTOR: The Hotspots for Multifarious Diseases in Alzheimer's Development. in Front Neurosci. (2019):10 (12);1-14.

58. Anubhav Das: Review on cytochrome C.in int.J.clinicopathological correlation; (2019):3 (1); 7-11.

59. Camilleri A, Zarb C, Caruana M, Ostermeier U, Ghio S, Högen T, Schmidt F, Giese A and Vassallo N: Mitochondrial membrane permeabilization by amyloid aggregates and protection by polyphenols. In Biochimica et Biophysica Acta (BBA)–Biomembranes. (2013): 1828 (11) ;2532-2543.

60. Li J, Zhang G, Meng Z, Wang L, Liu H, Liu Q and Buren B: Neuroprotective effect of acute melatonin treatment on hippocampal neurons against irradiation by inhibition of caspase 3. In Experimental and Therapeutic Medicine (2016):11(6); 2385-2390.

61. Corpas R, Griñán-Ferré C, Palomera-Ávalos V, Porquet D, García de Frutos P, Franciscato Cozzolino SM, Rodríguez-Farré E, Pallàs M, Sanfeliu C and Cardoso BR: Melatonin induces mechanisms of brain resilience against neurodegeneration. in J Pineal Res. (2018):65(4); 1-15.

62. Kim S, Chang KA, Kim Ja, Park HG, Ra JC, Kim HS and Suh YH. The preventive and therapeutic effects of intravenous human adipose-derived stem cells in Alzheimer's disease mice. in PLoS One (2012): 7(9) ;1-17.

63. Galieva LR, James V, Mukhamedshina YO and Rizvanov AA: Therapeutic potential of extracellular vesicles for the treatment of nerve disorders. in Front Neurosci (2019): 13(163);1-9.

64. Yuyama K., takahashi K., Usuki S., Mikami D., Hui Sun H., Hanamatsu H., furukawa J., Mukai K. and igarashi Y: plant sphingolipids promote extracellular vesicle release and alleviate amyloid-β pathologies in a mouse model of Alzheimer's disease. in Scientific Reports (2019): 9(16827);1-11.

65. Hossain F., Uddin S., Uddin S.G.M., Sumsuzzman D., Islam S., Barre to G.E., Mathew B. and Ashraf G: Melatonin in Alzheimer's Disease: A Latent Endogenous Regulatorof Neurogenesis to Mitigate Alzheimer's Neuropathology. in Molecular Neurobiology (2019): 56(12):8255-8276.

66. Yu G, Kubota H, Okita M and Maeda T: The anti-inflammatory and antioxidant effects of melatonin on LPS-stimulated bovine mammary epithelial cells. In PLoS One (2017); 12(5);1-17.

67. Lau WW, Ng JK, Lee MM, Chan AS and Wong YH. Interleukin-6 autocrine signaling mediates melatonin MT (1/2) receptor-induced STAT3 Tyr (705) phosphorylation. in J Pineal Res. (2012):52(4);477-489

68. Lou G, Chen Z, Zheng M, Liu Y. Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. Exp Mol Med. (2017): 49(6);1-9.

69. Rice AC, Keeney PM, Algarzae NK, Ladd AC, Thomas RR and Bennett JP Jr: Mitochondrial DNA copy numbers in pyramidal neurons are decreased and mitochondrial biogenesis transcriptome signaling is disrupted in Alzheimer's disease hippocampi. In Journal of Alzheimer's Disease: JAD; (2014):40(2);319-330.

70. Kireev R, Vara E and Tresguerres J: Growth hormone and melatonin prevent age-related alteration in apoptosis processes in the dentate gyrus of male rats. In Biogerontology (2013): 14(4);431–442.

71. Paredes SD, Rancan L, Kireev R, González A, Louzao P, González P, Rodríguez-Bobada C, García C, Vara E andTresguerres JA: Melatonin Counteracts at a Transcriptional Level the Inflammatory and Apoptotic Response Secondary to Ischemic Brain Injury Induced by Middle Cerebral Artery Blockade in Aging Rats. in Biores Open Access. (2015):4(1);407-416.

72. Zhi W, Li K, Wang H, Lei M and Guo Y: Melatonin elicits protective effects on OGD/R insulted H9c2 cells by activating PGC 1α/Nrf2 signaling. in Int J Mol Med (2020): 45(5);1294-1304.

73. Ansari Dezfouli M, Zahmatkesh M, Farahmandfar M and Khodagholi F: Melatonin protective effect against amyloid β-induced neurotoxicity mediated by mitochondrial biogenesis; involvement of hippocampal Sirtuin-1 signaling pathway. in PhysiolBehav; (2019): 204;65-75.

74. Lee M, Ban JJ, Kim KY, Jeon GS, Im W, Sung JJ and Kim M: Adipose-derived stem cell exosomes alleviate pathology of amyotrophic lateral sclerosis in *vitro*. in BiochemBiophys Res Commun. (2016):479(3);434-439.

75. Sooho L, Nhu H and Dongchul K: Melatonin alleviates oxidative stress-inhibited osteogenesis of human bone marrow-derived mesenchymal stem cells through AMPK activation. in Int.J. Med Sci (2018): 15(10):1083-1091.

76. Jin H, Zhang Z, Wang C, Tang Q, Wang J, Bai X, Wang Q, Nisar M, Tian N, Wang Q, Mao C, Zhang X and Wang X: Melatonin protects endothelial progenitor cells against AGE-induced apoptosis via autophagy flux stimulation and promotes wound healing in diabetic mice. in Exp Mol Med. (2018) ;50(11):1-15.

77. Reza-Zaldivar EE,Hernández-Sapiéns M. A., Minjarez B., Yanet K. Gutiérrez-Mercado Y.K., Márquez-Aguirre A.L. and Canales-Aguirre A.A: Potential Effects of MSC-Derived Exosomes in Neuroplasticity in Alzheimer's Disease. in Front Cell Neurosci (2018):12 (317);1-16.

الملخص العربى

العلاج بالحويصلات متناهية الصغر مع الميلاتونين في استهداف الاختلال الوظيفي للميتوكوندريا في نموذج مرض الزهايمر لإناث الجرذان البيضاء: دراسة هستولوجية وكيميائية حيوية

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الخلفية والاهداف: مرض الزهايمر هو اضطراب تنكس عصبي يصاحبه انخفاض كبير في الوظائف الإدراكية . هذه الدراسة هدفت الي دراسة تأثير الحويصلات متناهية الصغر و هرمون الميلاتونين على التكاثر الحيوي للميتوكوندريا وموت الخلايا المبرمج في نموذج لمرض الزهايمر في الجرذان.

نبذة مختصرة: قد شملت الدراسة الحالية علي ٤٥ انثي من الجرذان مقسمة إلي٥ مجموعات :(٩ جرذان في كل مجموعة): مجموعة ضابطة ،مجموعات تجريبية وقد اشتملت علي مجموعة جرذان الزهايمر،مجموعة جرذان مصابة بمرض الزهايمر تلقت علاج بالحويصلات متناهية الصغر ، مجموعةجرذان مصابة بمرض الزهايمر تلقت علاج بالميلاتونين و مجموعة جرذان مصابة بمرض الزهايمر تلقت علاج مشترك بين الحويصلات متناهية الصغر والميلاتونين . تم إحداث الزهايمر في المجموعات التجريبية عن طريق حقن LPS مرة واحدة داخل الغشاء البيروتوني بجرعة ٠,٨ مجم / كجم ثم بعد اسبوع واحد تلقت المجموعات المعالجة حقنة واحدة من الحويصلات المتناهية الصغر بجرعة ٠,٢ مجم / كجم بينما تم إعطاء الميلاتونين بجرعة ١٠ مجم / كجم يوميًا لمدة ٣ أسابيع او علاج مشترك بينهما. في نهايةالتجربة، تم استخدام اختبارات متاهة Y واختبارات المجال المفتوح لتقييم الوظائف الادراكية . تم اخذ عينات من النسيج الدماغي لاجراء التحليل النسيجي و الكيميائي المناعي لتقييم الشكل المورفولوجي.

الي جانب ذلك تم اجراء الاختبارات البيوكيميائية الخاصة بمسببات مرض الزهايمر و علامات التخليق الحيوي للميتوكوندريا ، ودلالات الالتهاب العصبي وموت الخلايا المبرمج.

النتائج: أظهرت مجموعة فئران الزهايمر ضعف في الوظائف الادراكية وزيادة ترسيب لويحات البيتا اميلويد في الانسجة الدماغية المصبوغة بصبغة الكونجو الحمراء ، وايضا هناك زيادة في مستويات البروتينات Aβ-42 و p tau ، وزيادة علامات موت الخلايا المبرمج ؛و ايجابيةالصبغة الهستوكيميائية المناعية ضد Casp-3 وزيادة مستوي Cyt-c.ايضا هناك انخفاض ملحوظ في علامات التخليق الحيوي للميتوكندريا والتعبيرات الجينية لـ Nrf- ، PGC-1α TFAM، 2 و Sirt-1 . هناك تاثر في التمثيل الغذائي للطاقة في الدماغ ممثلة بمستوى AMPK ، إلى جانب زيادة كبيرة في مستوى IL-6.

وقد اظهرت ان العلاج بالحويصلات متناهية الصغر او الميلاتونين او العلاج المشترك بينهم له دور علاجي ضد مرض الزهايمر وكانت افضل النتائج باستخدام العلاج المشترك.

الخلاصة: العلاج المشترك بين الحويصلات متناهية الصغر والميلاتونين افضل من العلاج الفردي لمرض الزهايمر.