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Therapeutic role of bone marrow mesenchymal stem cells in diabetic neuronal alternations of rat hippocampus

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Abstract

Background: As the hippocampus is the main brain region for many forms of learning and memory functions and is acutely sensitive to blood glucose changes, diabetes mellitus (DM); which is a serious metabolic disease; is often accompanied by learning and memory deficits. Through scientific literatures, mesenchymal stem cells (MSCs) promote functional recovery in rats with traumatic brain injury, so the present work was conducted to study MSCs as a possible treatment for the diabetic neuronal degeneration and functional impairment of rat hippocampus.

Material and methods: It was carried out using male albino rats; non-diabetic control groups (4, 8, 12 weeks) (n= 15), diabetic groups by i.v. injection of streptozotocin (STZ) for (4, 8, 12 weeks) (n= 15) and MSCs treatment to diabetic groups for (8, 12 weeks) (n= 10). Hippocampal learning and memory functions were assessed by the Morris Water Maze test (MWM) and its results were statistically analyzed. The rat hippocampal regions (CA1 and CA3) were subjected to histological, ultrastructural examination and morphmetrical analyze of pyramidal neurons.

Results: Neurons of the diabetic groups showed disturbed function and architecture; shrunken hyperchromatic nuclei and vacuolated eosinophilic cytoplasm (apoptotic changes) also MSCs treatment improved hippocampal learning and memory functions plus its architectural changes; increasing populations and normal regular distribution.

Conclusions: It can be concluded that diabetic hippocampal neuronal alternations and functional impairment can be ameliorated by MSCs treatment.
Key words: diabetes, mesenchymal stem cells, hippocampus, neuronal alternations, learning and memory

Introduction

Uncontrolled DM leads to severe complications of central nervous system usually associated with neuronal degeneration [31]. As the hippocampus is particularly sensitive to changes in blood glucose level specially CA1 and CA3 regions [21], DM produces hippocampal dysfunction that is involved in learning and memory processing enhancing the risk of Alzheimer's disease [3]. Through scientific literature, DM can affect the hippocampus through reducing its synaptic plasticity [22], changes glutamate neurotransmission [10] and augments enzymatic activity linked to oxidative stress in the hippocampus [29]. These changes are usually associated with sings of neuronal loss and apoptosis which are due to stimulation of caspase-3 activity, nuclear DNA cleavage and induction of proapoptotic genes in hippocampus especially with uncontrolled DM [19]. As STZ can cause pancreatic β-cell destruction, it is used experimentally to induce type 1 DM [17]. MSCs have been used experimentally in the treatment of the injured brain [1, 6, 11] as well as other damaged organs as in kidney [9], heart, etc. In the reviewed literatures there was obvious contradiction concerning the differention of MSCs into the functioning nerve cells. Several studies showed that MSCs transplanted into the intact, injured or diseased CNS environments do not differentiate or even a small portion of them produce neural phenotypes [8]. Even though, Chopp [6] demonstrated that, transplanted MSCs promote functional recovery in rats with traumatic brain injury through activation of endogenous angiogenesis, neurogenesis, and synaptogenesis. Moreover, other studies supported that; MSCs could differentiate into mature neuron-like cells and exhibiting neuronal properties [1, 11]. Also, Calió [4] discussed MSCs role on the injured brain by decreasing neuronal apoptosis and oxidative stress. The aim of the present work was to evaluate using MSCs as a possible treatment for diabetic neuronal degeneration and learning and memory alternations.

Material and methods

Mesenchymal stem cells preparation: MSCs were obtained from Medical biochemistry department, Faculty of Medicine, Cairo University. Bone marrow derived mesenchymal stem cells was isolated and cultivated for four weeks according to protocol of Jiang [13]. Next, cells were labeled with 5-bromo- 2′-deoxyuridine (BrdU). Fluorescence phase-contrast microscope (Axiocam MR R3, Carl Zeiss, Germany) was used to observe the rats mesenchymal stem cells every 2 or 3 days.

Animals: The experiment strictly adhered to all ethical guidelines regarding animal research and was approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC). Adult normoglycemic male Sprague-Dawley albino rats (150-200g) were used in the current study. Rats were kept in a temperature and humidity-controlled room, with free access to food and water and were placed 5/cadge.

Experimental design: The rats were divided into 3 groups: **Control group** (n=15) which was subdivided into 3 subgroups (n=5/subgroup) [20]: (Control 4wks: sacrificed after 4 weeks, Control 8wks: sacrificed after 8 weeks and Control 12wks: sacrificed after 12 weeks). **DM groups** (n=15) received single i.v. injection of STZ (STZ, Sigma–Aldrich, St. Louis, MO, USA) dissolved in sodium citrate 0.1ml buffer, PH 4.5 at a dose of 60 mg/kg [2]. This group was subdivided into 3 subgroups (n=5/subgroup) [20]: (4wks DM: sacrificed after 4 weeks, 8wks DM: sacrificed after 8 weeks, and 12wks DM: sacrificed after 12 weeks). Fasting blood glucose levels were measured in DM groups (using spectrophotometer-Beckman; USA) 72 h after STZ injection to confirm induction of DM (>200 mg/dL). This day was considered the first day of the experiment. **DM+ MSCs group** (n=10) received single i.v. dose of STZ and 4 weeks later a single i.v. dose of fluorescent MSCs 1 ml of about 2,000,000 stem cells in the rat tail vein [15]. This group was subdivided into two subgroups (n=5/subgroup) [20]: (DM+MSCs 8wks: sacrificed after 8 weeks from the start of experiment; 4wks after MSCs administration and DM+MSCs 12wks: sacrificed after 12 weeks from the start of experiment; 8wks after MSCs administration). Fasting blood glucose levels were measured in DM and MSCs groups before sacrificed.

Morris Water Maze (MWM): Learning and memory impairment induced by DM and improvement after MSCs treatment were evaluated through performing MWM (4, 8 and 12 weeks from the start of the experiment). It was performed according to previous studies Vorhees [32]. Four-day training (twice/day) learning and memory was assessed through measuring escape latency to rich hidden platform (recorded with video camera). The maze was structured of white

circle tank (diameter: 127 cm and height: 51cm) filled with water depth 45 cm (made opaque with liquid milk) and a white platform (12 cm^2) submerged 2 cm below the water surface in the northwest quadrant of the maze. Navigational landmarks in the form of external cues were placed to the room wall. Signs of retrieving such memory (by removing platform and animals were allowed to swim freely for 60 seconds) were assessed through a provisional trial by counting both the number of platform-site crossovers and percentage of time spent in the target quadrant compared with the other quadrants. Statistical analysis was done for the means of escape latency times/secs of the trails; and for the provisional trial.

Scarification and Histological staining: By the end of the experimental periods, animals were anaesthetized by I.V. injection of Ketamine-dylazine and cardiac perfusion fixation technique by formalin 10% was done. For brain extraction, animals were decapitated immediately rostral to the first cervical vertebra and brains were removed. The right hemispheres allocated for light microscopic examination stained with Hx. & E. Studying hippocampal both CA1 and CA3 regions, as those regions are the hub of memory and learning functions [18]. The left hippocampi were dissected and allocated for ultrastructural examination using transmission electron microscope JEOL TEM (JEM-1400, JEOL, Japan) at 80 kV. To detect labeled MSCs in the hippocampus, 2 sections of both MSCs subgroups were immunostained by anti-Brdu and goat anti-mouse Ig and examined by fluorescent microscopy (Fig 1).

Morphometric analysis: It was conducted by using hippocampal histological sections stained by Hx.& E., implemented for the numbers of the pyramidal neurons in CA1 and CA3 hippocampal regions in all subgroups (five sections par each) using total magnification of 400. These statistical assessments were performed using analysis of variance (ANOVA) and statistical package for social science (SPSS). P value > 0.05 was considered not significant (NS) and P value <0.05 was considered significant (S).

Results

Statistical analysis

Regarding to the escape latency parameter in MWM test, all control groups showed identical numerical data so they are considered as one control group. All DM subgroups tended to require significant more escape latency periods to find platform than control group and there was more significant difference between 12wks DM than after 8wks DM subgroups ($P<0.05$) (Fig 2a). On the other hand, MSCs treatment improved learning and memory deficit, as both MSCs subgroups

exhibited a significant decrease in the escape latency period (P<0.05) as compared to DM subgroups (Fig 2a) but still significantly higher numbers when compared to control groups (P< 0.05) (Fig 2a). Evaluation of provisional trial test, the mean numbers of the DM subgroups showed significantly lower numbers compared to control groups (P< 0.05). However, the outcomes of DM+MSCs subgroups were significantly improved when compared to DM subgroups but still lower than control groups (P< 0.05) (Fig 2b).

Concerning fasting blood glucose levels in all groups, the mean numbers were significantly increased in DM groups according to the extent of diabetic durations (P<0.05) but with MSCs treatment the numbers were significantly decreased according to the extent of treatment indicating improvement (P<0.05) (Table 1).

Control groups	4wks DM	8wks DM	12wks DM	DM+MSCs 8wks	DM+MSCs 12wks
82.7±5.65	224±6.91	248.5±35.08	273±13.93	134.5±22.59	117±7.91

Table (1): the mean numbers of fasting blood glucose level (mg/dl) in all groups

Regarding to the morphometrical statistical analyses of the pyramidal neurons mean numbers were used to assess diabetic neuronal degeneration and regeneration by MSCs of CA1and CA3 regions.

The numbers of both regions of DM subgroups were decreased when compared to control group. Pyramidal cell reduction of CA3 was significant in 8wks and 12 wks DM subgroups while the reduction of number of cells of CA1 was only significant in12wks DM subgroup (P< 0.05). Moreover, among DM subgroups, their mean numbers were decreased according to the extent of DM duration; this reduction was significant in rats of 12wks DM subgroup (P< 0.05). There was prominent neuronal numbers improvement in DM+MSCs subgroups. However, the numbers were still significantly lower in DM+MSCs 8wks subgroup when compared to control group (P<0.05). On the other hand, marked improvement in their numbers was demonstrated in rats of DM+MSCs 12wks subgroup which was non-significant in CA1 region and only significant in CA3 region when compared to the control group (P>0.05) (Fig 3).

Haematoxylin and Eosin stained sections:

All control groups showed identical histological architecture, so they were considered as one control group.

Examination of CA1 region: The control group revealed arranged pyramidal neurons of uniform size and shape with narrow neuropil in-between, each enclosing single, rounded, large and vesicular nucleus with prominent nucleolus. Many glial cells within the molecular layer were seen (Fig 4a). On examination of 4wks DM subgroup; most of the pyramidal neurons were comparable to the control, while other few neurons were shrunken with karyorrhectic and karyolytic nuclei. vacuolations were also seen (Fig 4b). On examination of the 8 wks DM subgroup showed more neuronal damage as many neurons had clogged marginated chromatin or hyperchomatic neurons (Fig 4c). Moreover, on examination of 12wks DM subgroup exhibited severe damage as apparent losses of the neuronal regularity; many shrunken hyperchromatic nuclei and severe clumping of the neuronal processes (Fig 4d). On the other hand on examination of DM+MSCs 8wks, there were variable degrees of improvement as neurons seemed normal comparable to the control, relatively few shrunken nuclei with vacuolated cytoplasm and small areas with clumping neuronal processes (Fig 4e). Moreover, examination of DM+MSCs 12wks demonstrated more improvement as increased population of pyramidal neurons with apparent compact and regular distribution, few shrunken neurons with hyperchromatic nuclei (Fig 4f).

Examination of CA3 region: The control group revealed arranged layers of pyramidal neurons represented with vesicular nuclei, well-formed basophilic cytoplasmic Nissl granules surrounded by narrow neuropil. The molecular layer showed glial cells (Fig 5a). However in 4wks DM subgroup; the pyramidal neurons represented normal nuclear appearance, while few nuclei with clogged marginated chromatin, relatively few shrunken neurons with vacuolated cytoplasm or with peripheral karyorrhectic nuclei and narrow neuropil with few vacuolations (Fig 5b). Moreover, on examination of 8wks DM subgroup there were pleopathological changes; as pyramidal neurons were haphazardly arranged with many pyknotic nuclei, neurons with peripheral homogenous nucleus and severe vacuolation with clumping processes (Fig 5c). Also, the 12wks DM subgroup represented more degeneration as neuronal disorganization with eosinophilic vacuolated cytoplasm and severe clumping processes (Fig 5d). While on examination of DM+MSCs 8wks subgroup, there were minimal regeneration as pyramidal neurons showed normal appearance and almost normal neuropil comparable to control group. However, others were represented with karyorrlytic or pyknotic nuclei and vacuolated cytoplasm. Extraneuron amyloid deposition was noticed (Fig 5e). Additionally, DM+MSCs 12wks subgroup had regeneration signs as most of the pyramidal neurons were comparable to control with vesicular

nuclei. Only few shrunken neurons represented with hyperchromatic nucleus and areas devoid of neurons were seen (Fig 5f).

Ultrastructural study of the pyramidal neurons:

The control groups revealed the normal neuronal ultrastructure; intact cell membrane, intact cytoplasmic mitochondria with normal cristae. Euchromatic nucleus with smooth nuclear envelope consisting of bilaminar layers and well-formed nuclear pores were seen (Fig 6a). On the other hand, the pyramidal neurons of 4wks DM subgroup showed dense neuron form with irregular out line, ballooning mitochondria with disrupted cristae and cytoplasmic deposition of electron-dense bodies. The nucleus showed rounded and distinctly chromatin clumps (Fig 6b). Pyramidal neurons of 8wks DM subgroup had more degeneration as nuclear condensation, ballooned mitochondria and more cytoplasmic lipofuscin particles (Fig 6c). Additionally, pyramidal neurons of 12wks DM subgroup had severe degeneration signs as apoptotic changes as nuclear membrane indentation, cytoplasmic condensation, few neurons with highly condensed dense chromatin and vacuolations within the neuropil (Fig 6d). While on examination of pyramidal neurons of DM+MSCs 8wks subgroup, there were some regeneration as normal neuronal form with euchromatic, central, and rounded nuclei. But few cells had abnormal dense form with shrunken and electron dense nuclei (Fig 6e). Moreover, pyramidal neurons of DM+MSCs 12wks subgroup, there was more regeneration as normal neuronal ultrastructure with intact mitochondria (Fig 6f).

Discussion

DM is a common serious metabolic disease and usually associated with cerebral alternations [31]. Several studies indicated that DM negatively affects hippocampal cellular morphology, proliferation and survival, indicating function impairment of this brain region [35]. MSCs treatment had been shown as a promising modality in the treatment of the injured brain [11], beside that there were contradictions concerning the differention of MSCs into the functioning nerve cells. In the present study, the MWM results of DM subgroups indicating learning and memory impairment which become worst with prolonged durations. Similar results were obtained by Malone [23] who further explained that DM decreased hippocampal neurotransmitters release; presynaptic synaptophysin and postsynaptic density protein which are the cause of memory and learning deficiency. However in MSCs treated subgroups, there was

prominent functional improvement which got better with prolonged treatment duration, indicating progressive improvement of neuronal function. These results were agreement with the findings of Chen [5] who detected that MSCs increase production of neorotrophic factors and enhancing neuronal plasticity of the injured brain.

Alongside with these functional impairment and improvements, the histological and ultrastructural examination showed hippocampal degenerative changes accompanying DM were variable regarding the affected region and also related to the duration of DM exposure. Minor apoptotic alternations were detected in 4wks DM subgroup in both CA1and CA3 regions, in agreement with findings Zhao [36] who study CA1 and dentate gyrus. But, more changes were mainly seen in CA3 region of 8 wks DM subgroup concurring to findings of Jafari [12], Kamal [14]. Furthermore, when DM was prolonged for 12 wks, progressive changes consequences were demonstrated mainly in CA3 region. These results were in harmony with findings of long-term study of Yang [33].

These apoptotic degenerative changes were confirmed by more accurate statistical morphometric data as all DM subgroups showed reduction of number of pyramidal neurons which was more significant in 12 wks than 4 wks and 8 wks indicating the progressive degenerative changes.

The findings of earlier diabetic neuronal apoptotic changes detected in the current work were explained by Zhao [36] and Orlovsky [27] as excessive production of nitric oxide in the hippocampal areas which has neurotoxic effect inducing neuronal damage and apoptosis. Also, the findings of progressive diabetic neuronal apoptotic changes were further explained by Yang [33] who established that long-term DM induces oxidative stress and lipid peroxidation with impairment of membrane functions. This occurs through decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. Additionally, Li [19] clarified neuronal cells' apoptosis, as diabetic hyperglycemia leads to opening of mitochondrial permeability transition pores which directs up expression of caspase 3 and caspase 8 that play a central role in the execution-phase of cell apoptosis.

Seeking for MSCs possible improving role, fluorescent-labeled MSCs were recognized in the hippocampus after their systemic injection according to each DM+MSCs subgroups. In the current work, the histological and ultrastructural examination of MSCs treated subgroups showed variable amelioration of the pathological changes and increase of neuronal numbers regarding the

extent of MSCs treatment and degree of damage produced by DM on the hippocampus. MSCs treatment for 4 wks after 4 wks of diabetic neuronal damage led to improvement of apoptotic changes especially more in CA1 region than in CA3 region. Moreover, this improvement was more apparent with prolonged duration of MSCs treatment to 8 wks after 4 wks of diabetic neuronal damage. There was obvious regression of neuronal apoptotic changes of CA1 and CA3 with relatively normal pyramidal neuronal structure.

Those histological changes were further documented by the statistical analysis of the morphometric counting of pyramidal neurons of both regions. There were increase in neuronal numbers especially with prolonged MSCs treatment in both regions, but this increase was still lower than control numbers. In the reviewed literatures, no studies examined the effect of i.v. MSCs administration on hippocampal neuronal diabetic degeneration. However, the MSCs neuronal improvement of CA1 region for short duration in the current work corresponds to findings reported by Kumar [16] who studied cold stress as a cause of hippocampal neuronal degeneration. Also, the improvement for longer duration of MSCs treatment was supported by the results obtained by Ye [34] who studied the neuronal degeneration in Parkinson disease, as there was neuronal recovery after 10 weeks of MSCs transplantation. While, the moderate improvement observed in the current work in CA3 region was in harmony with the findings of Matchynski-Franks [24] who studied MSCs effect after 10 weeks of transplantation in a mouse model of Alzheimer's disease.

Discussing the improving role of MSCs in the current study to ameliorate diabetic neuronal degeneration and functional impairment, many authors explained their effects in the injured brain; Mezey [25] concluded that human MSCs could enter the human and rat brains and generating neurons especially in the hippocampus and cerebral cortex. Moreover, Zhang [35] found that transplanted MSCs could engraft into brain tissue and differentiate in vivo into neurons and glial cells. In contradiction, Sanchez-Ramos [30] described that a small proportion of MSCs derived cells differentiated into neuron-like cells. Those findings explained by Crigler [7] and Munoz [26] as MSCs implantation into the hippocampus did not proliferate, but they greatly increased proliferation, migration and differentiation of the endogenous neuronal stem cells after eight weeks of neuronal degeneration. Prockop [28] described that MSCs could promote the neurogenesis of primary neural progenitors and survival of neural cells by expressing neurotrophic factors. Also, Calió [4] reported that MSCs could decrease neuronal oxidative stress and apoptosis

changes by enhancing the anti-apoptotic activity and antioxidant protection. These results were very promising for the development of a new therapeutic strategy for patient exhibiting diabetic neuronal degeneration and learning and memory impairment by using MSCs treatment.

Conclusions

MSCs administration ameliorates diabetic neuronal degeneration and learning and memory impairment mainly through promoting neurogenesis, alleviating oxidative stress and also through its anti-apoptotic effects. So, bone marrow derived MSCs treatment may exert a successful curative role in patients suffering from diabetic neuronal complications and improving their quality of life.

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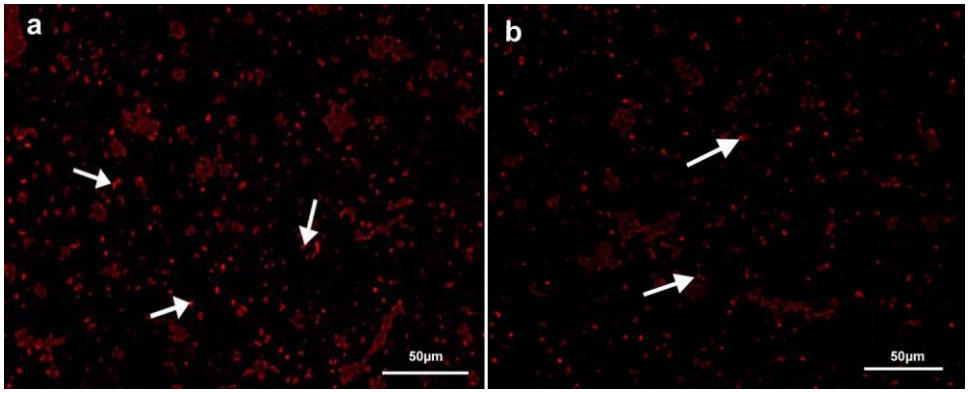

Figure 1. Hippocampal fluorescent-labeled MSCs, immunostained with BrdU antibody in: a: DM+MSCs 8wks group and b: DM+MSCs 12wks group Bar= 50µm.

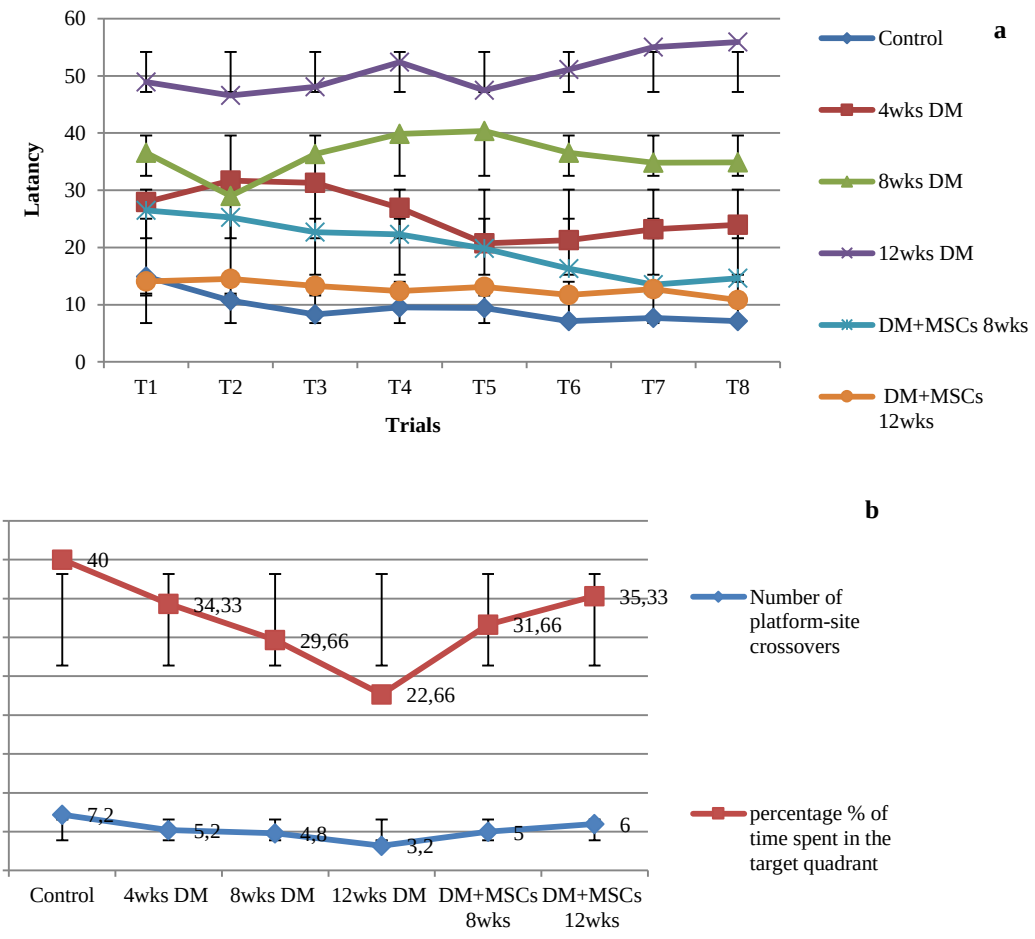

Figure 2. A. The means of escape latency periods during all MWM training trials in all subgroups; **B.** The provisional trial results (the mean numbers of the platform-sites crossovers and the percentage of time spent in the target quadrant) in all subgroups.

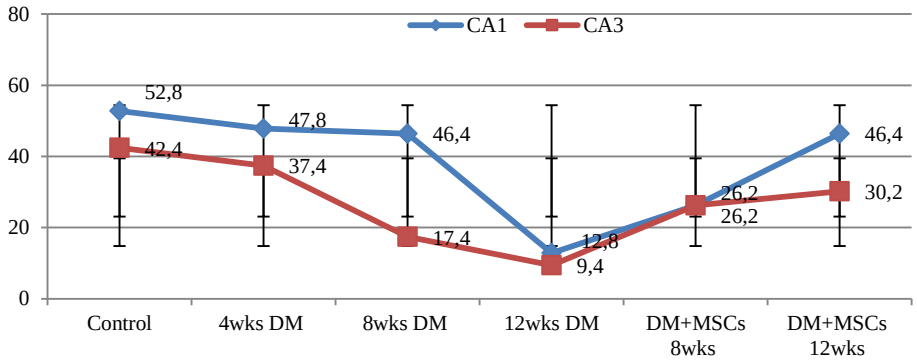

Figure 3. The mean numbers of the pyramidal neurons in regions (CA1 and CA2) in all subgroups.

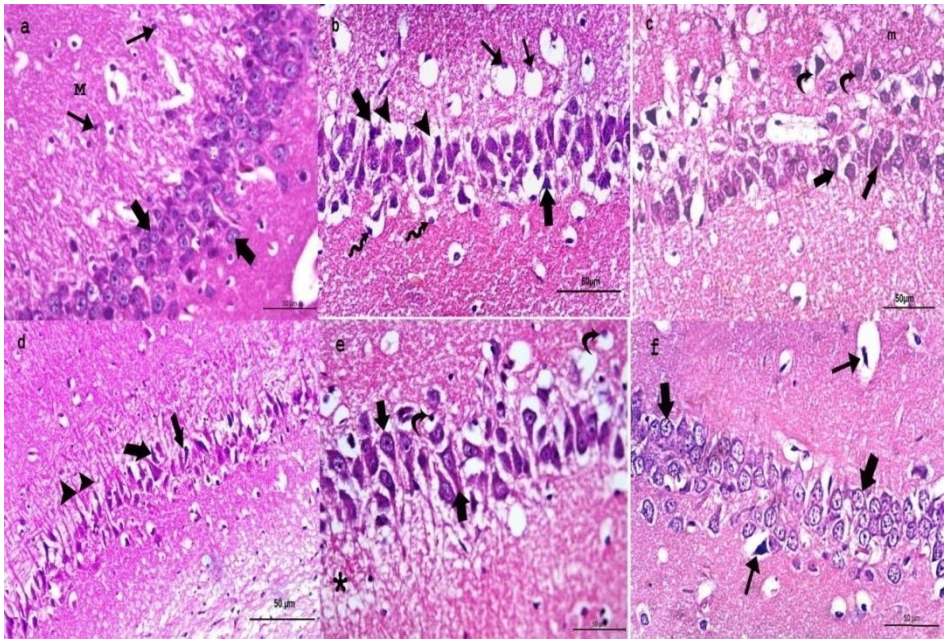

Figure 4. CA1 region a: control group: arranged pyramidal (P) with vesicular nucleus (thick arrows) and glial cells (thin arrows) in molecular layer (M). b: 4wks DM: karyorrhectic (thick arrows), karyolytic (zigzag arrow) and shrinkage nuclei (thin arrows), vacuolations (arrowheads). c: 8wks DM: clogged marginated chromatin (arrows) and hyperchomatic neurons (curved arrows) in molecular layer (M). d: 12wks DM: severe degeneration as, many shrunken nuclei (arrows) and

clumping neuronal processes (arrowheads). e: DM+MSCs 8wks: neuronal improvement (thick arrows), few shrunken nuclei (curved arrows) and clumping processes (asterisk). f: DM+MSCs 12wks: progressive neuronal improvement (thick arrows), few shrunken neurons (thin arrows) Bar= 50µm.

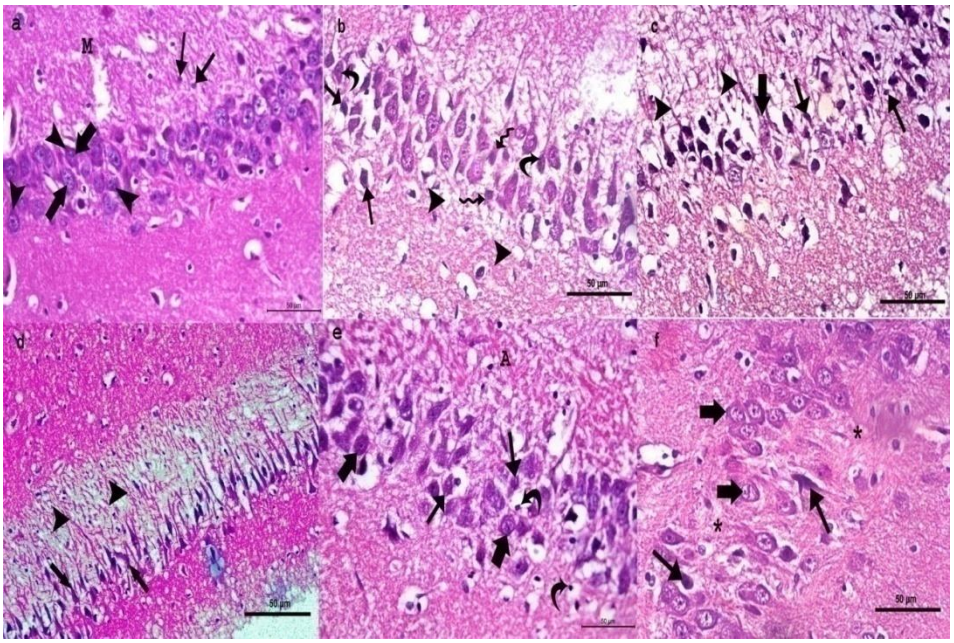

Figure 5. CA3 region a: control group: arranged pyramidal neurons (thick arrows), well-formed Nissl granules (arrowheads) and glial cells (thin arrows) in the molecular layer (M). b: 4wks DM: clogged marginated nuclear chromatin (curved arrows), karyorrhectic nuclei (zigzag arrows), vacuolated cytoplasm (thin arrows) and neuropil vacuolations (arrowheads). c: 8wks DM: pyknotic nuclei (thin arrows), neuron with homogenous nucleus (thick arrow) and clumped processes (arrowheads). d: 12wks DM: more degeneration as eosinophilic cytoplasm (thick arrows) and clumping processes (arrowheads). e: DM+MSCs 8wks: minimal regeneration as neuronal vesicular nuclei (thick arrows), karyorrlysis (thin arrows) or pyknotic nuclei (curved arrows) and amyloid depositions (A). f: DM +MSCs 12wks: more regeneration as pyramidal neurons with vesicular nuclei (thick arrows), areas devoid of neurons (asterisks) and few shrunken neurons (thin arrows) Bar= 50µm

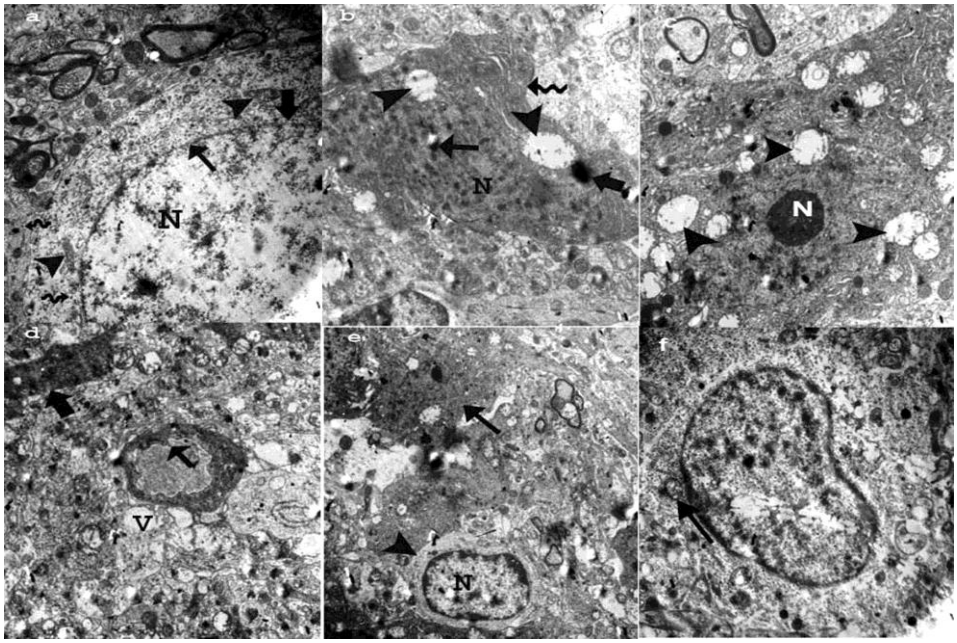

Figure 6. Pyramidal neurons a: control group: normal neuron with intact mitochondria (arrowheads), lysosomes (zigzag arrow), euchromatic nucleus (N), smooth envelope (thin arrow) and well-formed nuclear pores (thick arrow) Bar= 2µm. b: 4wks DM subgroup: irregular neuronal out line (zigzag arrow), ballooning mitochondria (arrowheads), electron-dense body (thick arrow), the nucleus (N) with chromatin clumps (thin arrow) Bar= 2µm. c: 8wks DM subgroup: more degeneration as nuclear condensation (N), many ballooning mitochondria (arrowheads) Bar= 2µm. d: 12wks DM subgroup: severe degeneration as condensation with membrane indentation (thin arrow), highly condensed dense chromatin neuron (thick arrow) and vacuolations (V) Bar= 2µm. e: DM+MSCs 8wks subgroup: some regeneration as normal neuronal form (arrowhead) with euchromatic nucleus (N), few abnormal dense neurons (arrow) Bar= 2µm. f: DM+MSCs 12wks subgroup: more regeneration as normal neuronal ultra-structures with intact mitochondria (arrow) Bar= 2µm