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ORIGINAL RESEARCH ARTICLE

Nanotubes impregnated human olfactory bulb neural stem cells promote neuronal differentiation in Trimethyltin-induced neurodegeneration rat model†

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Abstract

Neural stem cells (NSCs) are multipotent self-renewing cells that could be used in cellular-based therapy for a wide variety of neurodegenerative diseases including Alzheimer's diseases (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS). Being multipotent in nature, they are practically capable of giving rise to major cell types of the nervous tissue including neurons, astrocytes and oligodendrocytes. This is in marked contrast to neural progenitor cells which are committed to a specific lineage fate. In previous studies, we have demonstrated the ability of NSCs isolated from human olfactory bulb (OB) to survive, proliferate, differentiate, and restore cognitive and motor deficits associated with AD, and PD rat models, respectively. The use of carbon nanotubes (CNTs) to enhance the survivability and differentiation potential of NSCs following their in vivo engraftment have been recently suggested. Here, in order to assess the ability of CNTs to enhance the therapeutic potential of human OBNSCs for restoring cognitive deficits and neurodegenerative lesions, we co-engrafted CNTs and human OBNSCs in TMT-neurodegeneration rat model. The present study revealed that engrafted human OBNSCS-CNTs restored cognitive deficits, and neurodegenerative changes associated with TMT-induced rat neurodegeneration model. Moreover, the CNTs seemed to provide a support for engrafted OBNSCs, with increasing their tendency to differentiate into neurons rather than into glia cells. The present study indicate the marked ability of CNTs to enhance the therapeutic potential of human OBNSCs which qualify this novel therapeutic paradigm as a promising candidate for cell-based therapy of different neurodegenerative diseases. This article is protected by copyright. All rights reserved

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Introduction

Alzheimer's diseases (AD) is a devastating neurodegenerative diseases with no effective cure till now. AD is characterized by cognitive and memory impairments mainly due to loss of cholinergic neurons in the hippocampus, and brain cortex (1). The AD pathology involves the basal forebrain cholinergic system, which provide cholinergic input to the neocortex, the hippocampus (2) and the cholinergic neurons of the nucleus basalis of Magnocellurais (NBM) in rodent and nonhuman primates which is the analogous to nucleus basalis of Mynert (NBM) in human (3)**.** The loss of neurons and astrocytic gliosis that had been encountered in the aforementioned areas, are the main reasons that induce aggressive loss of memory function (4)**.**

Neural stem cells (NSCs) are multipotent cells resides in different brain regions such as sub ventricular, and sub granular zone of hippocampus (5), and they hold a great opportunity for the future of treatments of neurodegenerative diseases such as AD, PD, ALS, and MS (6).

Obtaining NSCs from adult human olfactory bulb (OB) would allow auto transplantation for traumatic and neurodegenerative diseases, and thus provide biosafety, histocompatibility, and does not raise the ethical issues due to the use of embryonic material (7).

In previous studies, we have isolated NSCs from the human OB. These OBNSCs we proliferated in culture, and then engrafted in three rat models for AD, PD, and spinal cord injury (SCI). These studies demonstrated the ability of engrafted human OBNSCs to proliferate, differentiated into different neuronal and glial elements, and to restore cognitive and motor deficits associated with AD (8), and PD (9). In our SCI study, despite the marker ability of the engrafted human OBNSCs to proliferate and differentiate following transplantation, no significant improvement was recorded in motor function (10). An important feature for the engrafted human OBNSCs in the aforementioned three independent studies was the absence of any tumor formation, a crucial finding that might qualify OBNSC for safe application at the clinical level (8-10). Engrafted NSCs in different neurodegenerative insults proliferated, migrated and differentiated into different neuronal subtypes including cholinergic neurons (10). Other positive influences induced by NSCs transplantation included their ability to enhance the survival of existing neuronal circuitry through immune modulation, neurotrophic influence (11, 12) and enzyme replacement, and migration (11-17).

Recently, the use of nanomaterials such as single carbone nanotubes (CNTs) have been suggested as effective substrate for different cellular sources. Such CNTs have been reported to provide a scaffolds to control the proliferation and differentiation potential of loaded cells. Moreover, such biological scaffolds

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could also be loaded with different effective molecules such as growth factors and cytokines (18). Specific to the central nervous system architecture and molecular uniqueness, neural cells adhere to specific extracellular matrix (ECM) formed of fibrillary proteins. This dynamic ECM interacts with different neuronal and glial elements to modulate cell growth, survival, and differentiation potential. Moreover, a loss of contact between the ECM and neuronal cellular elements may induce cell apoptosis (19). It is for this reason that attention has recently been given to different nanostructured biomaterials that mimic the unique histo-architecture of CNS tissue.

The unique properties of CNTs which are formed of single or multiple sheets of graphene, their mechanical strength, stability, light weight, and rich electronic criteria (20) have attracted the attention of several neuroscientists to use CNT as scaffolds for neural cell growth. CNTs could be either used alone or after addition of various chemical groups, and in both cases they are biocompatible with neuronal cell proliferation, growth and adhesion.

Besides neural cells, other cell types such as stem cells and glia cells can successfully grow on CNT scaffolds. A marked modulation in neuronal growth has been demonstrated by different types of functionalized CNTs. The neurite growth of hippocampal neurons was enhanced by growing on positively charged CNTs, and the mechanical properties and conductivity of CNTs have been shown to modulate neuronal morphology. Provision of CTNs to the sites of CNS injuries enhances the regeneration of lost synaptic connections, induce neurite outgrowth which might aid in the regeneration process (21). Despite promising outlook for such novel biological scaffolds as promising direction for enhancing cellular based therapy, assessment of such direction of therapeutic intervention for mammalian neurodegenerative changes is still far from complete (22).

Intraperitoneal injection of TMT selectively induces neuro-inflammations, neuronal death which is reflected clinically as cognitive impairment and seizures (23, 24). In particular, TMT-induced neurodegeneration has been found useful in the study of AD (25). In the present study, our previous findings regarding the marked ability of human OBNSCs to proliferate, differentiate, and restore lost neural functions in *in vivo* rat model of AD, and PD, and the ability of CNT to enhance neurite outgrowth have prompted us to investigate the therapeutic potential of nanotubes impregnated OBNSC and their ability to restore behavioral and cognitive deficit in a TMT-induced rat model of neurodegeneration.

Materials and Methods

Male Wister albino rats weighing 220 ± 25 g were used for the experiments. Animals were acclimatized to the animal house conditions (12:12 hr. light/dark cycle and under a temperature-

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controlled environment of 24 ± 1 ° C) for a week. Standard pelleted feed and water were provided *ad libitum*. All experimental procedures were conducted according to the Institutional Animal Care and Use Committee guidelines at the University of Mansoura, Egypt. The animals were divided into three groups.

Groups	**Experimental protocol**	**Animals**
Group-I	Control	10
Group-II	Lesioned in which 6mg/kg body wt. of Trimethyltin (TMT) was injected intraperitoneally	16
Group-III	Lesioned with Nanotubes impregnated adult human olfactory bulb neural stem cells (CNT/OBNSCs) transplantation	20

Experimental induction of trimethyltin chloride (TMT) cognitive dysfunction model

Trimethyltin chloride (TMT) (Sigma, Germany) was dissolved in 0.9% saline and intraperitoneal (ip) injected at a dose of 6mg/kg body weight according to previously reported method (26)**.** The rat returned to its cage and allowed to rest at 12:12 hr. light/dark cycle and under a temperature-controlled environment of 24 ± 1 ° C for 4 weeks. Standard pelleted feed and water were provided *ad libitum*. The testing of different variants such as memory and exploratory task were conducted at the end of the 4 weeks.

Behavioural Assessment (Morris Water Maze Test)

The learning and memory functions of TMT-treated and CNTs/OBNSCs engrafted rats were assessed using Morris water maze (MWM) test (27). To exclude any effects of TMT on the visual acuity of rats, we used a visible platform that was extended 1 cm above the water level of MWM. Rats were habituated to the MWM swimming pool by allowing them to swim freely (four trials from different directions North, West, East and South) for 60 seconds to reach and climp the visible platform.

Next, three blocks of five trials (a total of 15 trials) for cue training were conducted during which no cues were provided for the rats except a guide for the platform. Two scores were recorded for each trial: a. Escape latency (time needed to reach submerged platform), and b. Quadrant time (time spent in the platform's quadrant after removal of the platform). The time window used for recording these scores were 7th day post-training, four and twelve weeks after induction of cognitive dysfunction by TMT, and after CNT/OBNSCs engraftment.

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Culturing of Human Olfactory Bulb NSCs

Frozen vials of human OBNSCS were obtained as gift from Institute of Neurosurgery, Catholic University, Rome, Italy. Informed consent was obtained according to protocols approved by the Ethical Committee of the Catholic University. After thawing, the cells were dissociated using 1% Accutase (Invitrogen), and were cultures in DMEM/F12 (1:1) serum-free medium (Invitrogen, Carlsband, CA) containing L glutamine 2 mM, glucose 0.6%, putrescine 9.6 ug/ml, progesterone 0.025 mg/ml, sodium selenite 5.2 ng/ml, insulin 0.025 mg/ ml, apo-transferrin sodium salt 0.1 mg/ml, sodium bicarbonate 3 mM, Hepes 5 mM, BSA 4 mg/ml, heparin 4 ug/ml, human recombinant EGF (20 ng/ml; PeproTech, Rocky Hill, NJ), human recombinant bFGF (10 ng/ml; PeproTech), and LIF (20 ng/ml; Immunological Sciences, Rome, Italy) (28).

Transfection and infection

Lipofectamine reagent (Invitrogen) were used to transiently transfect human embryonic kidney (HEK)-293T cells in log-phase growth with LV-GFP plus helper plasmids to produce virions (29). Two days after transfection, media containing virions was collected and transferred directly onto OBNSCs. Lentiviral infection was performed in the presence of polybrene solution at 8 mg/ml (Sigma–Aldrich). Antibiotic G418 (Euroclone) was added to the cells at 400 mg/ml over time for OBNS/PC selection and maintenance.

CNT/OBNSCs preparation

After reaching 80% confluence, the formed OBNSCs neurospheres were collected, and dissociated into single cells by accutase treatment. They were then suspended in artificial CSF (Sigma-Aldrich) at 50,000 cells/µL, and were kept on ice until transplantation. Cell viability was checked by trypan blue exclusion test, and the total number of cells was determined using a hemocytometer.

Multiwalled Carbon Nanotubes "MWCNT" (Chengdu Organic Chemicals Co., Ltd, Chinese Academy of Sciences) with outer diameter "OD"~50-60 nm and length ~10-20 µm, dissolved in Phosphate buffer saline (PBS) at concentration up to 100µg/ml. (As MWCNTs are hydrophobic so, we need to make a homogenous suspension of them using mechanical way “magnetic stirrer” to be equally distributed in the solution without using any chemical chargeable solvent for dispersion the MWCNT which may affect the cellular viability)

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Four weeks following TMT administration, the lesioned rats were anesthetized and stereotaxically injected at CA1region with 4µL of CNT/OBNSCs mixture(1µl MWCNT+3µl OBNSCs"150,000cells" at the following coordinates: (±2.5mm (ML), -3.5mm (AP), 2.5 mm (VD)).

Animals and surgery for CNT/OBNSCs transplantation

Atropine sulfate was given as pre-anesthetic medication, and the rats were anaesthetized by i.p. injection of Ketamine (60 mg/kg body weight) and Xylazine (20 mg/kg body weight). The animals were fixed into a stereotaxic frame, and an incision was made to expose the skull. A bilateral infusion of CNT/OBNSCs (4µl) were injected into the CA1 region using 10-µl Hamilton syringe at the following coordinate from bregma: ± 2.5mm medio-lateral (ML), -3.5 mm anterio-posterior (AP), 2.7 mm ventro-dorsal (VD) according to the brain atlas of (30). The injection was given over a 5 minutes timeframe, and needle was left in the injection site for 2 minutes to avoid oozing of injected cells. Rats we subjected to a post-operative care regime that included I.M. injection of antibiotics (penicillin (20,000 U), analgesic (nalorphine), I.P. injection of glucose for 3 days. The rat returned to its cage and allowed to rest with special post-operative care and fully controlled temperature and adjusted light /dark cycle with fine and easily digestible food and dry, clean and soft litter. Clean drinking water *ad lipitum.*

Animals receiving grafts were immunosuppressed via daily subcutaneous injections of cyclosporine (10 mg/kg bw; Biomol), started one day before grafting and finished on the day of sacrifice.

Samples collection

The lesioned group samples were collected ; three rats were euthanized after 2, 4, 8 and 16 weeks post TMT administration

The CNT/OBNSCs engrafted group samples were collected; four rats were euthanized after1, 2, 4, 6 and 8 weeks following CNT/OBNSCs transplantation. The brain was dissected, and the hippocampi were collected and fixed in 4% paraformaldehyde, and were processed for paraffin sectioning.

Histological Analysis

Tissue fixation and processing for light microscope (LM)

Collected hippocampi were fixed in 10% neutral buffered formalin, and 4% paraformaldehyde in 0.1 M phosphate buffer for 24h, dehydrated in ethanol, cleared in xylene and impregnated and embedded in paraffin. Five to seven µm section were cut on a rotatory microtome and mounted on

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glycerol-albumin-coated glass slides. The slides were stained with haematoxylin–eosin (HE) for general histological studies, Cresyl Violet stain for Nissl substance **(9).**

Enzymatic Immunohistochemical Assessment

Ten micron (μm) thick serial brain sections were prepared starting from the needle entry site to 1.0 mm anterior and posterior to injection site. Tracing of GFP-labelled human OBNSCs following engraftment was conducted using Anti-GFP (1:2000, rabbit, N-Terminal, Sigma-Aldrich). Visualization of the primary antibodies was done using Biotin-conjugated goat anti rabbit secondary antibody labeled with poly-horseradish peroxidase (HRP) conjugate and metal enhanced DAB to develop the signal (brown color).

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Stereological quantification

The stereological quantification was performed according to our previous studies (8). In brief, the engrafted GFP-OBNSCs/CNTs were counted using light microscope using enzyme immunohistochemistry against GFP. The types of differentiated cells was recognized based on nuclear morphology after heamatoxylin nuclear counterstain. The GFP positive cells were counted 1 mm segments rostral and caudal to the injury site. Cells were counted in four consecutive sections using a 40X objective. The number of labeled cells was counted for each type using ImageJ Version 10.2 software. The numbers of labeled cells was expressed as a percentage of the total number of GFP-positive cells counted in each individual type. For each cell analyzed, the values were averaged together to get the final percentage.

Statistical analysis

SPSS 16.0 software package was used to process all collected data, and the *t* test was used for comparisons between 2 groups. Multiple group comparisons were achieved by single-effect analysis of variance (one-way ANOVA) (Student-Newman-Keuls test). All data are presented as mean ± standard deviation (SD). Statistical significance was defined as $p<0.05$.

Results

The TMT treated animals showed inflammatory changes, death of neurons and glia cells (Fig 1. A, B, C). Two weeks post TMT administration, the total number of pyramidal neuron revealed loss of cellular architecture (Fig 1. D, E). Some granule cells showing pyknotic nuclei, cell bodies are shrunken, most cells showing light and not densely packed nuclei (Fig. 1 F, G). CA3 neurons showed vacuolation, and neuronal death with glia cells proliferation (Fig. H). Dissolution of Nissl granules was also revealed (Fig. 1 I).

Behavior results (Morris Water Maze Task)

The mean latency time in four consecutive days to find and locate the hidden platform in TMT-Lesioned group was increased significantly ($p<0.05$) after four weeks. While compared to TMT lesioned group after twelve weeks that show significance improvement in memory with decrease escape latency. The mean escape latency for non-lesioned rat after training ~3.9 seconds while for

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TMT lesioned group after four weeks~19.3seconds ,but after twelve weeks post TMT injection the escape latency improved to ~ 7.8 seconds (Fig 2)**.**

The total time that rats spent in goal quarter during probe trial on the fifth day of testing (the platform has been removed) was decreased significantly ($p < 0.05$) in TMT group after one weeks when compared with TMT group after twelve weeks. The mean quadrant time for non-lesion rats after training was ~32.9 seconds while for TMT lesioned group after four weeks was ~24.2 seconds, and, ~29.6 seconds after twelve weeks post TMT injection (Fig 2)**.** To be certain that the TMT didn't impairing the animals' vision or changing the motivation to escape from water; in which affect the task results, the two groups were run on cued version of the task.

MWM Test for CNTs/OBNSCs engrafted Group

The results of the test is not reliable because the significance improvement of learning ability and memory of rats due to endogenous neurogenesis pathway before engraftment of OBNSCs/CNTs (Supplementary Figure S1). So, we cannot prove that the improvement in memory and cognition from our engrafted OBNSCs/CNTs because there were two different variables, the endogenous and exogenous neurogenesis. Based on this observation, we restricted our assessment to test the ability of engrafted OBNSCs/CNTs to survive, proliferate, and differentiated into different types of neuronal, and glial elements using immunohistochemical protocol. In our opinion assessment of the survival, proliferation and differentiation potential of the engrafted GFP-OBNSCs/CNTs is the most important and decisive findings if we want to provide a conclusive evidence about the potential use of OBNSCs/CNTs for cell-based therapy. Here, we traced our engrafted OBNSCs/CNTs based on their positive reactivity to GFP, and we differentiated between neuronal and glial cells based on nuclear morphology which is one important criterial to differentiate between neuronal and glial cells at LM level.

Immunohistochemical Assessment for the Engrafted GFP-OBNSCs/CNTs

In the present study, we had genetically engineered the human OBNSCs to overexpress green fluorescent protein (GFP) to trace the engrafted OBNSC, and to differentiate between our engrafted (exogenous), and endogenous neuronal and glial elements. The performed immunocytochemical using anti-GFP antibodies confirmed the ability of engrafted cells to survive, proliferate, and differentiated into different types of neuronal and glial cells following engraftment of GFP-OBNSCs/CNTs in the CA1 region of hippocampus. The GFP-OBNSCs/CNTs survived in the lesion environment for more than eight weeks after implantation, and no tumor formation was recorded during the lifetime window of the eight weeks.

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Brain sections were processed for immunostaining to determine the grafted cells by using Anti-GFP. Using enzymatic immunohistochemistry microscopy, GFP-expressing cells were found to migrate from injection site into the deeper layers of the tissue. One week post GFP-OBNSCs/CNTs engraftment, GFP-expressing cells were detected in various depths of hippocampus where they appeared as GFP-positive dark brown granules in their cytoplasm (Fig 3. A, B, C). After two weeks, GFP-positive cells with morphological criteria suggestive of mature neurons, astrocytes, and oligodendrocytes were identified. **(Fig. 3 D).** The number of these cells increased by 4 weeks, and by six weeks, GFP neuronal and glial cells (astrocytes, and oligodendrocytes) were identified within different hippocampal fields indicating their migration **(Fig. 3 E, F). By** eight weeks post the engraftment, GFP-positive cells with morphological criteria suggestive of mature neurons were recognized **(Fig. 3 G), and (Figs. 4 A, B, C, D, E, F, G, H, I).** During the eight weeks' time window of the present study, 60, 17, and 23% of the engrafted cells were differentiated into neurons, oligodendrocytes, and astrocytes (supplementary Figure 2).

Histological assessment following engraftment of GFP-OBNSCs/CNTs revealed gradual restoration of pyramidal neuron within CA1 normal with very little necrotic neurons with mild improvement in pyramidal layer thickness**.** By eight weeks restoration of normal architecture and pyramidal cell layer were observed **(Fig. 5 A, B, C, D, E, F).** The granular layer of DG restored its normal architecture and cellular content and thickness **(Fig. 5 G, H, I).**

Four weeks post the Engraftment of GFP-OBNSCs/CNTs, the CA3 neurons and oligodendrocyte increase its number with remnant of necrotic cells **(Fig. 6 A). By** eight weeks restoration normal structure were seen **(Fig. 6 B).** By Eight weeks, restoration of pyramidal cell layer thickness (increase in thickness) to normal and restore cellular architecture with blue stain cytoplasm due to Nissl granules **(Fig. 6 C, E, F, G, H, I, J, K, L)**

Discussion

Here, we developed an animal model of Alzheimer's disease (AD) using TMT to elucidate the potential utility of human olfactory bulb NSCs carried on carbon nanotubes as scaffolds for cellular-based therapy of neurodegenerative changes, and their ability to increase the survival of grafted NSCs.

The OBNSCs were infected with lentivirus transducing GFP gene, according to protocol set up by (29). Martinez-Serrano and Bjorklund (31) and Brustle et al. (32) demonstrated that NSC have the capacity to grow indefinitely and are able to differentiate into three major cell types of CNS,

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neurons, astrocytes and oligodendrocytes. In human, existence of NSCs with multipotent differentiation capability has also been reported in embryonic and adult human brain (33, 34).

In many of the previous studies, the beneficial effects of NSCs transplantation had never reached the full histological and functional recovery. Several factors such as insufficient number, inability of the engrafted NSC to reach the intend site of injury, and inappropriate niche or microenvironment at the site of lesion might contribute to this limitations. This had prompted us to combine several technology with the aim to enhance the regenerative capacity of NSCs. Toward this objective, previous studies had infected NSCs with human nerve growth factor (hNGF) which were reported to enhance the proliferation capability of NSCs. Administration of nerve growth factor (NGF) in the CNS tissue is a potential treatment for preventing degeneration of basal forebrain cholinergic neurons in AD (35).

To tackle the problem of in ability of NSCs to reach the intended pathological site, we co-engrafted our cells with CNTs which would provide a scaffolds to carry the NSCs to the site of injury providing a mechanical support and help the adhesion and differentiation of cells. It could also allow the transitions of electrical impulses and connectivity between cells. Finally, we have also infected our OBNSCs with GFP as a tracer marker to follow up the proliferation, and differentiation ability of engrafted OBNSCs.

To test the efficacy of OBNSCs/CNTs to restore neurodegenerative changes, we developed a TMT-neurodegeneration model by intraperitoneal administration of TMT in our rat model (26). After two weeks of TMT administration, the neurodegenerative changes were associated with a pronounced inflammatory response, neuronal death, loss of Nissl granules, gliosis, and congestion of blood capillary. Extensive cellular necrosis of pyramidal neuron of CA1 and granular layer of DG and neuron of CA3 were also revealed. The mechanism by which TMT induce neuronal necrosis is still under dispute. TMT might induce its neurotoxic effects through glutamate excitotoxicity, intracellular calcium overload and impairment of neurotransmission (36, 37), along with oxidative stress and HPSP activation (38-41).

In the present study, The TMT-lesioned rats have shown a profound Morris water maze deficit after 4 weeks post TMT injection. TMT administration was associated with impairment of spatial memory and cognitive functions creating cytotoxic neurodegeneration model ready to be used for assessment the therapeutic potential of OBNSC/CNTs-based therapy, and their ability to restore cognitive and memory deficit induced by TMT administration.

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To avoid any problems with the niche or microenvironment of the NSCs we start the engraftment of OBNSCs/CNTs after subsidence of the inflammatory phase post TMT injection to avoid the inflammatory mediators and any substances or the remnants of the neurotoxicant that may affect the implanted cells. The present study revealed that following engraftment, the OBNSCs/CNTS survived in the lesion environment for more than eight weeks. Moreover, no tumor formation was recorded during the lifetime window of the current study.

Next, we performed histological and immunohistochemical assessment to evaluate the way by which engrafted GFP-OBNSCs/CNTs were able to restore the hippocampal histo-architecture. The transplanted OBNSCs/CNTs were assessed 1, 2, 4, 6 and 8 weeks following implementation into hippocampal formation. Sections from the two hippocampi were processed for double immunostaining to trace the fate of the engrafted GFP-OBNSCs by using Anti-GFP and cell type-specific markers. Using enzymatic immunohistochemistry, GFP-expressing cells were localized initially near the site of injection then migrate from injection site into the deeper layers with time.

Two weeks later, GFP-positive cells with morphological criteria suggestive of mature neurons, astrocytes, and oligodendrocytes were identified within the stratum pyramidale of the hippocampus, beside normal endogenous non GFP-positive neuronal and glial cells. Six week after transplantation, positive GFP neuronal and glial cells (astrocytes, and oligodendrocytes) within different hippocampal fields were identified indicating their migration away from the primary site for engraftment.

The mechanism by which the transplanted GFP-OBNSCs/CNTs have exerted a positive influence both at cognitive and histo-architecture level are still not fully understood. In the present study, the modified GFP-OBNSCs survived and differentiated into different types of neuronal and glial elements including neurons, astrocytes, and oligodendrocytes. The transplanted cells migrate within damaged areas and promote repair or neuroprotection via cell replacement, integration and/or neuroprotection. Also the endogenous pathway of neurogenesis reported to improve memory and cellular architecture following TMT injection (42).

The role of hNGF in enhancement the regenerative capacity of NSC has been described before. Engraftment of fibroblasts encoding NGF gene in the primate brain had rescued the degenerating basal forebrain cholinergic neurons. So, genetic engineering of different cell sources to over-express the hNGF genes seems to be a promising strategy for direct intraventricular delivery of hNGF into

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the brain tissue (43). In our previous study, human olfactory bulb neural stem cells (OBNSC) with transgenic expression of human nerve growth factor (hNGF) were demonstrated to restore cognitive deficit and neuronal losses associated with ibeitonic acid (IBO) AD rat model. The OBNSCs were stably transduced with hNGF and enhanced green fluorescent protein (eGFP) genes (GFP-OBNSCs) by using a recombination lentiviral expression vectors (8).

Here, the combined use of OBNSCs with CNTs seems to enhance the proliferation and differentiation potential of OBNSCs as revealed by their ability to restore cognitive and neurodegenerative changes associated with ATM administration. Previous studies demonstrated that the physical and mechanical support provided by CNTs help the seeded NSCs to adhere and differentiate at the site of lesion (21). The CNTs allowed for a favorable substrate for the neural stem cells for adherence so, keeping them in the local environment that they could be stimulated to differentiate into neurons by the conductive carbon nanotubes (44). These findings were in harmony with those reported in the present study where we found that most of the engrafted cells were differentiated into neuron. CNTs are not biodegradable so it could be used as an implant where long-term molecular cues for neurite outgrowth are necessary, such as in regeneration after spinal cord or brain injury. Moreover that, their chemical prosperities are not interfering the cellular proliferation and differentiation (45). The use of multiwalled CNTs reduced glial cells (gliosis) specially to astrocytes which is the major component of glial scar and this lead to overcome the major obstacle in tissue repair and regeneration after injury in CNS (45). This could explained the marked tendency of our engrafted GFP-OBNSCs to differentiate into neurons rather than astrocytes. The use of CNTs coated with neurotropic factor like NGF would help in neurite outgrowth of neuron (46). Finally the unique electrical prosperities of CNTs might help interaction and allow electrical impulses to pass through tissue engineered scaffold, and thus help to restore function (47). Taken together, the present study revealed that human OBNSCs expressing NGF carried on CNTs scaffolds are promising candidates for the cell-based gene therapy for neurodegenerative diseases.

To underlie the effects of nanotube alone or cells alone, we compared the outcome of the additive/synergistic effects of the two modalities with our previous findings (8, 9) regarding the use of human OBNSCs alone without CNTs scaffolds. The enhanced neuronal differentiation potential observed here might confirm our findings regarding the role of CNTs scaffolds in enhancing the tendency of engrafted GFP-OBNSCs to differentiate into neurons rather than astrocytes.

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Conclusion

Using a combined treatment of human olfactory bulb neural stem cells (OBNSCS), and CNTs, the present study demonstrated the marked potential of OBNSCs/CNTs for cell-based therapy of TMT neurodegenerative rat model. The engrafted OBNSCS/CNTs were traced using GFP, and their differentiation into different neuronal and glial cells were assessed based on their nuclear morphology. Such protocol proved its reliability and provided a strong morphological and immunohistochemical evidence for successful integration of engrafted OBNSCs/CNTs with existing neuronal circuitry. Using CNTs as scaffold was very helpful for support and promote NSCs implantation and promote its differentiation and functions. OBNSCS impregnated in CNT increased the differentiation of NSCs to neurons and decreased astrocytes formation, a finding that indicate the marked ability to restore TMT-neurodegenerative lesion, and qualify them as promising candidates for the cell-based for different neurodegenerative diseases.

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Legends of Figures

Figure 1: Photomicrograph of a section of rat's hippocampus two weeks post TMT-administration showing congestion of blood capillary, edema with degenerative changes in CA1 pyramidal neurons (A), neuronal necrosis (B, C), proliferation of glial cells (D, E), neuronal death in granular layer of DG and CA3 (F, G, H, I). H&E and cresyl violet.

Figure 2: Chart for escape latency and quadrant time before TMT, after one week and after 12 weeks post TMT administration to evaluate the impairment of memory. Note impairment of memory after four weeks followed by improvement of memory after 8 weeks. The chart indicates active auto-neurogenesis which made assessment of improvement due to engrafted OBNSC unreliable.

Figure 3: Photomicrograph of a section from rat's hippocampus (one week post OBNSC/CNTs engraftment) stained for GFP through indirect detection using Biotin-Conjugated Goat Anti Rabbit Secondary Antibody, Poly-Horseradish peroxidase (HRP) conjugate and Metal Enhanced DAB to develop the signal (brown color) (arrows). Note, the GFP-positive neurons within the stratum pyramidale indicating the survival of OBNSC (A, B, C), and their differentiation into neurons, oligodendrocyte and astrocyte like GFP- positive cells (arrows) (D). By six week, GFP neuronal and glial cells (astrocytes, and oligodendrocytes) were identified within different hippocampal fields indicating their migration **(Fig. 3 E, F).** By eight weeks post the engraftment, GFP-positive cells with morphological criteria suggestive of mature neurons were recognized **(Fig. 3 G), and Figure 4 A, B, C, D, E, F, G, H, I).** HRP-enzyme IHC.

Figure 4: Photomicrograph of a section from rat's hippocampus (eight-week post GFP-OBNSC/CNTs engraftment) showing GFP- positive cells (arrow) (A), GFP- positive cells (arrow) astrocyte like cells (B), GFP- positive cells (arrows) oligodendrocyte like cell (C, D, E), GFP-positive cells (arrow) neuron (N), oligodendrocyte (O) like cells (F), GFP- positive cells (arrow) (G, H), GFP- positive cells "neuron" (arrow) (I).

Figure 5: Photomicrograph of a section of rat's hippocampus eight weeks post (OBNSC/CNTs engraftment) showing normal pyramidal neurons of CA1 (A, B, C, D, E, F). The granular layer of DG restored its normal architecture and cellular content and thickness **(Fig. 5 G, H, I).** H&E.

Figure 6: Photomicrograph of a section of rat's hippocampus four weeks post (OBNSC/CNTs engraftment) showing pyramidal normal neurons of CA1 with clear Nissl granules (A), reduction of necrotic cells (B). By Eight weeks, restoration of pyramidal cell layer thickness (increase in

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thickness) to normal and restore cellular architecture with blue stain cytoplasm due to Nissl granules **(Fig. 6 C, E, F, G, H, I, J, K, L)**. cresyl violet stain.

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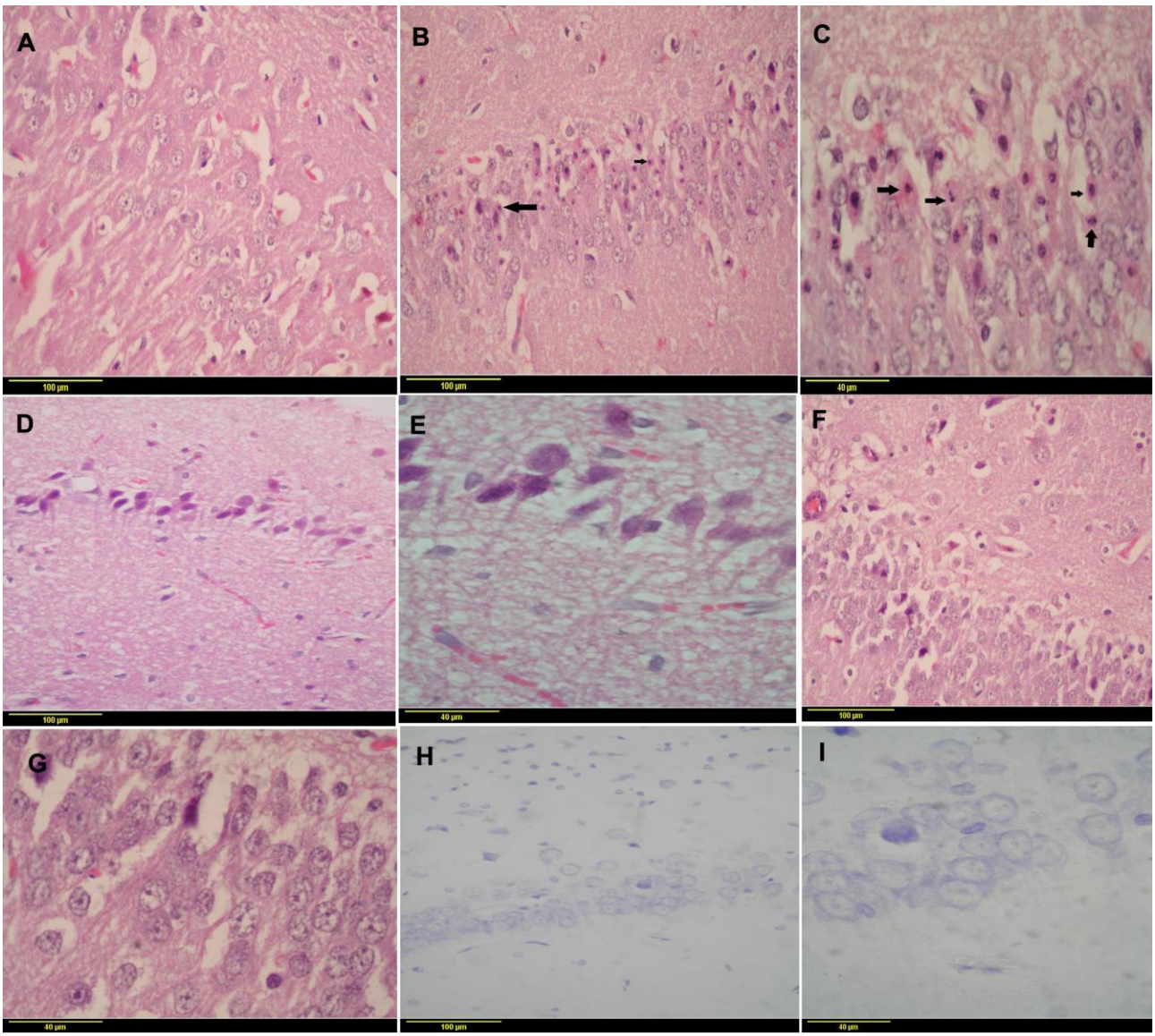

Figure 1

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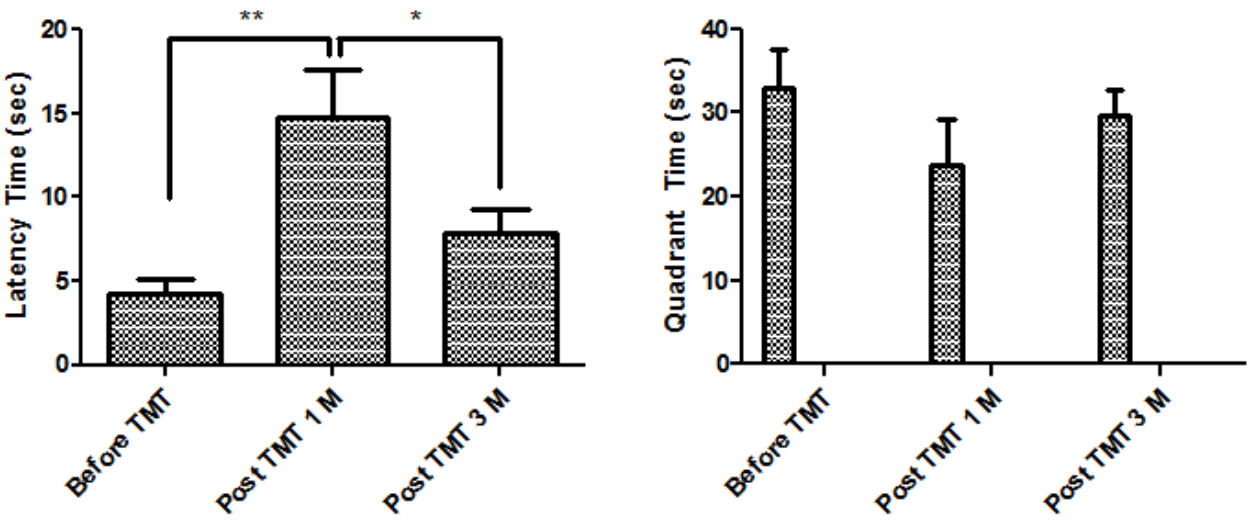

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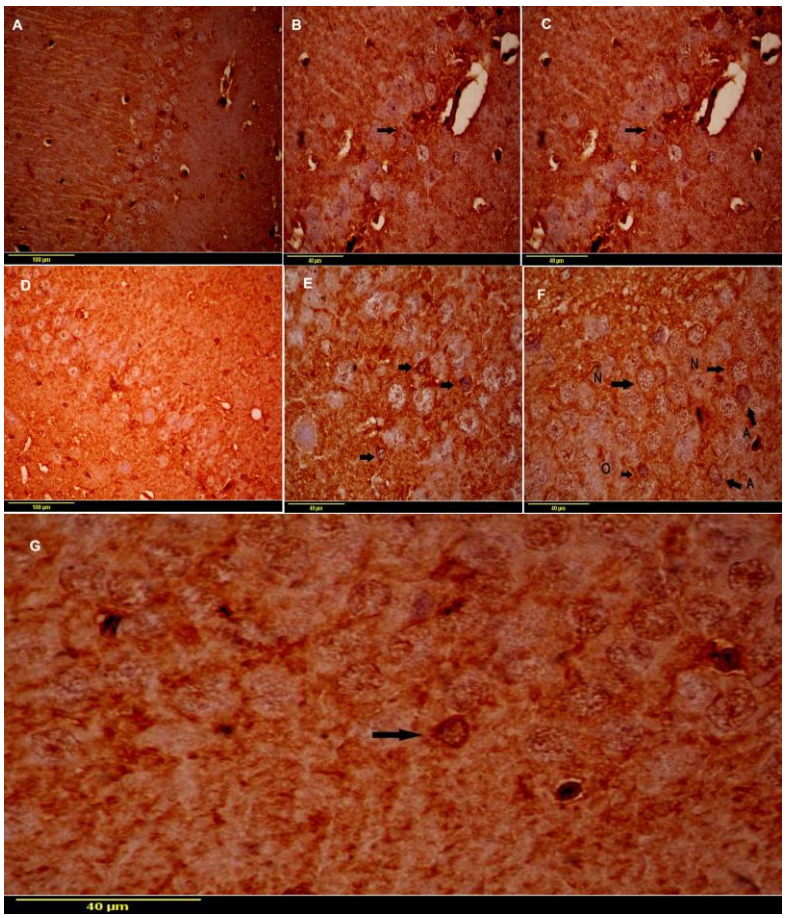

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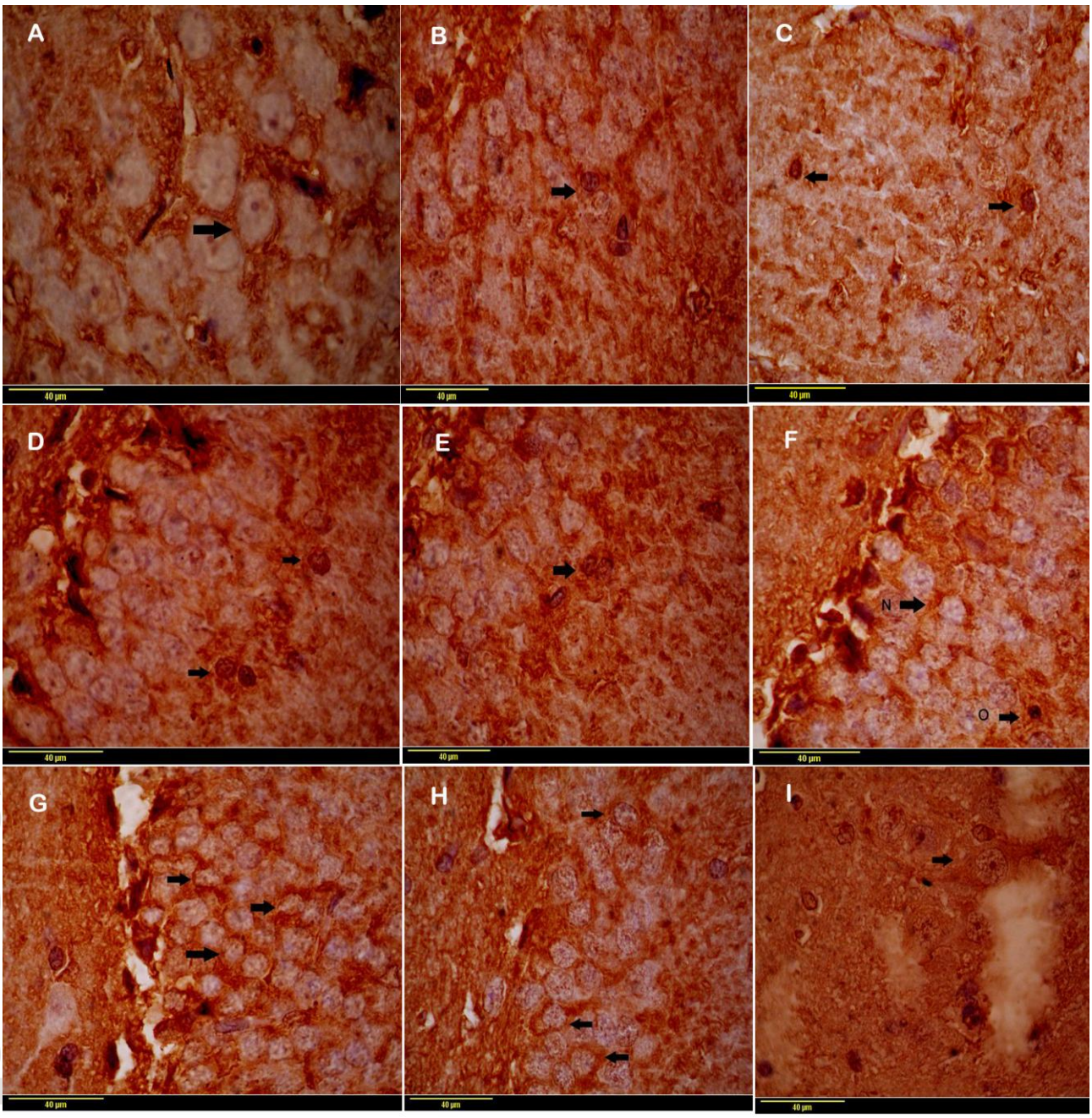

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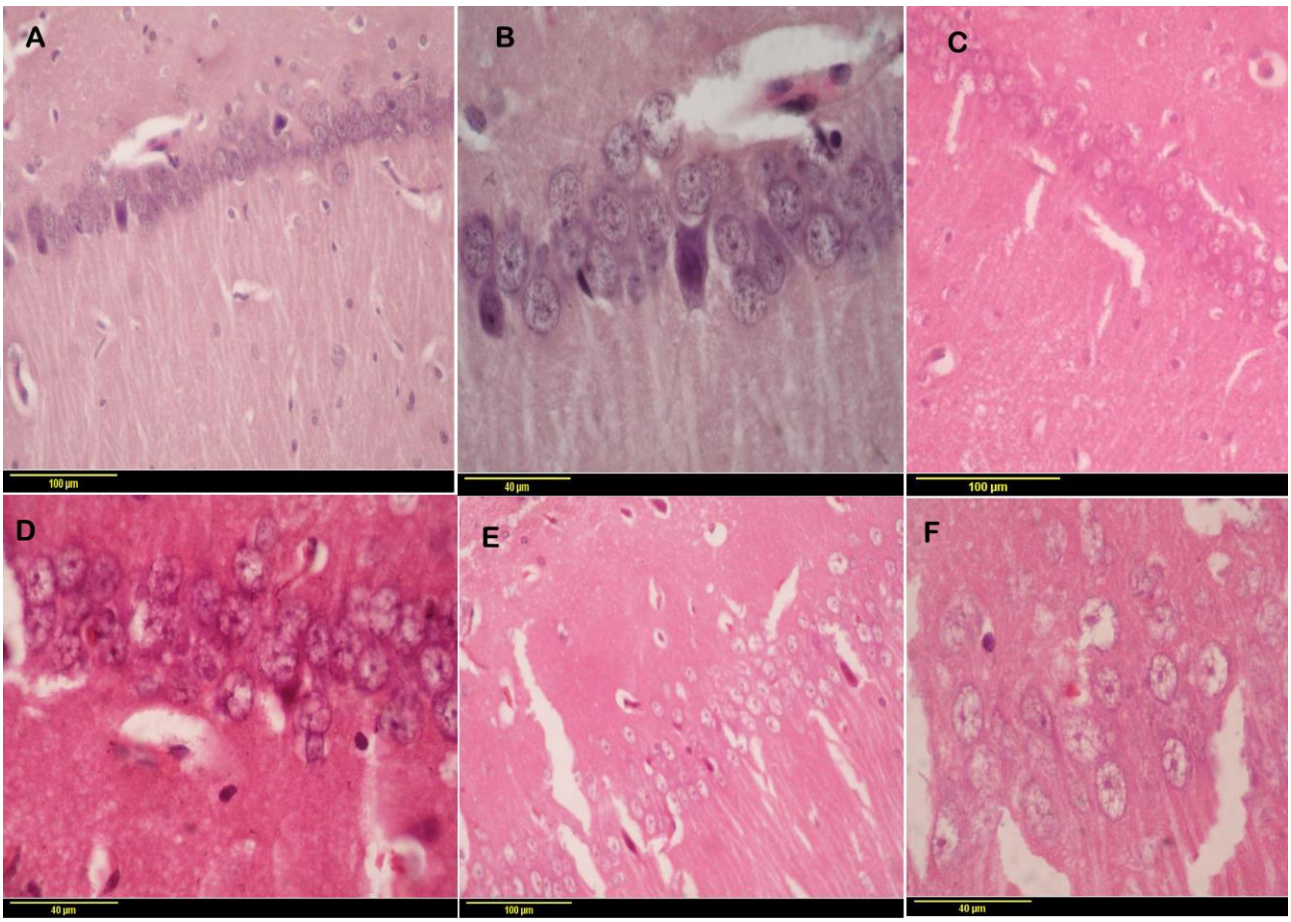

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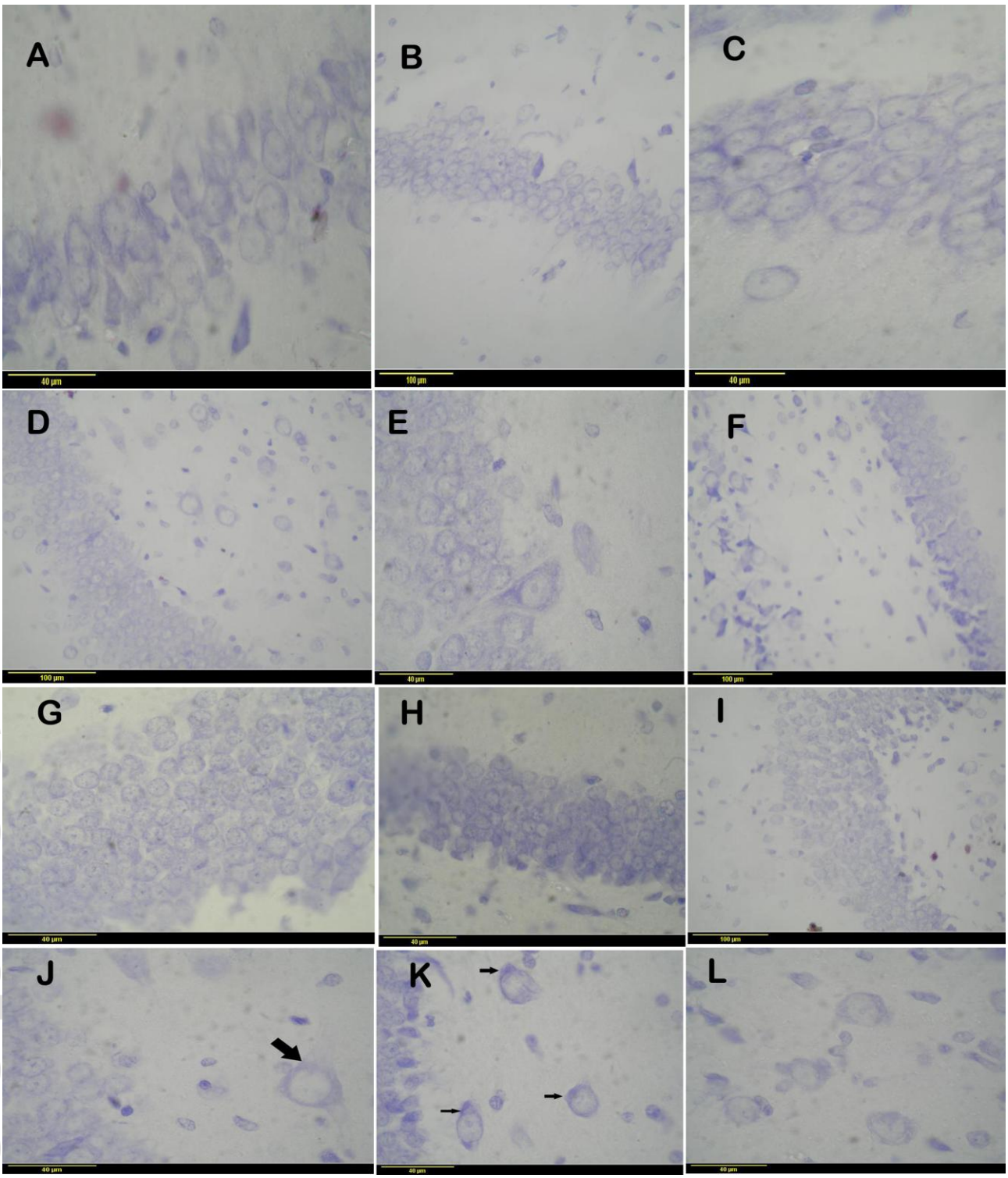

Figure 6

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