



Spatial learning and memory of young and aging rats following injection with human Wharton's jelly-mesenchymal stem cells

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SUBMITTED 16 March 2021 REVISED 10 May 2021 ACCEPTED 18 May 2021

ABSTRACT Human Wharton's jelly-mesenchymal stem cells (hWJ-MSC) are an emerging potential source of stem cells derived from the umbilical cord. Previous studies have shown their potential as treatment for traumatic brain injury and Parkinson's disease. However, no study has yet investigated the effect of hWJ-MSC injections in countering spatial learning and memory impairment in aging rats. The effect of hWJ-MSC injection on young rats is also unknown. The objective of this research was to analyze the effect of an hWJ-MSC injection on spatial learning, memory, density of putative neural progenitor cells (pNPC), and neuronal apoptosis in the dentate gyrus (DG) of young and aging rats. Injection of hWJ-MSC did not change spatial learning and memory in young rats until two months post-injection. This might be due to retained pNPC density and neuronal apoptosis in the DG of young rats after injection of hWJ-MSC. In contrast, injection of hWJ-MSC promoted both spatial learning and memory in aging rats, a finding that might be attributable to the increased pNPC density and attenuated neuronal apoptosis in DG of aging rats during the two months post-injection. Our study suggests that a single injection of hWJ-MSC might be sufficient to promote improvement in long-term learning and memory in aging rats.

KEYWORDS Human Wharton's jelly-mesenchymal stem cells (hWJ-MSCs); learning; memory; neurogenesis; stem cells

1. Introduction

Aging is process of gradual deterioration across multiple organs which is attributed to decreasing regenerative ability (Lazarov et al. 2010). The brain is one of the organs affected by the aging process. Brain aging can cause spatial learning and memory impairment (Drapeau et al. 2003; Terry et al. 2011). Spatial learning is the process of encoding information regarding position based on environmental cues into the cognitive map to guide navigational process (Floresco 2010); and memory is encoded and stored information which can be recalled when needed (Smith 1980).

Spatial learning and memory are regulated by neurogenesis and apoptosis in the hippocampus (Dupret et al. 2007). Neurogenesis is the formation of newborn neurons by neural progenitor cells (Deng et al. 2010). Newborn neurons are important to encoding new memories in the learning process (Deng et al. 2010; Kempermann 2002).

Neurons in the hippocampus also undergo apoptosis, a process of programmed cell death (Elmore 2007). Progressive cell death in the hippocampus can cause impairment in spatial learning ability.

Previous studies have shown many attempts to counter neuronal damage caused by aging in the hippocampus such as dietary regulations, exercise, and injection of young blood (Galli et al. 2002; Speisman et al. 2013; Villeda et al. 2014). Another alternative to prevent neuronal degradation is stem cells injection. Stem cells are undifferentiated cells which are able to self-renew and differentiate into many types of cells (Pera et al. 2000). Stem cells could be a potential anti-aging agent because they could directly repair damaged tissue by differentiating into many cell types (Boyette and Tuan 2014). Stem cells also produce growth factor, which could inhibit apoptosis and induce proliferation of progenitor cells (Bali et al. 2016).

Wharton's jelly-mesenchymal stem cells (WJ-MSC) are a new emerging potential source of stem cells. Pre-

vious studies have showed that WJ-MSC can be isolated robustly, have a higher proliferation rate than other mesenchymal stem cells, and multipotent ability to differentiate into chondrocyte adipocyte, osteocyte, as well as trans-differentiate into hepatocyte, cardiomyocyte, glia, and neurons (Mitchell et al. 2003; Nekanti et al. 2010; Wang et al. 2004; Weiss et al. 2006). WJ-MSC also have immunosuppressive ability, which can reduce immune rejection upon injection (Weiss et al. 2008; Zhou et al. 2011). Moreover, the isolation process is ethically unchallenged because the umbilical cord is often regarded as post-birth waste (Wang et al. 2004; Weiss et al. 2006).

Human Wharton's jelly-mesenchymal stem cells (hWJ-MSC) has been used in pre-clinical tests as treatment for traumatic brain injury and Parkinson's disease model (Cheng et al. 2015; Weiss et al. 2006). However, to our best knowledge, no study has shown the effect of hWJ-MSC injection to counter spatial learning and memory impairment in aging rats. The effect of hWJ-MSC on young rats is also yet to be known. Moreover, the effect of hWJ-MSC injection on the cellular conditions in dentate gyrus of young and aging rats also unknown. Therefore, this study aimed to analyze the effect of hWJ-MSC injection to: spatial learning, memory, density of putative neural progenitor cells (pNPC), and neuronal apoptosis in dentate gyrus (DG) of young and aging rats.

2. Materials and Methods

2.1. Experimental procedures

This research has been approved by Animal Care and Use Committee (ACUC), Educational Animal Hospital, Faculty of Veterinary Medicine, Bogor Agricultural University. All rats used in this research were from Unit Pengelola Hewan Laboratorium (UPHL), Faculty of Veterinary Medicine, Bogor Agricultural University. This research used 9 young rats (\pm 5 month old) and 9 aging rats (\pm 24 month old) (*Rattus norvegicus*) strain Sprague-Dawley, each divided into 3 groups: 1 control group and 2 treatment groups. Each group consisted of 3 rats. Control group was given 0.5 mL saline (vehicle). Treatment groups were given 1.5×10^6 hWJ-MSC cells/rat in vehicle. hWJ-MSC was prepared in Stem Cell and Cancer Institute (Indonesia). Isolation and characterization of hWJ-MSC used in this study has been described in Antoninus et al. (2012). All treatments were given via tail intravenous injection. Rats were anesthetized with ketamine 10 mg/kg and xylazine 1 mg/kg of body weight. Treatment groups were divided based on euthanasia period: 1 month (SC1) and 2 month (SC2) post-injection to assess long-term effect of hWJ-MSC injection.

2.2. Spatial learning test

Spatial learning ability was measured using Y-maze alternation test. Test protocol was based on Juliandi et al. (2015). Spatial learning ability was measured in 3 time-points: before treatment (7 d before treatment), 1-month

post-treatment (M1), and 2 month post-treatment (M2). Y-maze alternation test was done using Y-arm maze apparatus which consisted of 3 identical arms ($40 \times 10 \times 30$ (H) cm) at 120° away from each other. The center platform had 10 cm side-length. Y-maze alternation test was performed by counting rat's alternation in Y-arm maze for 5 min. Alternations were counted through video analysis. One alternation was defined as 3 consecutive arms entered by rats. Alternation was considered correct if rats entered 3 different arms continuously which is based on rats' instinct to explore new environment. The apparatus was cleaned with ethanol 70% after each session to minimize possible olfactory cue. Spatial learning ability was measured by analyzing correct alternation percentage, which is percentage of ratio between total correct alternation and total alternation.

2.3. Object recognition memory test

Rat's memory was measured by novel object recognition test (NORT). NORT protocol was based on Bevins and Besheer (2006). This test was based on rats preference to novel object. NORT was done in an acrylic compartment ($30 \times 30 \times 32$ (H) cm). NORT consisted of two phase: acquisition and test phase. Acquisition phase was done 1 d before treatment. In acquisition phase, each rat freely explored the apparatus which had two identical objects for 10 min. The identical objects used in this experiment are two plastic yellow balls with 10 cm diameter. The purpose of this acquisition phase was to familiarize each rat with those two identical objects which referred as familiar objects. After acquisition phase, rat was put in their house cage and given 3 delay time-points to test the memory retention. Test phase in this research was done in 1 d (D1) post-treatment to assess short-term memory, 1 month (M1), and 2 month (M2) post-treatment to measure long-term memory. In test phase, each rat explored the apparatus which has one of the familiar object replaced with novel object for 3 min. Novel object used in this research was a stuffed animal (chicken) ($10 \times 8 \times 9$ (H) cm). Apparatus and 2 objects were cleaned after each session with ethanol 70% to decrease olfactory cue. Rat's memory was measured using discrimination ratio which is percentage of ratio between time to explore novel object with total to explore both novel and familiar object. Time to explore was measured using video analysis. Rat was considered exploring one object if rat's snout was within 3 cm from the object.

2.4. Hematoxylin-Eosin staining

Rat's brain was isolated using perfusion method. Rat's brain was fixed in buffered neutral formalin (BNF) 10% for 4 d. Coronal trimming was done in 3 mm posterior bregma. Brain section containing hippocampus was made into $5 \mu\text{m}$ paraffin sections. Paraffin sections were stained with hematoxylin-eosin and observed with light microscope which connected to camera. Photomicrograph of dentate gyrus sections were captured using IndomicroView software (Indomicro, Indonesia) for further anal-

ysis. Putative neural progenitor cells (pNPC) and apoptotic neuron in dentate gyrus (DG) were identified based on Han et al. (2008) and Hashem et al. (2010). Counting of pNPC and DG apoptotic neurons density were done using ImageJ 1.50i software (NIH, USA).

2.5. Immunohistochemistry

Antigen retrieval was conducted either by incubation in L.A.B. Solution (Polysciences) for 15 min at room temperature (RT) or by autoclaving in Target Retrieval Solution (Dako) for 15 min at 105 °C. After 3 washes with PBS, the sections were incubated for 1 h at RT in blocking solution (PBS containing 3% FBS and 0.1% Triton X-100). They were then incubated overnight at 4 °C with the appropriate primary antibodies. The following primary antibodies were used: mouse anti-human Ki67 (1:500, BD Pharmingen) and goat anti-active caspase3 (1:500, R&D Systems). After 3 washes with PBS, the sections were incubated for 2 h at RT with the appropriate secondary antibodies. The following secondary antibodies were used: Cy3-conjugated donkey anti-mouse and Cy5-conjugated donkey anti-goat (1:500, Jackson ImmunoResearch). After 3 washes with PBS, nuclei were stained for 15 min at RT with Hoechst 33258 (Nacalai Tesque). Sections were mounted on cover slips with Immu-Mount (Thermo Scientific). Cell counting was conducted manually and photographed using LSM 710 confocal microscope (Zeiss) equipped with a camera and appropriate epifluorescence filters. The total number of positive cells was counted in every twelve section (480 µm apart). Positive cells were counted throughout the rostro-caudal extent of the granule cell layer (GCL), and the derived numbers were multiplied by 12 (slice series) to obtain total cell number per GCL.

2.6. Data analysis

Spatial learning and memory were analyzed with one-way ANOVA, two-way ANOVA and independent t-test. Analysis on pNPC density and neuronal apoptosis in DG were done using one-way ANOVA with post-hoc test using Tukey honest significant difference (HSD). Statistical analysis was performed in SPSS 22 (IBM, USA) with minimum significance level $p < 0.05$.

3. Results and Discussion

3.1. Spatial learning ability

Young rats in control group had an increased spatial learning ability from before treatment up to 2 month post-treatment ($p=0.046$, one-way ANOVA) (Figure 1a). In comparison, aging rats in control group had a consistent spatial learning ability throughout 2 month ($p=0.858$, one-way ANOVA) (Figure 1b). This result shows that young rats were able to learn the Y-maze apparatus. In comparison, aging rats did not learn the apparatus throughout test period.

Injection of hWJ-MSC did not alter spatial learning of young rats compared to control group ($p=0.118$, two-way

ANOVA) (Figure 1a). Shift in spatial learning ability was not observed in young rats injected with hWJ-MSC from before treatment to 2 month post-injection ($p=0.063$, one-way ANOVA). Conversely, injection of hWJ-MSC promoted spatial learning ability of aging rats ($p=0.013$, two-way ANOVA) (Figure 1b). Increased spatial learning ability was observed in aging rats injected with hWJ-MSC ($p=0.0052$, one-way ANOVA). Rats injected with hWJ-MSC had higher spatial learning ability in 1 month post-injection (M1) ($p=0.0098$, independent t-test) and 2 month post-injection (M2) ($p=0.021$, independent t-test), compared with control group. This result suggests that hWJ-MSC had a long-term effect on promoting spatial learning of aging rats.

3.2. Object recognition memory

All rats had 50% discrimination ratio in the acquisition phase (Figure 2). This result indicates that all rats were exploring both familiar objects on equal proportion. Injection of hWJ-MSC did not alter object recognition memory of young rats ($p=0.108$, two-way ANOVA) (Figure 2a). Retained object recognition memory were observed on both young rats in control group ($p=0.788$, one-way ANOVA) and young rats injected with hWJ-MSC ($p=0.110$, one-way ANOVA).

Treatment with hWJ-MSC promoted object recognition memory of aging rats ($p=0.0013$, two-way ANOVA) (Figure 2b). Compared to control group, object recognition memory of aging rats in hWJ-MSC group was significantly higher in 1 d (D1) ($p=0.042$, independent t-test) and 1 month (M1) ($p=0.0097$, independent t-test) post-injection. However, no significant differences were observed in object recognition memory between aging rats in control group and hWJ-MSC group in 2 month (M2) post-treatment ($p=0.110$, independent t-test).

No significant changes were observed in the control group of aging rats ($p=0.345$, one-way ANOVA). In contrary, significant increase was observed in aging rats injected with hWJ-MSC ($p=0.0026$, one-way ANOVA). Object recognition memory of aging rats in hWJ-MSC group was increased and peaked in 1 d post-injection (D1) ($p=0.042$, Tukey HSD) compared to acquisition phase. This result shows that hWJ-MSC promoted short-term memory of aging rats. Significant increase also observed in 1 month post injection compared to acquisition phase ($p=0.029$, Tukey HSD). Object recognition memory in 1 month post-injection (M1) was not significantly different compared to 1 d post-injection (D1) ($p=0.252$, Tukey HSD). This result indicates memory retention from short-term to long-term. However, significant decrease in object recognition was observed in 2 month post-injection (M2) in comparison to 1 d post-injection (D1) ($p=0.252$, Tukey HSD). This result shows that injection of hWJ-MSC did not produce permanent effect on object recognition of aging rats.

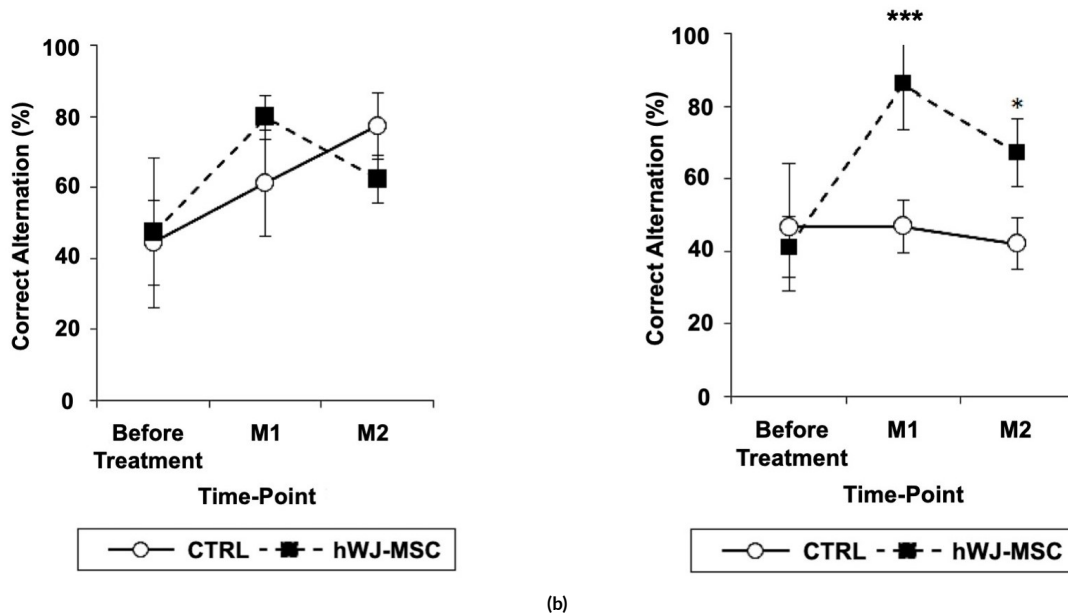


FIGURE 1 Spatial learning of (A) young rats and (B) aging rats following human Wharton’s jelly-mesenchymal stem cells injection. CTRL: control group, M1: 1 month post-treatment, M2: 2 month post-treatment. * $p < 0.05$ and *** $p < 0.005$, independent t-test, compared to control group.

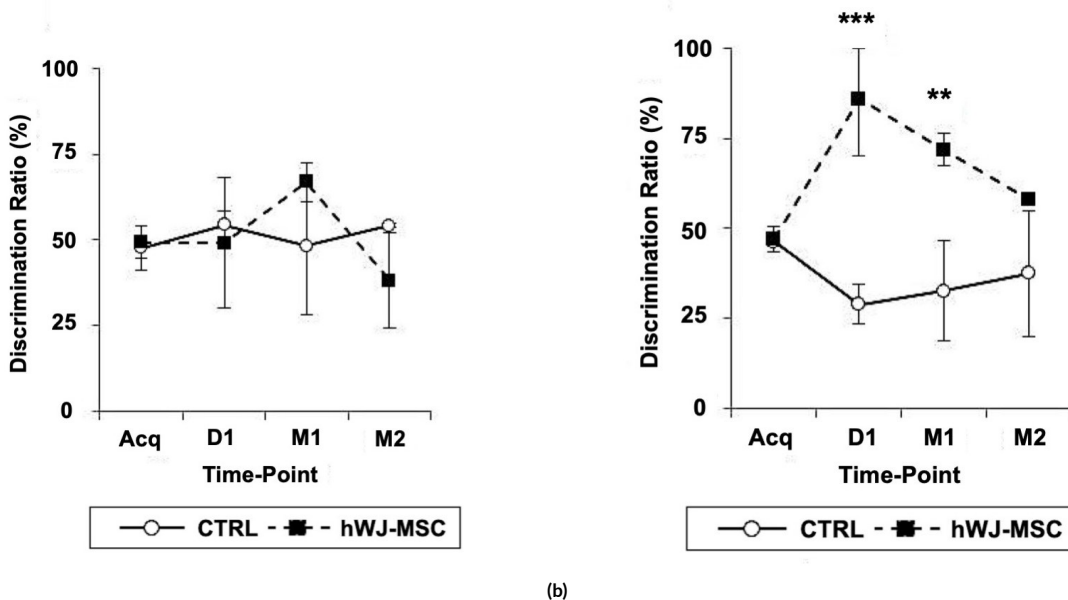


FIGURE 2 Effect of human Wharton’s jelly-mesenchymal stem cells injection to object recognition memory of (A) young rats and (B) aging rats. CTRL: control group, Acq: Acquisition phase, D1: 1 d post-treatment, M1: 1 month post-treatment, M2: 2 month post-treatment. ** $p < 0.01$ and *** $p < 0.005$, independent t-test, compared to control group.

3.3. Putative neural progenitor cells density and neuronal apoptosis of young rats

Photomicrograph of rat’s brain coronal section showed dentate gyrus as part of hippocampus (Figure 3a). Photomicrograph of dentate gyrus showed putative neural progenitor cells (pNPC) which identified by the non-granular morphology, dark cytoplasm, and resides in the deepest part of granular cell layer (GCL) or the subgranular zone (SGZ) (Figure 3b). Apoptotic neurons in dentate gyrus were identified by vacuolation, shrunken cytoplasm, and

pyknotic nucleus (Figure 3c).

Injection of hWJ-MSC increased pNPC density of young rats ($p = 0.025$, one-way ANOVA) (Figure 3a). Density of pNPC was higher at 1 month post-injection (SC1) compared to control group ($p = 0.023$, Tukey HSD). However, pNPC density at 2 month post-injection (SC2) was not significantly different compared to control group ($p = 0.697$, Tukey HSD).

Neuronal apoptosis in dentate gyrus of young rats was consistent despite treatment with hWJ-MSC ($p = 0.522$,

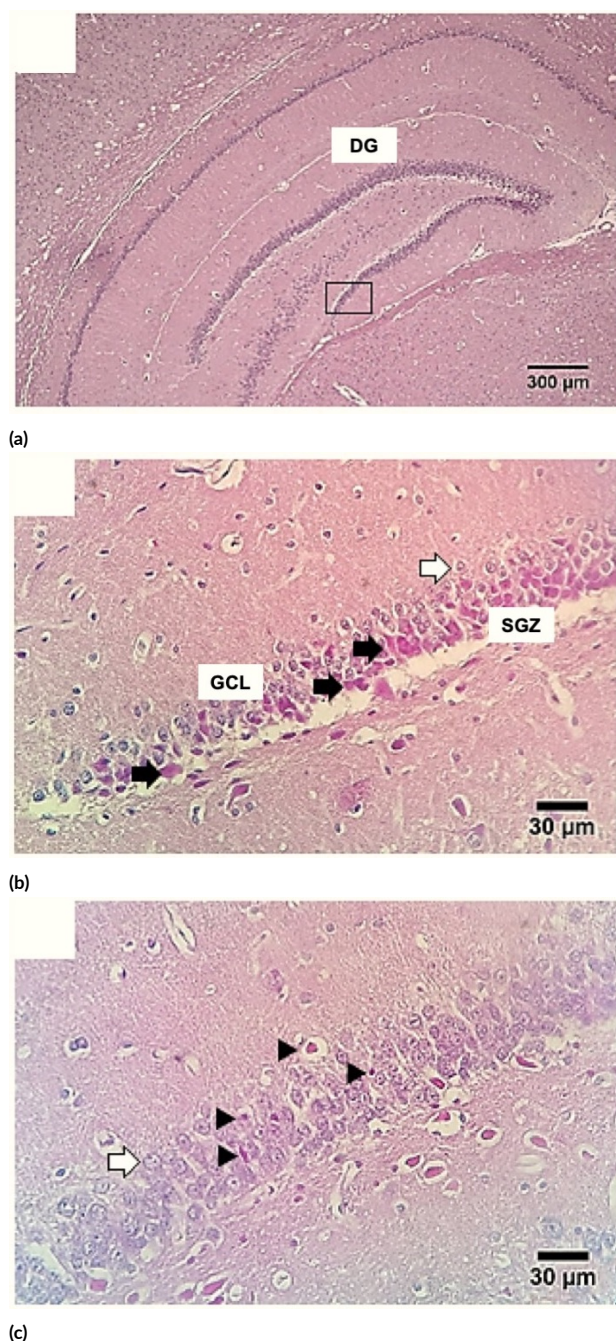


FIGURE 3 Photomicrograph of dentate gyrus with hematoxylin-eosin staining. (a) Photomicrograph showing dentate gyrus as part of hippocampus. (b) Dentate gyrus section. (c) Apoptotic neurons in dentate gyrus. DG: dentate gyrus, GCL: granular cell layer, SGZ: subgranular zone. White arrow: granular neuron, black arrow: putative neural progenitor cells, black arrow-head: apoptotic neurons.

one-way ANOVA) (Figure 4b). Increased pNPC density without significant change on neuronal apoptosis showed that injection of hWJ-MSC induce proliferation of neural progenitor cells in dentate gyrus.

3.4. Neural progenitor cells density and cell apoptosis of aging rats

Treatment with hWJ-MSC promoted pNPC density of aging rats ($p=0.023$, one-way ANOVA) (Figure 5a). Rats in hWJ-MSC group had an increased density of pNPC on 1 month post-injection (SC1) compared to control group ($p=0.032$, Tukey HSD). Increased in pNPC density was also observed in 2 month post-injection (SC2) despite statistically not significant ($p=0.091$, Tukey HSD). Injection of hWJ-MSC attenuated neuronal apoptosis in aging rats ($p=0.037$, one-way ANOVA) (Figure 5b). Decreased neuronal apoptosis was observed in 1 month post-injection (SC1), but this decreased trend was not statistically significant ($p=0.131$, Tukey HSD). Neuronal apoptosis was significantly decreased in 2 month post-injection (SC2) in comparison to control group ($p=0.039$, Tukey HSD). hWJ-MSC injection also increases Ki67+ (red) neural progenitor cells number in both young and aging rats (Figure 6a-b), and decreases activated Caspase3+ (white) apoptotic cells number in dentate gyrus of aging rats (Figure 6c-d).

3.5. Discussion

To our knowledge, this is the first report on the effect of human Wharton's jelly-mesenchymal stem cells (hWJ-MSC) xenogeneic transplantation in vivo to both aging and young rats. Injection of hWJ-MSC produced age-specific effect. Treatment with hWJ-MSC to young rats did not change the spatial learning (Figure 1a) and object recognition memory (Figure 1a). In comparison, significant change was observed in spatial learning (Figure 1b) as well as object recognition memory (Figure 2b) of aging rats. This age-specific effect might be attributable to difference in proliferation rate of young and aging rats.

Previous studies have presumed that brain might has certain neurogenesis threshold to retain spatial learning ability and memory (Drapeau et al. 2003; Juliandi et al. 2015). Rats with NPC proliferation above threshold, such as in young rats, would not produce higher spatial learning and memory despite induction of NPC proliferation. This research supports this hypothesis. Injection of hWJ-MSC to young rats did increase pNPC density (Figure 4a), which reflect NPC proliferation. However, this induction was not followed by change in spatial learning and object recognition memory. We presumed that formation of newborn neurons which stores new memories are already sufficient or above the threshold, so induction of NPC proliferation after hWJ-MSC injection did not change the spatial learning and object recognition memory.

Rats with neurogenesis below threshold, such as caused by aging, showed spatial learning impairments (Drapeau et al. 2003). Aging process is known to decrease NPC proliferation due to increasing quiescence of NPC (Heine et al. 2004; Hattiangady and Shetty 2008). This age-related decline could cause reduction in newborn neurons formation which is vital in memory and learning process (Bernal and Peterson 2004). Indeed, in this study, aging rats in control group had lower pNPC density,

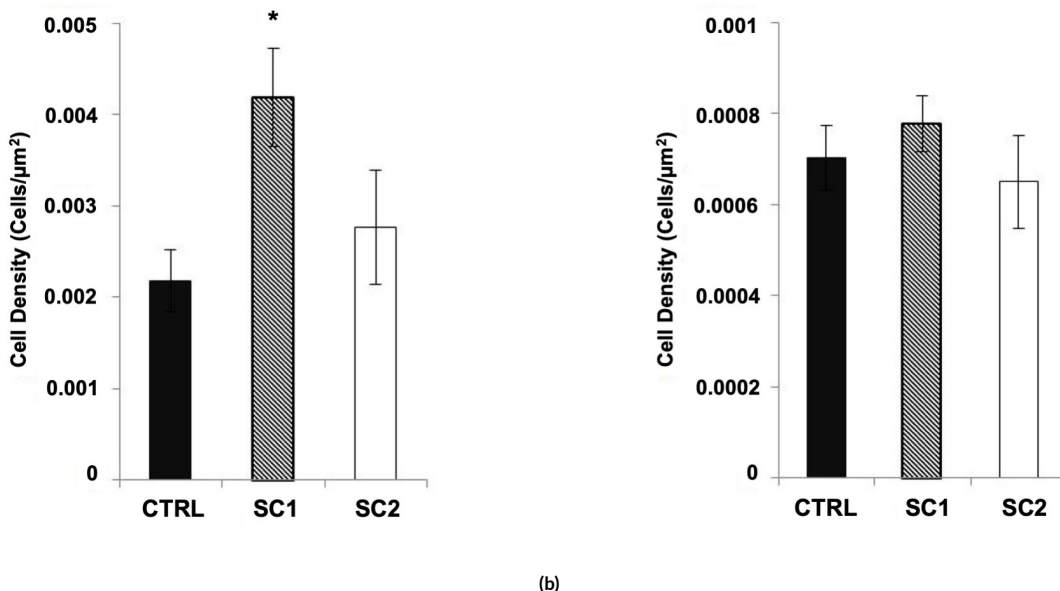


FIGURE 4 Effect of human Wharton’s jelly-mesenchymal stem cells to (a) putative neural progenitor cells density and (b) neuronal apoptosis in dentate gyrus of young rats. CTRL: control group, SC1: hWJ-MSC group 1 month post-injection, SC2: hWJ-MSC group 2 month post-injection. * $p < 0.05$, Tukey HSD, compared to CTRL.

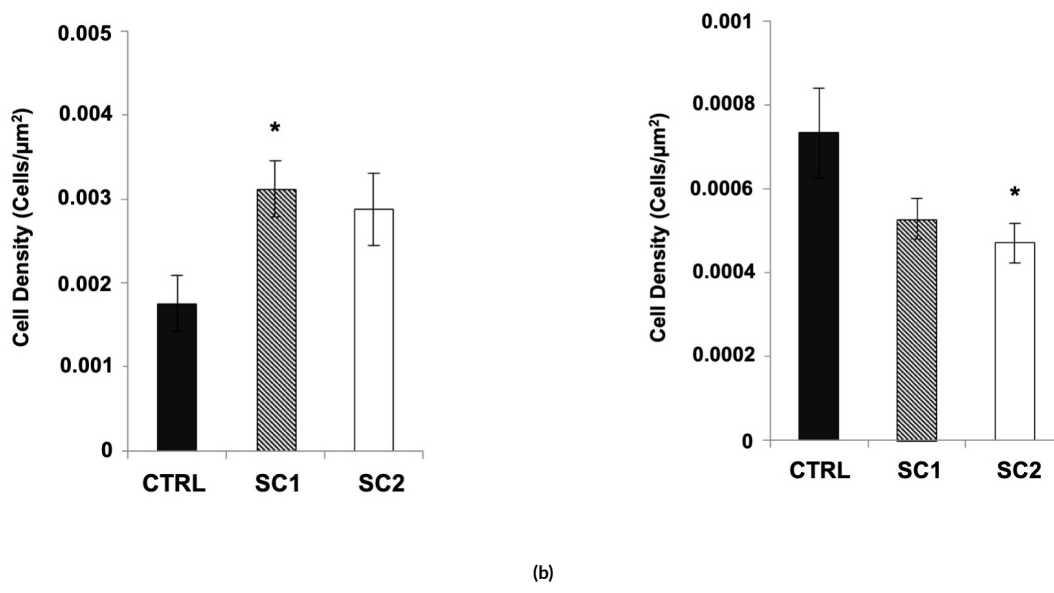


FIGURE 5 Human Wharton’s jelly-mesenchymal stem cells injection’s effect to (A) putative neural progenitor cells density and (B) neuronal apoptosis in dentate gyrus of aging rats. CTRL: control group, SC1: hWJ-MSC group 1 month post-injection, SC2: hWJ-MSC group 2 month post-injection. * $p < 0.05$, Tukey HSD, compared to CTRL.

spatial learning ability, as well as memory. In line, aging rats injected with hWJ-MSC had higher pNPC density which showed induction of proliferation as well as spatial learning and memory. Therefore, we presumed that hWJ-MSC injection might be able to induce NPC proliferation to above threshold which resulted in increased spatial

learning and memory.

We also found that hWJ-MSC could attenuate neuronal apoptosis in DG. Inhibition of neuronal apoptosis in DG is important to retain stored memories and spatial learning ability (Ramírez et al. 2005). Inhibition of neuronal apoptosis might also reflect increased newborn

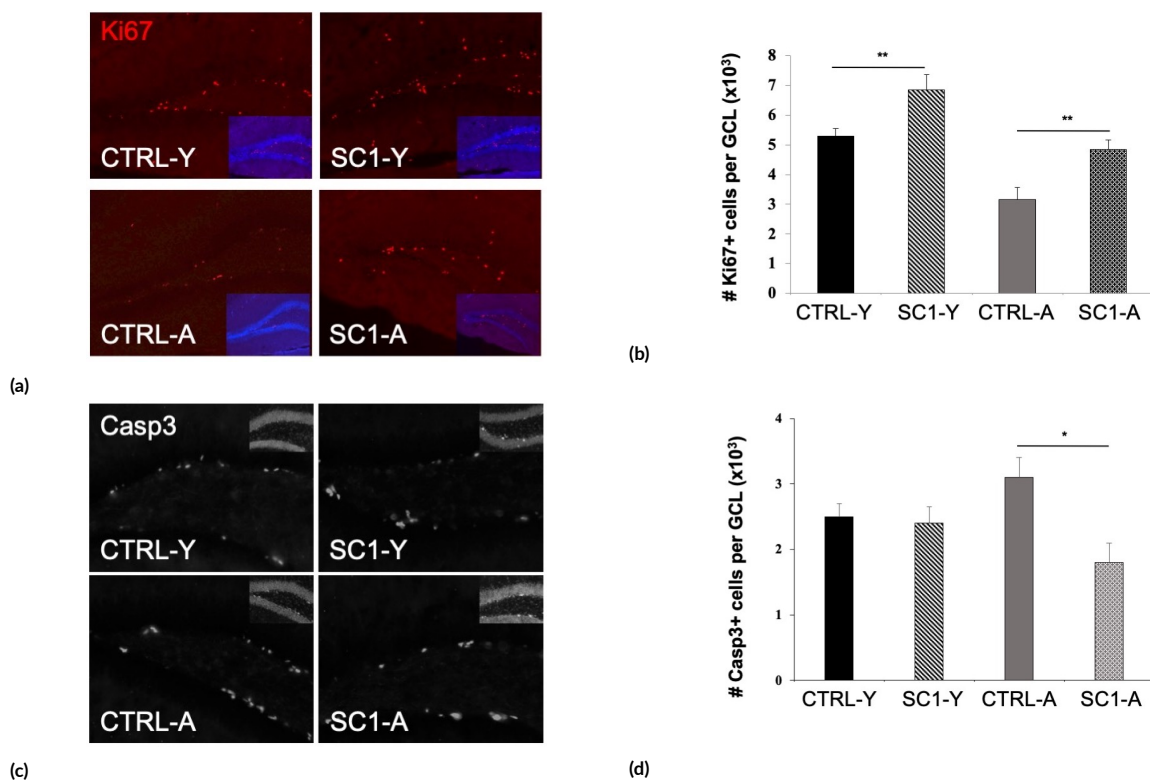


FIGURE 6 Human Wharton’s jelly-mesenchymal stem cells injection increases Ki67+ (red) neural progenitor cells number in both young and aging rats (a-b), and decreases activated Caspase3+ (white) apoptotic cells number in dentate gyrus of aging rats (c-d). CTRL-Y: control group of young rats, CTRL-A: control group of aging rats, SC1-Y: hWJ-MSC group 1 month post-injection of young rats, SC1-A: hWJ-MSC group 1 month post-injection of aging rats. * $p < 0.05$ and ** $p < 0.01$, independent t-test, compared to control group.

neurons survival. Approximately 9,000 newborn neurons were produced daily in young rats (Heine et al. 2004). Integration of newborn neurons in massive numbers could cause disruption in hippocampal circuitry, which leads to seizures and forgetting (Meltzer et al. 2005). Therefore, 80-90% of newborn neurons produced were removed by apoptosis (li Ming and Song 2011; Sierra et al. 2010; Kempermann et al. 2003).

Injection of hWJ-MSC in this study increased proliferation of NPC. Induced NPC proliferation should have been followed by increased neuronal apoptosis. However, rats injected with hWJ-MSC showed reduced neuronal apoptosis in DG of aging rats, which suggests increased survival of newborn neurons. Similar result was found in previous studies in which aging rats were given exercise treatment (Kim et al. 2010; Lee et al. 2005). Combined, this results also suggest that acquisition of newborn neurons is more selective in aging rats compared to young rats. Increased survival of newborn neurons in aging rats also could promote spatial learning and memory (Drapeau et al. 2003).

The precise mechanism on how hWJ-MSC could affect cellular condition in dentate gyrus and learning ability is unknown. Previous study has shown that porcine WJ-MSC could integrate and proliferate in substantia nigra and ventral tegmental area until 8-weeks post-injection in Parkinsonian rat model (Medicetty et al. 2004). Porcine WJ-MSC injected directly into rat’s brain also migrate into region in ventral of corpus callosum on 6-weeks post-

injection (Weiss et al. 2003). In general, WJ-MSC could survive in rats even long after injection period. However, none of the above uses systemic injection as we did in this study.

Indeed, the main challenge in systemic injection of stem cells is whether they could penetrate blood brain barrier (BBB) which tightly regulates substances going into the brain (Goncharova et al. 2014). Elahy et al. (2015) showed that increased capillary permeability happens due to BBB breakdown in normal aged mice (24 month) which is the same age as rats used in this study. Furthermore, Montagne et al. (2015) showed that aging can cause BBB breakdown specifically in hippocampus. This might open up possibility of hWJ-MSC integration into aging hippocampus and attenuates neuronal degeneration either via cell-to-cell contact or cytokine secretion. This study cannot provide data to show whether hWJ-MSC is indeed could migrate into dentate gyrus. Therefore, further studies using specific marker such as human nuclei antibody are needed to confirm the integration and survival of hWJ-MSC injected in aging rats.

Regardless, we presumed that induction of NPC proliferation and inhibition of neuronal apoptosis in DG might be attributable to secretion of growth factor by hWJ-MSC. Yang et al. (2008) showed that hWJ-MSC could produce vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) which are well-known to increase NPC proliferation (Jin et al. 2002; Lin et al. 2015).

Neuroprotective effect of hWJ-MSC might due to secretion of VEGF and granulocyte-colony stimulating factor (G-CSF) (Koh et al. 2008; Yang et al. 2008). Both G-CSF and VEGF are known to inhibit neuronal apoptosis (Yata et al. 2007; Solaroglu et al. 2009; Sun et al. 2003). However, as aforementioned, further research is needed, especially to confirm whether the effect is indeed caused solely by growth factor secreted by hWJ-MSC, or by promotion of endogenous growth factor produced in SGZ, or even both

4. Conclusions

In conclusion, this study has provided information regarding the effect of hWJ-MSC injection to spatial learning and memory of young and aging rats. Intravenous injection of hWJ-MSC did not alter both spatial learning and memory of youth rats. In contrast, hWJ-MSC promoted spatial learning as well as memory of aging rats. Increased pNPC density and attenuation of neuronal apoptosis in DG might be responsible for the promotion of both spatial learning and memory of aging rats.

Acknowledgments

We would like to thank Drh. Alif Iman Fitrianto and Tini Wahyuni for their help in conducting the experiment. We also thank Prof. Sheena Gardner of Coventry University, United Kingdom, for her contribution in English editing and careful reading of the manuscript.

Authors' contributions

BJ, WM, DA, AB designed the study. BJ, WM, DA, MS carried out the laboratory work. BJ, WM, DA, AB, MS, IB, HM, KY, BS analyzed the data. BJ, WM wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

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