# Wharton' jelly mesenchymal stem cells and insulin effect on BDNF expression in CA1 and CA3 regions of rats' hippocampus after chronic hypoxia

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**Objectives** Brain is vulnerable to deprivation of oxygen supply during hypoxia, and therefore undergoes neurodegeneration and cognitive dysfunction. Regarded to Regenerative capacities of Wharton's jelly mesenchymal stem cells (MSCs) and insulin at the site of injury, we were aimed to evaluate the effect of Wharton's jelly MSCs and insulin on degenerative consequences induced by chronic hypoxia.
**Methods** 36 male rats were randomly divided into six groups: Control (C), Sham1-saline (Sh1), Sham2-surgery (Sh2), Hypoxia (H), Hypoxia + Insulin (HI), Hypoxia + MSCs (HCs). Animals were exposed to hypoxic chamber (8% O2, 92% N2) for 30 days (4 hours/day) in H, HI and HCs groups. Intranasal insulin and stereotaxical MSCs in HI and HCs were used, respectively. Spatial learning and memory were analyzed using the Morris water maze task. Brain-derived neurotrophic factor (BDNF) gene expression was studied in the hippocampus by real time-PCR.
**Results** BDNF had the significant depletion in HI group and magnification in HI and HCs groups comparing with C and Sh groups ($P < 0.05$). Insulin and MSCs improve hypoxia's signs such as BDNF gene expression fallen and memory impairment.
**Conclusion** In conclusion, we indicated that use of insulin hormone and MSCs as neuroprotective and stimulating factors for neurogenesis, could be beneficial in neurodegenerative damage induced by hypoxia.
**Keywords** BDNF, hypoxia, insulin, Morris water maze, Wharton's jelly mesenchymal stem cells

## Introduction

Chronic hypoxia stress as much as biological ageing processes have been reported to play a crucial role in neurons degeneration, spatial memory impairment and decreased cognitive performance.[1] Brain regions such as the hippocampus, particularly its dorsal part, along with the neocortex and piriformis cortex are the most sensitive to the harmful effects of hypoxia.[2] Many researchers have demonstrated that hippocampus is the most vulnerable area of the brain in anoxia.[3–5] Global cerebral hypoxia has been extensively studied for its effects on learning and memory by diminishing of partial pressure of oxygen in the atmosphere. It leads to reduction in cerebral oxygenation that effects neuro-signaling mechanisms and cognitive performance.[1] Previous studies have shown the hippocampal CA3 and CA1 neurons that participates in the transmission of outside information to the nerve centers,[6,7] in particular are more susceptible to hypoxic damage.[2,8] Morphological changes, excitotoxicity, decreased SOD activity, mitochondrial dysfunctions , neuroglobin expression and apoptosis in these areas have been ascribed as governing factors for cognitive deficits in hypoxic rats.[1,9]

Earlier studies have confirmed the brain capability of neurogenesis, and targeted this potential in designing therapeutic strategies.[10] Neural stem cells embedded in the olfactory bulb,[11,12] subventricular zone (SVZ) and subgranular zone (SGZ),[13] with the capability of endogenous neurogenesis[11] would be proliferated to adult-functional neuron and emigrated to injured regions, hippocampus and cerebral cortex.[14] Although the limited endogenous neurogenesis potential, it should be stimulated by mediators.[15] Regarded to recent studies, one of the most remarkable source of these molecules are originated from secretome which leaks from MSCs and act as neurotrophins like brain-derived neurotrophic factor (BDNF) and could be associated with neurogenesis regulation.[13,14]

In line with recent observations, it has shown that administration of MSCs in nervous system because of their secretion, a wide range of molecules (neurotrophins and cytokines), rather than their ability to differentiate into neuronal or glial cells, can be beneficial as an alternative to central nervous system repair.[16,17]

Both endogenosis and exogenosis NSCs proliferation are insulin and/or IGF dependent.[18] Many studies have demonstrated that insulin administration, more than its physiological level, activates not only insulin receptors but also IGF-receptors which stimulates neurotrophin secretion in order to growth of neural stem cells,[19,20] cell-cycling, self-renewal and niche of stem cells proliferation.[19]

This study aimed at knowing on possible occurrence of structural changes in CA1 and CA3 hippocampal neurons after exposure to chronic global hypoxia. Besides that, any possible relationship between the administration of insulin hormone or Wharton's jelly mesenchymal stem cells and expression of neurotrophin, BDNF, in hippocampus of hypoxic rats were also investigated.

## Materials and Methods

### *Animals and experimental design*

A total number of 36 male Wistar albino rats (200–256 g, 6-week-old) were purchased from Pharmacy Faculty of Tehran University of Medical Sciences, Tehran, Iran. All animals were maintained in a clean and hygienic environment, on a 12-h light and dark cycle and 23 ± 2°C temperature, and had access to food and water *ad libitum*. All procedures were performed in accordance with the guidelines of the Iranian Council for

use and care of animals and approved by Ethical Committee of Tehran University of Medical Sciences.

Subjects were randomly divided into six different groups: hypoxia group (H) which exposed to 8% oxygen, nitrogen 92% for 30 days in hypoxic chamber (4 hours/day), hypoxia + insulin group (HI) which after hypoxia period received insulin, intranasaly, hypoxia + mesenchymal stem cells (HC) which after hypoxia period received MSCc intraventricularly, sham1 group (Sh1) which after exposing to 21% $O_2$ for 30 days in hypoxic chamber (4 hours/day) received normal saline intranasal, sham2 group (Sh2) which after exposing to 21% $O_2$ for 30 days in hypoxic chamber (4 hours/day) received PBS intraventricularly, and control group.

All subjects were housed in keeping cages in the resting time.

### Hypoxia model

The model used in this study was based on the modified Rice-Vannucci model.[21] Animals were exposed (8% $O_2$ at normal barometric pressure) by placement of rats in a Plexiglas chamber for 30 days (4 hours/day). The hypoxic environment in the chamber was archived by inflow of $N_2$ gas. Oxygen level in hypoxic chamber was controlled by $O_2$ meter (lutron DO-5510 $O_2$ meter, Taipei, Taiwan). Control animals were kept in normoxic condition.

### Serum analysis

To determine whether the blood serum phosphorylated-IRS-1 pathway and pro-inflammatory factors like interleukin-1beta (IL1β) and tumor necrosis factor-α (TNF-α) was affected by hypoxia, at the end of hypoxic exposure (day 30), blood was sampled from left ventricle of animals and centrifuged for 10 minutes at 20,000 rpm and plasma was separated for biochemical analysis. We determined above mentioned factors in serum by using enzyme-linked immuno absorbent assay (ELISA) Kit (zellBio-GmbH).

### Morris water maze test

After exposing to hypoxic condition (day 30), spatial learning and memory of animals was assessed by Morris Water Maze (MWM) test and based on the previously described method.[22] It consisted of a circular water tank (160 cm diameter, 60 cm height) filled with water (25 ± 1°C) to a depth of 25 cm. A non-toxic water dispersible emulsion was used to render the water opaque. Four equally spaced locations around the edge of the pool (North, South, East, and West) were used as start points which divided the pool into four quadrants. An escape platform (10 cm in diameter) was placed in the pool 2 cm below the surface of water. The escape platform was placed in the middle of one of the randomly selected quadrants of the pool and kept in the same position throughout the entire experiment (north–east for this study). One month after probe test 1 (day 34), probe test 2 (day 64) was done to identify if learning and memory skills were interrupted or not. The time spent in the target quadrant indicated the degree of memory consolidation which had taken place after learning.

### Intranasal administration of insulin hormone

After exposure to chronic hypoxia, rats (HI group) were administered (5 IU/rat/day) insulin hormone (human recombinant, Sigma) through intranasal route for six consecutive days. All stages of experiment are shown in Figure 1.

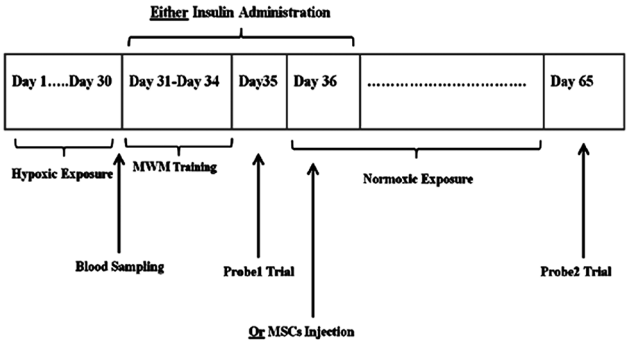


Fig. 1 **All stages of experimental design.**

### Isolation and cultivation of WJ-MSCs

Umbilical cords were obtained with the signed permission of the Arash Hospital (Tehran, Iran) patients. According to previous study in our lab, Explant method was used to isolate MSCs. WJ was cut into 2-mm pieces and cultured for 1 week in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 15% fetal bovine serum (Gibco, USA), 1 µg/ml amphotericin B (Gibco, USA), 100 U/ml penicillin (Gibco, USA) and 100 µg/ml streptomycin (Gibco, USA), and placed in a 5% $CO_2$ incubator at 37°C. Then, WJ pieces were removed, and medium was renewed twice weekly. WJ cells were collected with 0.25% trypsin–ethylenediaminetetraacetate (Gibco, USA) upon achieving 90% confluence, and cells in passage three to four were used for stereotaxic injection into lateral brain ventricles of animals.[13] Data are not shown in present study.

### Perfusion fixation

Rats were euthanized 30 days hypoxia. Rats were deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (25 mg/kg) (both Razi Co., Iran) intraperitoneally and perfused through the ascending aorta by 500 ml of ice-cold normal saline then 4% paraformaldehyde (freshly made from paraformaldehyde) in 0.16M phosphate buffer (pH 6.9). Immediately after fixation, brains were removed and stored in a 10% formaldehyde solution prior to sectioning.

### Nissl staining

Sections were stained with 0.1% cresyl violet (Merck, Germany) and dark neurons were quantified in CA1 and CA3 regions of the hippocampus in at least three sections of each rat. The process was repeated for at least three times in each section and its average was taken as the final value.

### RNA isolation and real time RT-PCR

Rats were deeply anesthetized with mixture of ketamine (100 mg/kg) and xylazine (25 mg/kg) intraperitoneally. After removing the brain, the hippocampal formation was dissected. Tissue pieces were immediately frozen in liquid nitrogen and maintained at −80°C until RNA isolation. Expression of Seladin-1 and APP genes were measured by qRT-PCR. By using TRIzol® reagent, (Cinnagen, Iran) total RNA was isolated from hippocampus, then mRNA (1 µg) converted to cDNA through reverse transcription using First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Specific primers along with cDNA and PCR reagents (polymerase, dNTP, magnesium and buffer; 5× HOT FIREPol® EvaGreen® qPCR Mix Plus [ROX] 1 ml, Solis Bio Dyne,

Estonia) were placed on the three-color real-time PCR machine (Applied Biosystems Step One, USA) for further analysis.

At first, incubation of samples were performed at 95°C for 15 minutes for initial polymerase activation. Then, samples underwent the three subsequent phases: denaturation, at 95°C for 15 s; annealing, at 60°C for 20 s; and elongation, at 72°C for 20 s. Finally, $\Delta\Delta C_t$ technique was used for relative quantification of data and further normalization to GAPDH and fold change comparison to the control.[13] The nucleotide sequences of primers are listed in Table 1.

### Statistical analysis

All data were analyzed through one-way analysis of variance (ANOVA) and the post-hoc Tukey's; and two-way ANOVA and the Bonferroni's multiple comparison tests were applied for behavioral data belonging to learning and memory skills using GraphPad PRISM version 6 Software (San Diego, California, USA).

Results were represented as mean ± SEM and $P \leq 0.05$ was considered significant.

## Results

### Serum analysis

Pro-inflammatory cytokines were measured in blood serum. Hypoxia group in comparison to the control and sham groups revealed a considerable increase in levels of TNF-α and IL-1β at day 30 after hypoxia ($P < 0.05$) (Fig. 2, Table 2). There were no significant differences in the levels of cytokines in control and sham groups.

### Behavioral studies

The MWM test was performed to examine spatial learning and memory related to hippocampus. As shown in Figures 3a and 3b, spatial memory testing in rats that were exposed to chronic hypoxia was reduced significantly comparing with control group ($P < 0.05$). During the 4 days of training, insulin administration ameliorates animals performance indicated by reduced escape latency and also travelled distance to reach the platform compared to the hypoxia group (Figures 3a and 3b) suggesting a protective effect of insulin in spatial learning and memory skills.

As introduced in Figure 2c, time in the target quadrant both in hypoxia and hypoxia + insulin groups were significantly different from the control group ($P < 0.05$).

During the probe 2, where rats tried to find the hidden platform, hypoxic rats spent less time in the target area than in any of the other three quadrants comparing with control group ($P < 0.05$). In contrast, both hypoxia + insulin and hypoxia + MSCs rats spent, significantly, more time in the target quadrant in comparison with hypoxia group ($P < 0.05$) proposing beneficial influent of insulin and MSCs in long term (Figure 3d).

Figure 3e demonstrates that all groups showed a declining latency time to reach the escape platform during the training period with a slightly stronger improvement in the hypoxia + insulin group compared to data shown in Figure 3a.

The swimming speed did not differ between all groups (data are not shown).

Table 1. **Primers and expected length of products**

| Primer | Sequence | Length (bp) |
|---|---|---|
| GAPDH | For 5'-AAGTTCAACGGCACAGTCAAGG-3' | 147 |
| | Rev 5'-CATACTCAGCACCAGCATCACC-3' | |
| BDNF | For 5'-GGCGAGCAGAGTCCATTCAG-3' | 298 |
| | Rev 5'-ACCCAGTATACCAACCCGGA-3' | |

Forward (For); Reverse (Rev); base pair (bp).

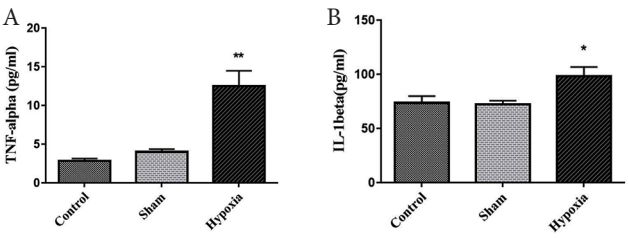


Fig 2. **Evaluation of both (A) Tumor necrosis factor-α (TNF-α) and (B) interleukin-6 (IL-1β) level to confirm hypoxic model. *$P < 0.05$, **$P < 0.01$ compared with control group. $n = 6$ in each group.**

Table 2. **Quantitative evaluation of both Tumor necrosis factor-α (TNF-α) and interleukin-1beta (IL-1β) level to confirm hypoxic model in blood serum**

| | TNF-α (pg/ml) | IL-1β (pg/ml) |
|---|---|---|
| Control | 2.83 ± 0.75 | 98.83 ± 8.10 |
| Sham | 4 ± 0.81 | 112.66 ± 15.98 |
| Hypoxia | 12.5 ± 4.84** | 156.25 ± 18.89* |

TNF-α: Tumor necrosis factor-α, IL-1β: interleukin-1beta, pg/ml: Picogram/milliliter. *$P < 0.05$, **$P < 0.01$ compared with control group. $n = 6$ in each group.

### Nissl staining

Nissl staining was performed to quantify the density of Nissl-stained neurons in the CA1 and CA3 pyramidal cell layer of hippocampus in different groups (Figures 4 and 5). Our results showed dark neurons with enriched Nissl bodies were abundant in hypoxia group in CA1 (12 ± 3.944053) and CA3 (10.3 ± 4.967673) in comparison with control group in CA1 (2.571429 ± 1.272418) and CA3 (2 ± 0.707107), respectively. In contrast, Nissl bodies in the pyramidal cell layer of hippocampus in hypoxia + insulin (HI) group in CA1 (3 ± 1.732051) and CA3 (4 ± 0.894427) and hypoxia + MSCs group in CA1 (4.5 ± 2/073644) and CA3 (4 ± 0/894427) were significantly deceased or disappeared, respectively, indicating that insulin hormone improved neurons arrangement. However, there were negligible differences in sham groups in CA1 and in CA3 with control group (Figure 6).

### Gene expression

Expression of BDNF mRNA was assessed by real-time RT-PCR (Figure 7). Significantly ($P < 0.05$) reduced levels of BDNF transcription were observed in the hypoxia group in comparison to the control group. Both Insulin administration and MSCs transplantation following long term hypoxia exposure, showed significant BDNF expression amelioration, protectively ($P < 0.05$).

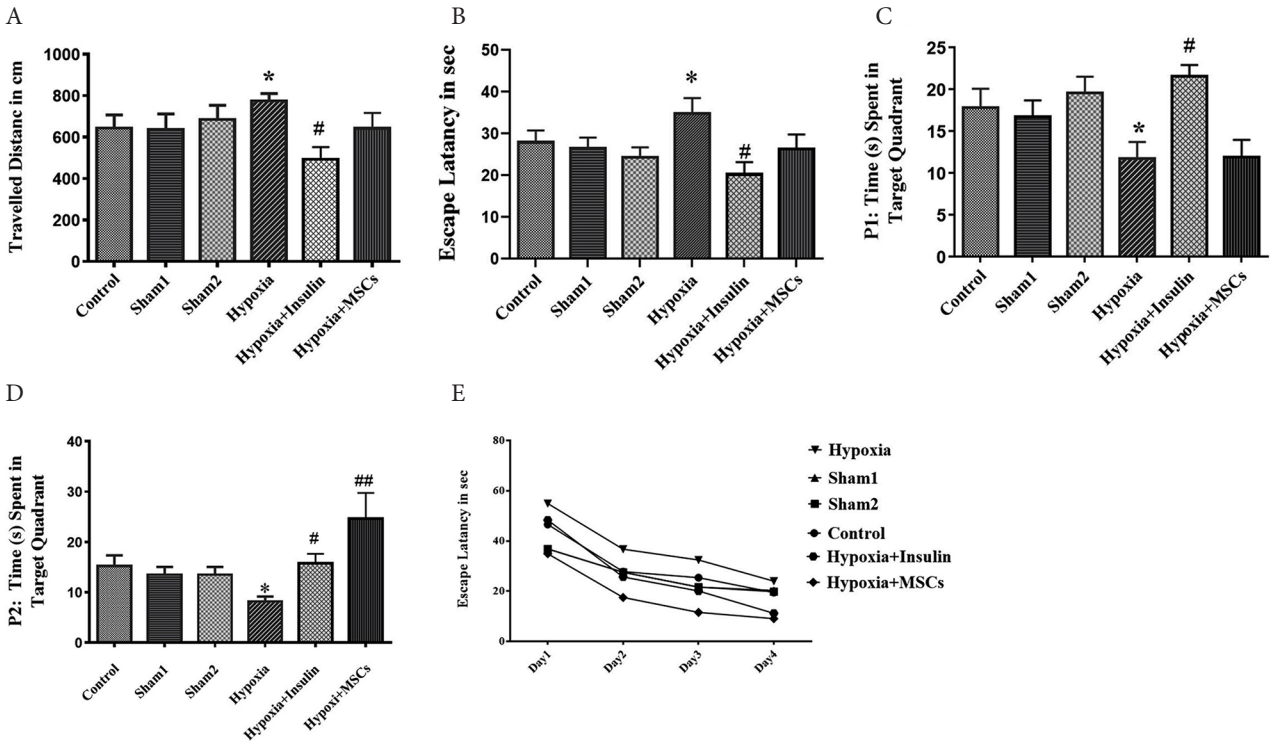


Fig. 3 **Effect of either insulin or MSCs on behavioral performance. (A) Escape latency and (B) travelled distance both after 4d, (C) time spent in the target quadrant in day probe 1, (D) time spent in the target quadrant in day probe 2, and (E) escape latency during 4d training period. Time of escape latency experiments expressed from animals per group (D) shows that in all groups a reduction can be observed during the training period with worst improvement in the hypoxia group. *$P < 0.05$ hypoxia vs control, #$P < 0.05$ hypoxia + insulin vs hypoxia, ##$P < 0.01$ hypoxia + MSCs vs hypoxia.**

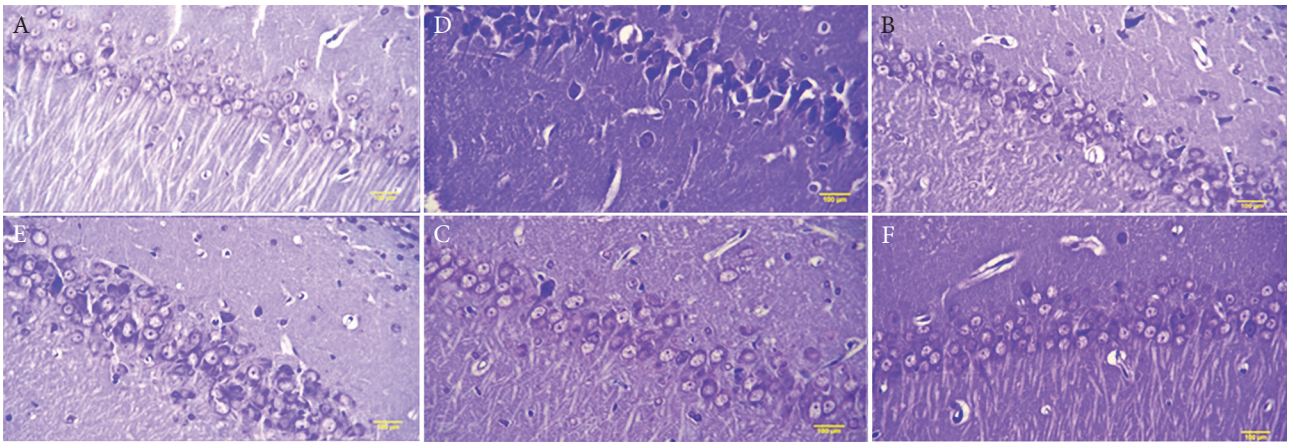


Fig. 4 **Nissl staining (cresyl-violet) of neurons in the CA1 pyramidal cell layer of hippocampus. (A) Control group with any intervention. (B and C) Sham groups with no significant difference by control group. (D) Hypoxia group. Dark neurons in the CA1 of hippocampus rats were dramatically increased. (E and F) Hypoxia + insulin and hypoxia + MSCs: Dark neurons were decreased or disappeared in the CA1 of hippocampus. All figures: 40×, Scale bar: 100 µm.**

## Discussion

This study demonstrated that hypoxic condition, due to oxygen supply decreasing of brain, causes structural CA1 and CA3 fragmentation,[1,23,24] parts of brain that are contributing for cognitive failure and facilitating aging process.[1,25] These structural damage and slow-downing in memory skills are parallel with what others have observed in their study.[1] We saw spatial retrieval memory and learning was significantly affected by long term hypoxic exposure, especially in second probe trial, 1 month after the first probe trial. MWM parameters were confirmed by histological findings of our study. Nissl bodies in pyramidal layer of CA1 and CA3 did not increase immediately in the end of 30 day-period-hypoxia (data are not recorded) but boosted, meaningfully, after 1 month of this period. It suggests that brain tissue is injured by secondary chronic hypoxia consequences.

On the other hand, when we administrated either intranasal insulin or stereotactical MSCs, our data in memory recovery was improved and animals spent more time in goal quadrant. Furthermore in morphological analysis, we found that the betterment had happened in hippocampus structure and Nissl bodies had lessened.

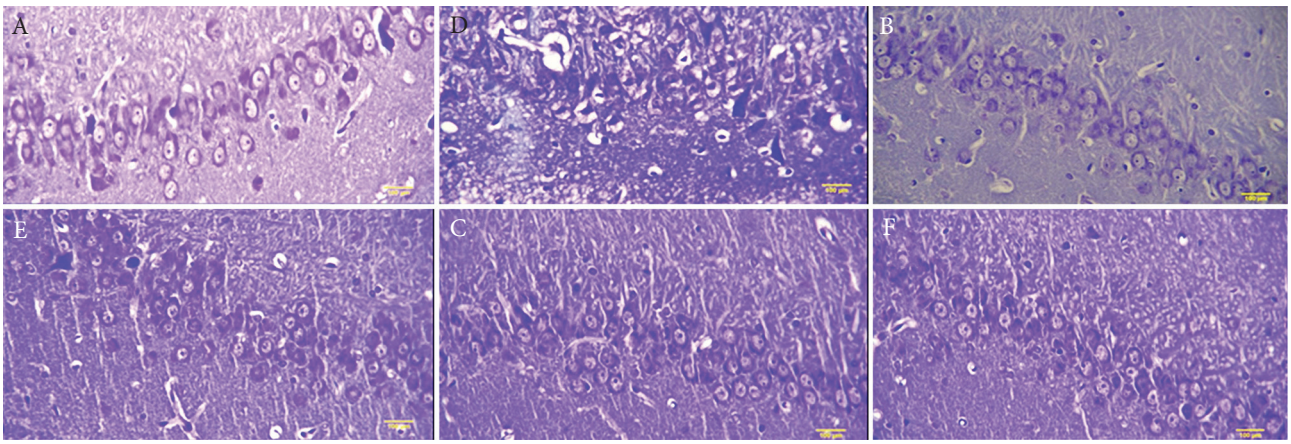


Fig. 5 **Nissl staining (cresyl-violet) of neurons in the CA1 pyramidal cell layer of hippocampus. (A) Control group with any intervention. (B and C) Sham groups with no significant difference by control group. (D) Hypoxia group. Dark neurons in the CA1 of hippocampus rats were dramatically increased. (E and F): Hypoxia + insulin and hypoxia + MSCs: Dark neurons were decreased or disappeared in the CA1 of hippocampus. All figures: 40×, Scale bar: 100 µm.**

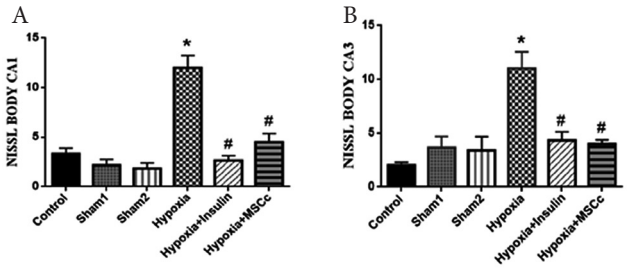


Fig 6. **(A) CA1 and (B) CA3 represent the mean ± SEM number of dark neurons with rich Nissl bodies in cytoplasm, n = 6 in each group.*$P < 0.05$ hypoxia vs control or vs. sham (a); #$P < 0.05$ hypoxia + insulin and hypoxia + MSCs hypoxia. There is no significant difference between hypoxia + insulin and hypoxia + MSCs.**

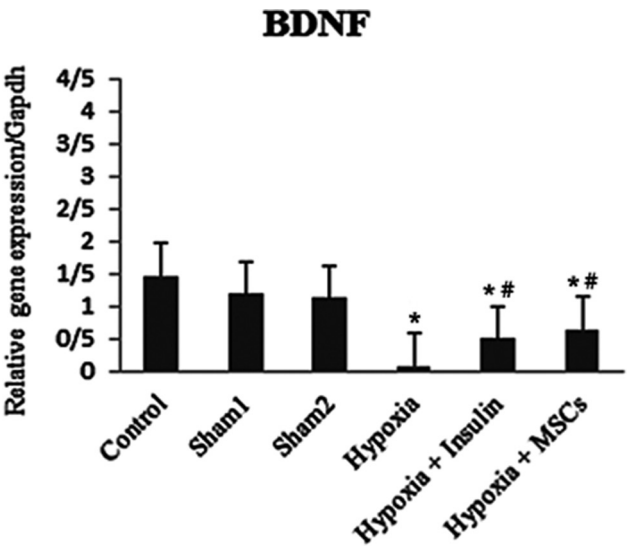


Fig 7. **Effects of either insulin or Wharton's jelly mesenchymal stem cells (WJ-MSCs) in combination with on fold change expression of brain-derived neurotrophic factor (BDNF) mRNA analyzed by quantitative real-time PCR (qRT-PCR) in hypoxic rats. Data were normalized to GAPDH and represented as mean ± SD of six rats. *$P < 0.05$ hypoxia vs control or vs sham (a); #$P < 0.05$ hypoxia + insulin and hypoxia + MSCs vs hypoxia. There is no significant difference between hypoxia + insulin and hypoxia + MSCs.**

It is ascribed that damaged CNS tissue could not be repaired because neurogenesis was only seen during development.[15] But what we saw in both memory improvement and hippocampus arrangement was in contrast with that claim. Several explanations could be suggested to explain this observation by means of stem cells recovery power, not only due to their proliferation and migration to injured side but also by secretion of neurotrophic factors such as VEGF, GDNF, GFAP and BDNF.[16,26,27]

Given the preceding our observations, CNS inflammation induced by hypoxia can promote endogenous neurogenesis.[28,29] Nowadays numerous investigators are trying to discovery of neural stem/progenitor cells (NSPCs) niches in the adult brain to stimulation of endogenous proliferation processes, as a therapeutic goal in neural diseases treatment. It has become increasingly clear that SGZ of hippocampal formation is one of those islands with neural stem cells,[29,30] that are stimulated for proliferation process strongly by the expression of BDNF receptor (TrkB).[14] Thereby, SGZ cells are affected directly by BDNF deprivation. BDNF acts its neurogenesis effects through improvement of neuronal survival in the SGZ and risen the number of recently produced neurons in the olfactory bulb.[31] These correlations may uncover why when we injected MSCs in the brain lateral ventricle, BDNF level had a significant amplification in the injured region. It can be supposed that the curative effect of WJ-MSCs are in production of several pro-angiogenic cytokines, chemokines, interleukins and trophic factors predominately participate in neurology, angiogenesis, hematopoiesis and neurogenesis.[32,33] Producing significantly higher messenger RNA (mRNA) and protein levels of the neurotrophic factors BDNF by MSCs, they bind to TrkB sites in SGZ and motivate proliferation and migration of new neuron to become functional in damaged sites.[14] As study by Shirayama et al.[34] clearly demonstrated that cell proliferation in the SGZ was stopped when BDNF receptors (TrkB) were blocked.[34] This observation strongly support our results, reinforcing the idea that BDNF is a key molecule for NSCs differentiation.[35]

In contrast to MSCs, it is less known about the insulin as a neuro-proliferating-growth factor. Many functional studies have focused on insulin signaling in growth, cell cycling and

proliferation of NSCs in mentioned niches.[20] Accumulating data in *in vivo* and *in vitro* show that physiological level of insulin (25 ng/ml) activates insulin receptors and co-cooperates in cell growth and survival but higher level (4–5 µg/ml) stimulates both insulin and insulin-like growth factors (IGF) receptors.[36] It is a well-established IGF system through Ras-Raf-MAPK and PI3K-Akt pathways required for IGF-stimulated neural proliferation.[37] In agreement with a previous observation by Shirayama et al., in our study we also observed the beneficial influence of insulin administration on structural arrangement and memorial skills enhancing induced by BDNF expression and mightily[38] neuronal differentiation in SVZ, respectively. One may further guess is that the intranasal insulin stimulates proliferation of neural stem cells, which would be embedded in the olfactory region, to adult-functional neuron and emigrate into injured regions, hippocampus and cerebral cortex.[39]

To confirm our results, researchers thought that there is a high correlation between insulin and its signaling deprivation in the brain and pathological features such as neural malfunction, synaptic failure and learning and memorial efficiency.[40,41] Additionally Kernie et al.,[42] speculated SVZ NSCs-derived neuroblasts migrate along the rostral migratory stream toward the olfactory bulb (OB) following a hypoxic–ischemic insult.

## Conclusion

Taken together, we suggest an extreme plasticity of hippocampal formation that up-regulated by transplantation of MSCs and insulin administration which can dramatically improve both BDNF expression in order to induce NSCs proliferation and cognitive function in chronic model of hypoxia.

## Acknowledgment

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## Conflict of Interest

The author declares no conflict of interest.

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