

Effects of maternal thyroid hormone deficiency on differentiation of mesenchymal stem cells in CSF-exposed neonatal Wistar rats

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Cerebrospinal fluid (CSF) contains growth and neurotrophic factors which regulate proliferation, differentiation, and neurogenesis. Thyroid hormones play a crucial role in the development of the nervous system and hypothyroidism during development of embryos leads to defects in the nervous system. This study aimed to survey the effects of rat neonatal CSF collected from induced hypothyroid mothers on differentiation of bone marrow mesenchymal stem cells (BM-MSCs). We hypothesized that hypothyroidism affected levels of growth factor in CSF. To induce hypothyroidism, pregnant Wistar rats received methimazole at the third day of gestation. BM-MSCs were obtained from rat femurs and tibias and cultured in medium. CSF was collected from the cisterna magna of newborn rats, and cells were subsequently exposed to CSF with concentrations of 5, 7, and 10 /100 (v/v) for 72 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and real time polymerase chain reaction (RT-PCR) were used to quantify the cell viability and analyze the expression of neural markers, respectively. Our morphological studies showed that treatment with hypothyroidism CSF (HTH-CSF) resulted in a significant decrease in neurite growth and proliferation as compared to normal CSF (N-CSF). RT-PCR analysis also showed a significant decrease in expression of neural markers (i.e., *Nestin*, *Neurod-1*, *NeuN*) in cells treated with HTH-CSF as compared with the N-CSF group. The most effective concentration of CSF for BM-MSC differentiation was 5% (V/V). Our results showed a significant decrease in differentiation of BM-MSCs in the presence of neonatal CSF of hypothyroid mothers compared with neonatal CSF of healthy mothers. Thus, thyroid hormones are essential in neural development and hypothyroid defects can affect development of the neonatal brain.

Key words: cerebrospinal fluid, bone marrow mesenchymal stem cells, thyroid hormones, neural differentiation, hypothyroidism

INTRODUCTION

Thyroid hormone (thyroxine, T₄) and its active deiodinated derivative, 3,5,3-triiodo-L-thyronine (T₃) are important regulators for neural development of vertebrates which function in the brain by binding to T₃ nuclear receptors (Chatonnet et al., 2011). In humans, the fetal thyroid gland is not formed until the second trimester of gestation and in rodents it is not functional until the 17th day of pregnancy. Importantly, thyroid hormones pass easily through the placenta and the growing embryo relies on the mother for thyroid

hormones during the first half of pregnancy (Sahay et al., 2012). When pregnancy is accompanied by hypothyroidism, an array of unexpected problems are possible for both the mother and fetus (Sahay and Nagesh, 2012). T₄ and T₃ ligand regulate neural differentiation and control dendritic and axonal growth, neural cell migration, synaptogenesis, myelination, and signaling (Bernal, 2015). Previous investigations have reported that hypothyroidism during critical phases of brain development in fetal and neonatal life lead to various changes in the pattern of migration, and result in reduced cell number and myelination in cerebellum, neocortex, and

hippocampus (Mussa et al., 2001). Other studies have revealed an interaction between thyroid and growth factors during development (Ahmed, 2018), which, according to our research, suggests that thyroid hormone deficiency affects growth factors level in the CSF. CSF contains cytokines, retinoic acid (RA), and diffusible factors such as transforming growth factor- β (TGF- β), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), insulin-like growth factor, and neurotrophin-3 which can regulate the survival, proliferation, and differentiation of neuroepithelium. Neurotrophins modulate various developmental processes such as neural differentiation in the adult and developing nervous system of vertebrates (Nabiuni et al., 2015). These factors have been shown to be involved in some brain functions such as connectivity of neurons and neurite outgrowth (Yoshi et al., 2010). Meanwhile, the effect of thyroid hormone on development of nervous system is mediated via growth factors such as NGF and epidermal growth factor (Fisher et al., 1982). CSF has a prominent function in regulating the behavior of neuroprogenitor cells (Yari et al., 2012). Self-renewal and lineage commitment of mesenchymal stem cells have shown to be influenced by several factors, including morphogenetic factors, TGFs, RAs, and other small molecules (Nava et al., 2012). As mentioned above, hypothyroidism has negative effects on both mother and fetus. Thus, it is important to study the effects of maternal hypothyroidism on CSF of neonates and also the effects of CSF from hypothyroidism mothers on neural cell proliferation and differentiation. The main aim of our study was to investigate the effect of neonatal CSF obtained from hypothyroid mothers on neural differentiation of bone marrow mesenchymal stem cells (BM-MSCs), to better understand the abnormality of hypothyroid CSF.

METHODS

Animals

Wistar rats were bred in the Animal Center and Cellular and Molecular Research Laboratory, Kharazmi University. The animals were kept in standard conditions at a constant temperature with a 12-hour light/dark cycle and free access to food and water. All animals were treated in compliance with the guidelines for Ethical Committee for the care and use of animals approved by our university, in accordance with the principles of laboratory animal care (NIH Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Washington, D.C). Mating was induced by housing individual females with a male (200 - 250 g) in a separate

mating cage. Day zero of gestation (GD) was determined by observation of the vaginal plaque. CSF was collected from newborn rats following our previous protocol (Nabiuni et al., 2012).

Preparation of hypothyroid rats

In this study, pregnant rats (n=40) from the third day of gestation (GD3) to the first day of postpartum (PN0) were allocated into one of two groups: euthyroid (normal control) and hypothyroid rats. Hypothyroidism was induced by administration of Methimazole (MMI) (Darupakhsh, Iran) in drinking water (50 ppm) from the third day of gestation to the first postpartum day (GD3-PN0). Control rats received only tap water.

CSF collection from newborn Wistar rats

CSF specimens were collected from the cisterna magna of euthyroid and hypothyroid newborn rats using glass micropipettes into sterile tubes. Specimens were centrifuged at 1400 rpm for 5 min to remove the remaining cells and debris. Supernatant was transferred into another sterile micro tube and immediately stored at -80°C (Nabiuni et al., 2012).

Isolation and expansion of BM-MSCs

BM-MSCs were harvested from the femur and tibia of 4 to 6 week old male rats weighing 100 - 150 g. Briefly, stromal cell suspension of bone marrow was prepared by flushing the femurs and tibias in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 1% pen/strep using a syringe needle. The cell suspension was then centrifuged at 15000 rpm for 5 min, and subsequently cultured in 25 cm² flasks. The adhered cells from the third passage were used to be treated with CSF (Shokohi et al., 2017).

Treatment of BM-MSCs using CSF

BM-MSCs were seeded in 96-well plates with a cell density of $2 \times 10^4/\text{cm}^2$ and the media renewed every two days. Then, cells were treated using N-CSF and HTH-CSF. To determine the most effective concentration of CSF, cells cultured in DMEM, 3% FBS and 1% pen/strep were exposed to different concentrations of CSF [i.e., 5, 7 and 10 /100 (v/v)] for a week. One concentration was selected as yielding the optimum CSF concentration (Shokohi et al., 2017).

Morphological properties and neurite-like processes assessment

To evaluate the morphology and growth rate of BM-MSCs, cell culture plate photography was performed using an inverted microscope (Olympus, Tokyo, Japan) and the images were analyzed by ImageJ software (NIH, version 1.51). The cell morphology and length of neurite-like processes was compared between cell treatments (Shokohi et al., 2018).

Cell proliferation assay

MTT assay is a colorimetric test for measuring cell proliferation and metabolic activity. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a yellow tetrazolium dye that in responding to activated mitochondrial dehydrogenases changes to dark blue formazan crystals. The effective CSF dosage and survival rate of cells were evaluated. BM-MSCs were exposed to HTH-CSF and N-CSF with concentrations of 0, 5%, 7%, 10 % (v/v) for 72 h. Next, the cells were incubated with MTT (5 mg/mL in PBS, Merck, Germany) at 37°C for 3 h. The formed formazan crystals were then dissolved in dimethyl sulfoxide (DMSO) (Sigma) and absorbance was measured at 570 nm via a plate reader (Stat fax 2100, USA) (Shokohi et al., 2018).

Quantitative real-time PCR (qRT-PCR)

To investigate the neural differentiation of BM-MSCs, real time PCR was performed to evaluate the expression of neural markers. Total RNA was isolated from treated and non-treated cells and neural markers were evaluated using RNX Plus TM (Cinagen, Iran). First-strand cDNA was synthesized by cDNA Synthesis Kit (Fermentas, Lithuania). Quantitative PCR amplification was performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) in a thermocycler (Applied Biosystems). The amplification procedure was carried out for 40 cycles, first at 95°C for 10 min, then at 95°C for 15 sec and finally at 60°C for 1 min in each

cycle (Zheng et al., 2017). The mRNA expression of neural markers including *Nestin*, neurogenic differentiation 1 (*Neurod-1*) and neuronal nuclei (*NeuN*) or (*BRFOX3*) was examined using the primer sets outlined in Table I.

Statistical analysis

Statistical analysis was performed using SPSS Software and $p < 0.05$ was considered significant. Data are represented as the mean $\pm$ standard error of the mean (SEM). One-way ANOVA and Tukey *post hoc* tests were used to analyze the data.

RESULTS

MTT assay

Results of the MTT test showed higher cell viability in control and N-CSF groups as compared with the HTH-CSF group. More importantly, cells that were treated with 5% HTH-CSF showed a more significant increase in viability as compared to cells treated with 7% and 10% HTH-CSF (Fig. 1).

Morphological study

Morphological analysis of BM-MSCs showed long spindle-shaped cells with oval-shaped nuclei. While N-CSF treatment resulted in long and thin neurites in BM-MSCs after a week, HTH-CSF showed a less similar morphology to neural-like cells. Thus, HTH-CSF induced less proliferation and greater cell degeneration as compared to N-CSF. Microscopic observations showed cell death in cells treated with 7% and 10% (V/V) HTH-CSF (Fig. 2).

Measurement of neurite outgrowth

ImageJ analysis showed minimum neurite outgrowth in non-treated BM-MSCs (i.e., controls). The

Table I. Primer sequences for qRT-PCR.

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (3'-5')	Product length
<i>Neurod-1</i>	CCTACTCTACCACTCCCT	CAAACTGGATGGTTCG	228
<i>Nestin</i>	GTTTGGGTAAGAGCTGGCC	TCCTAGCCCCATACT	232
<i>NeuN (BRFOX3)</i>	AGTGSSTSTGCCCCACCTC	GGTGGTTGCTGGCTATCT	209
<i>GAPDH</i>	CAAATTCATGGCACCGT C	TCTCGCTCCTGGAAGATGGT	62

BM-MSCs exposed to HTH-CSF had significantly less neurite outgrowth, on average, as compared to the N-CSF-treated cells. Maximum neurite outgrowth was observed in N-CSF-exposed cells (Fig. 3).

qRT-PCR

Expression of *Neurod-1* was significantly lower in BM-MSCs exposed to HTH-CSF as compared with controls ($P \leq 0.001$). *Nestin* expression, in contrast, was significantly higher in BM-MSCs exposed to HTH-CSF as compared to controls ($P < 0.01$), and no significant changes in *NeuN* were observed as compared with controls. Altogether, HTH-CSF resulted in a significant decrease in the expression of *Nestin*, *Neurod-1* and *NeuN* as compared with N-CSF ($P \leq 0.001$). As compared to controls, cells treated with N-CSF showed an increased in

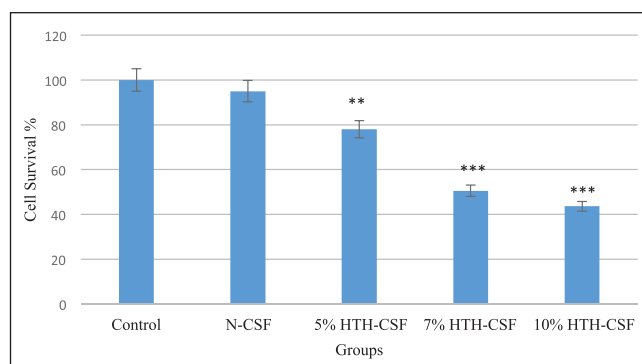


Fig. 1. The effects of N-CSF and HTH-CSF on viability of BM-MSCs. To define the most effective dose, different concentration of HTH-CSF (5%, 7% and 10% v/v) were determined via MTT assay in 72 h. Data are represented as mean $\pm$ SEM (** $P \leq 0.001$, ** $P \leq 0.01$) compared to controls.

mRNA expression (i.e., $P \leq 0.001$ for *Nestin* and *NeuN* and $P \leq 0.01$ for *Neurod-1*) (Fig. 4).

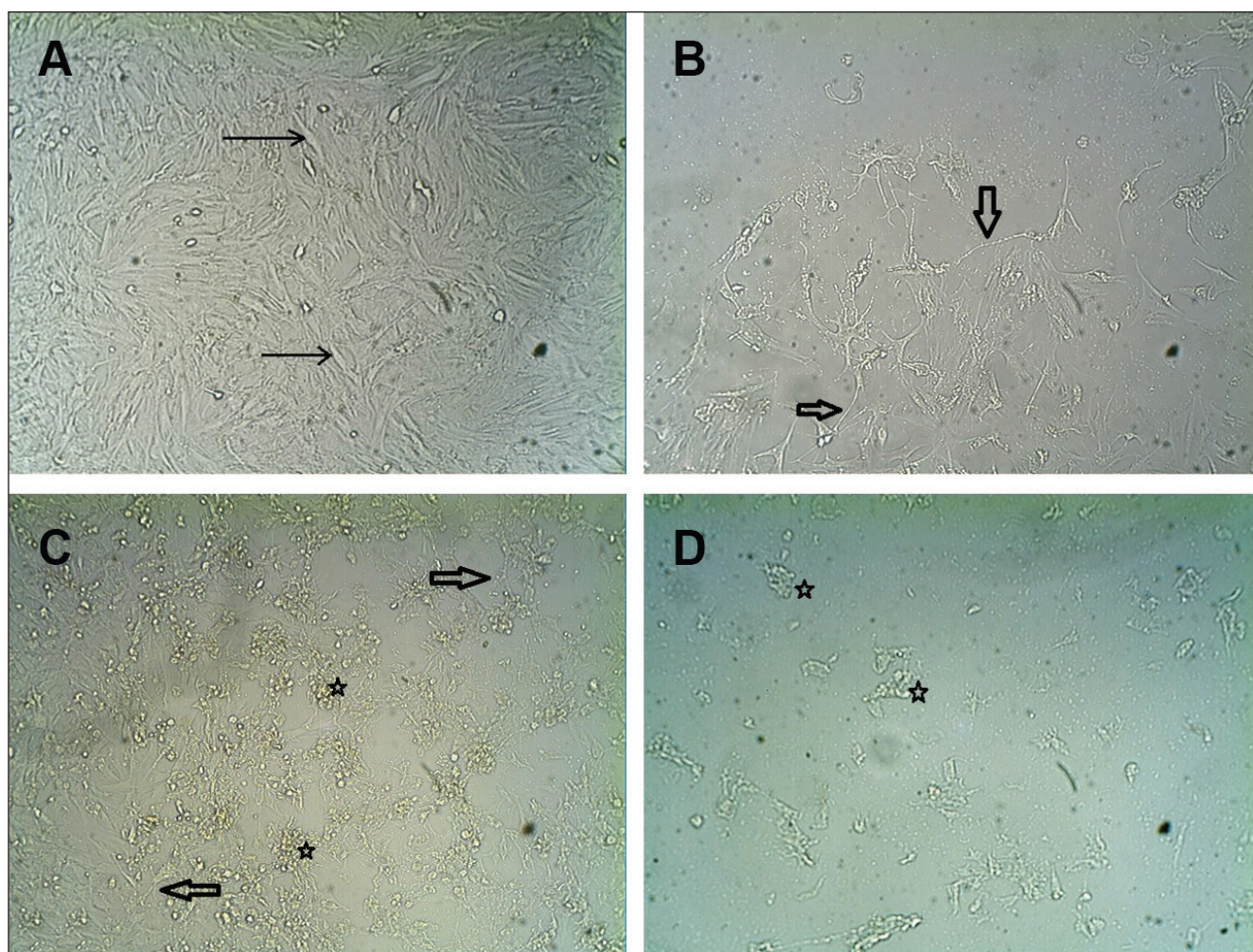


Fig. 2. Morphology of bone marrow mesenchymal stem cells (BM-MSCs) treated with N-CSF and HTH-CSF after 7 days. Non-treated BM-MSCs (A), BM-MSCs exposed to N-CSF (5% v/v) (B), BM-MSCs exposed to HTH-CSF (5% v/v) (C), and BM-MSCs exposed to HTH-CSF (10% v/v) (D), (x100). Arrow: spindle-shaped MSCs, thick arrow: neurite outgrowth, star: degenerated cells.

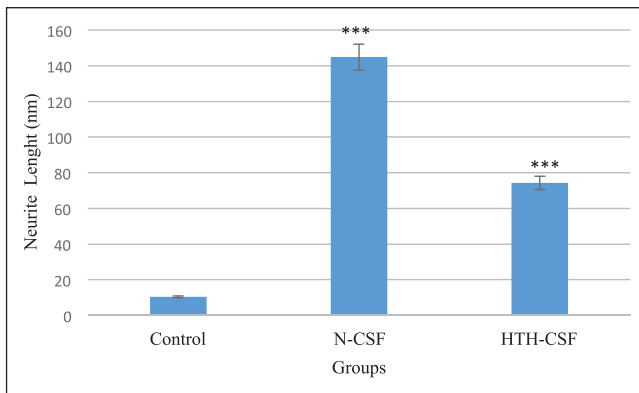


Fig. 3. Neurite outgrowth of BM-MSCs. Cells were cultured for a week without CSF treatment (i.e., control condition), and then exposed to N-CSF and HTH-CSF (5% v/v). Neurite length was measured in each group. Treatment with HTH-CSF resulted in a significant decrease in neurite length as compared with N-CSF. Data are represented as mean $\pm$ SEM (*** $P \leq 0.001$, ** $P \leq 0.01$) compared to the control condition.

DISCUSSION

Numerous studies have demonstrated that thyroid hormones play an essential role in brain development and neurogenesis. Hypothyroxinemia in mothers during the gestational and early postnatal period has been shown to result in behavioral impairments and neurological defects in children (Miranda and Sousa, 2018). In the present study, we demonstrated that maternal hypothyroidism is associated with reduced neurogenesis in BM-MSCs. Previous studies have shown that maternal thyroid hormone deficiency leads to decreased proliferation and impaired neurogenesis in the developing neo-cortex of the fetus (Mohan et al., 2012). Our results indicate that hypothyroidism suppresses neural differentiation in Wistar rat BM-MSCs. Shafiee and colleagues (2016) have demonstrated that hypothyroidism during the fetal and early postnatal periods is associated with an impairment in spatial learning and memory, and a reduction in hippocampal BDNF levels in both male and female rat offspring. Our results add to these data by showing that HTH-CSF induces morphological changes and decreased neurite growth in BM-MSCs. Previous studies have shown that maternal hypothyroidism during pregnancy restricts neurite outgrowth and induces abnormal neuronal location and synaptic impairment (Miranda and Sousa, 2018). Moreover, TH deficiency leads to morphological alterations in the cortex which may be due to altered development of axodendritic processes wherein cells become smaller and more closely aggregated than under typical conditions (Ahmed et al., 2008). Our morphological assessments showed an aggregation of cells

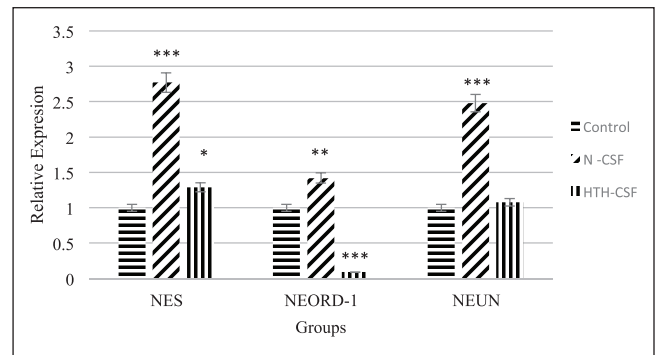


Fig. 4. Expression of neuronal markers in BM-MSCs. The expression of (A) *Nestin* [(5% v/v) N-CSF, (5% v/v) HTH-CSF], (B) *Neurod-1* [(5% v/v) N-CSF and (5% v/v) HTH-CSF] and (C) *NeuN* [(5% v/v) N-CSF and (5% v/v) HTH-CSF] are shown. Data are represented as mean $\pm$ SEM (*** $P \leq 0.001$, ** $P \leq 0.01$, * $P < 0.05$) compared to the control.

and shorter dendrite process among cells exposed to hypothyroidism CSF. In the current study, neural differentiation was characterized by expression of neural markers. In particular, *Nestin* expression is used as a marker for neural stem cells, predominantly during early development in embryonic and adult brain tissue. Stem and neural progenitor cells have been shown to express *Nestin* during proliferation (Liu et al., 2015). Our results demonstrated that *Nestin* expression decreased in BM-MSCs treated with HTH-CSF as compared with N-CSF. *Neurod-1* is a basic helix-loop-helix (bHLH) transcription factor that is expressed in the central and peripheral nervous systems late in development, and plays an important role in embryonic brain development and adult neurogenesis (Pataskar et al., 2016). Our findings show that expression of *Neurod-1* significantly decreases in BM-MSCs treated with hypothyroidism CSF as compared with N-CSF and control conditions. Neuronal nuclei (*NeuN*) is a neuron-specific marker that is used to examine the differentiation of stem cells. *NeuN* appears during early embryogenesis in postmitotic neuroblasts and remains in differentiating and terminally differentiated neurons (Gusel'nikova et al., 2015). We found a downregulation of *NeuN* in cells exposed to HTH-CSF as compared to those exposed to N-CSF. Thus, the effects of thyroid hormones on neural differentiation and survival is mediated by controlling the expression of neurotrophins. Furthermore, previous research shows that interactions between thyroid hormone and neurotrophic factors such as NGF are important for growth and maintenance of cholinergic neurons in the basal forebrain (Bernal 2015). Our results reveal that induction of maternal hypothyroidism can inhibit neural differentiation in rat BM-MSCs. CSF contains neurotrophic and growth factors which

have been shown to support the viability, proliferation, and differentiation of BM-MSCs. Results of the present study suggest that levels of neural markers in hypothyroidism CSF were lower than in N-CSF, and this reduction inhibited neural differentiation of BM-MSCs.

CONCLUSION

Previous studies have demonstrated the importance of both thyroid hormones and CSF for neurogenesis and development. In this study, we demonstrated that maternal hypothyroidism CSF can decrease neural differentiation of mesenchymal stem cells and disrupt neurogenesis and brain development of offspring.

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