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RESEARCH ARTICLE

Neural differentiation of choroid plexus epithelial cells: role of human traumatic cerebrospinal fluid

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Abstract

As the key producer of cerebrospinal fluid (CSF), the choroid plexus (CP) provides a unique protective system in the central nervous system. CSF components are not invariable and they can change based on the pathological conditions of the central nervous system. The purpose of the present study was to assess the effects of non-traumatic and traumatic CSF on the differentiation of multipotent stem-like cells of CP into the neural and/or glial cells. CP epithelial cells were isolated from adult male rats and treated with human non-traumatic and traumatic CSF. Alterations in mRNA expression of *Nestin* and microtubule-associated protein (*MAP2*), as the specific markers of neurogenesis, and astrocyte marker glial fibrillary acidic protein (*GFAP*) in cultured CP epithelial cells were evaluated using quantitative real-time PCR. The data revealed that treatment with CSF (non-traumatic and traumatic) led to increase in mRNA expression levels of *MAP2* and *GFAP*. Moreover, the expression of Nestin decreased in CP epithelial cells treated with non-traumatic CSF, while treatment with traumatic CSF significantly increased its mRNA level compared to the cells cultured only in DMEM/F12 as control. It seems that CP epithelial cells contain multipotent stem-like cells which are inducible under pathological conditions including exposure to traumatic CSF because of its compositions.

Key Words: *nerve regeneration; choroid plexus; cerebrospinal fluid; stem cells; Nestin; microtubule-associated protein 2; glial fibrillary acidic protein; neurogenesis; central nervous system; neural regeneration*

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Introduction

Well-delineated function of choroid plexus (CP) is the production of cerebrospinal fluid (CSF). As a matter of fact, CP epithelial cells secrete a wide range of proteins and signaling molecules into the CSF in which they are essential for development of the brain and its preservation against damaging elements (Strazielle and Ghersi-Egea, 2000). Furthermore, CSF not only guards the central nervous system (CNS) physically by providing a fluid cushion, but also protects the brain chemically and immunologically (Skipor and Thiery, 2008).

Particularly, CSF contents are considerably similar to blood plasma, however, it appears to contain less protein concentration and some modified amount of electrolytes, vitamins and growth factors (Hühmer et al., 2006). It is quite important to mention that CSF components are not invariable and based on the pathological conditions of the CNS, the composition of the CSF can change (McLean et al., 2006). According to the reports, there has been a growing concern for developing novel biomarkers for neurological diseases using biological components of CSF (Craig-Schapiro et al., 2011). Indeed, the protein level of neurogranin, a calmodulin-binding postsynaptic protein which regulates synaptic plasticity, increases in CSF of Alzheimer's disease patients (Janelidze et al., 2016). In addition to application of CSF for diagnostic tests, it has been reported that due to the brain-derived proteins, CSF can be a source of neurotrophins and growth factors alongside its protein profile originated from the plasma (Patterson et al., 1993; Romeo et al., 2005). Huttner et al. (2008) reported that human CSF contains membrane particle-associated prominin-1/CD133, a somatic stem cell marker. Furthermore, variations in the protein profile and content of CSF have been documented in distinct neuropathological situations indicating that the protein profile of CSF significantly changes based on the condition (Nordinet al., 2007).

Unlike the traditional view, identification and isolation of neural stem cells (NSCs) from adult CNS not only indicates the developmental abilities of adult brain, but also opens a

contemporary therapeutic window for neurological disorders. As a matter of fact, the distinct molecular mechanisms by which undifferentiated neural stem cells progress into the differentiated functional neurons have not been fully understood. Absolutely, a series of components of the neurogenic niche are needed for NSC differentiation including cell-cell interaction and secretory factors (Wurmser et al., 2004; Walker et al., 2009; Gattazzo et al., 2014). Due to the existence of various neurotrophic, mitogenic, and morphogenic factors, CSF could be considered as a fundamental media providing proper signals for prenatal and postnatal neurogenesis (Guerra et al., 2015).

The purpose of the present study was to evaluate the influence of non-traumatic (normal) and traumatic CSF on neurogenesis markers *Nestin*, microtubule-associated protein (*MAP2*) and glial fibrillary acidic protein (*GFAP*) in the cultured CP epithelial cells.

Materials and Methods

Isolation and culture of CP epithelial cells

CP epithelial cells were isolated and cultured according to the protocol as described previously (Aliaghaei et al., 2014). Briefly, adult male albino Wistar rats (Pasteur institute, Tehran, Iran), weighing 150–200 g, were decapitated under deep anesthesia and their brains were removed (n = 8). All the procedures described in this study were consistent with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 80-23, revised 1996), approved by the Animal Care Committee of Shahid Beheshti University of Medical Sciences, Iran. CP tissues were removed from the ventricles and incubated with 0.25% trypsin solution for 20 minutes at 37°C. Afterwards, the fetal bovine serum (FBS) (10%) was added and following the centrifugation, the pellets were transferred into a culture media containing DMEM/F12, FBS (10%), and antibiotic. After reaching the maximal confluency (about 2 weeks), CP epithelial cells were stained immunocytochemically with antibody against transthyretin (*TTR*), a CP epithelial cell marker and visualized with a fluorescence microscope (Olympus, IX71, Japan). CP epithelial cells were cultured in DMEM/F12 media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin (1%) for treatment (**Figure 1**).

CSF collection and treatment

Adult male patients with acute traumatic brain injury, aged 30–45 years, were included in this study. They were admitted to the Department of Neurosurgery of Shohada Tajrish Hospital, Tehran, Iran within 48 hours after the onset of symptoms. The normal CSF was obtained from patients who did not suffer from any neurological disorders and were selected from the other departments of Shohada Tajrish Hospital. The study protocol was approved by the local ethics committee (Ethics code 9020878), and written informed consent was obtained from each participant or from the close family members prior to enrollment. Ten patients with severe traumatic brain injury were included in this study. All patients had a Glasgow Coma Score ≤ 8 and showed abnormal imaging findings including obvious midline shift, effacement of the lateral ventricle and shape of hemorrhage. Intracranial hematomas were surgically evacuated if it was needed. After successful surgery, the patients were transferred to the intensive care unit (ICU) and treated according to the standardized protocol. The CSF was drained *via* intraventricular catheter (IVC) when intracranial pressure (ICP) exceeded 15 mmHg; furthermore, CSF samples were screened for bacterial infection every 3 days to ensure the absence of any infection factors. Catheters were removed when ICP was constantly remained below 15 mmHg for 24 hours. All patients were fed by gastric tube; nonetheless, no parenteral feeding was needed. Distinctly, in the control group, the CSF samples were collected by lumbar puncture from seven patients who received spinal anesthesia. These patients were checked by an independent medical doctor to rule out any neurological diseases through monitoring the recent medication. A subsample of each CSF sample was sent to a clinical laboratory for routine CSF diagnostics.

CSF samples (6–8 mL for each) were collected using polypropylene tubes. The samples were filtered and split into 1 mL aliquots and stored at –80°C prior to use. CP epithelial cells were treated with normal CSF and/or traumatic CSF (volume ratio of CSF to DMEM/F12 medium = 4:1, respectively). In the control group, CP epithelial cells were cultured only with DMEM/F12.

Reverse transcription polymerase chain reaction

The total RNA in CP epithelial cells was extracted using a high pure RNA isolation kit according to the manufacturer's instructions (Roche, Basel, Switzerland). RNA was quantified according to the absorbance at 260 nm using an Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then, 1 μg of total RNA was transcribed into cDNA using murine leukemia virus (MuLV) reverse transcriptase (Fermentas, Walthman, MA, USA) in the presence of random hexamers and RNase inhibitor. Afterwards, the obtained cDNA was amplified by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 40 seconds and extension at 72°C for 45 seconds using specific primers (**Table 1**). PCR products were size fractionated by 1.5% agarose gel electrophoresis and the images were analyzed for the density of DNA bands using US-Scan-It Gel Analysis Software (Skill Scientific Inc., Orem, Utah, USA).

Quantitative real-time PCR

For quantitative real-time PCR (qPCR) analysis, specific primers for *TTR*, *Nestin*, *GFAP*, *MAP2* and hypoxanthine phosphoribosyl transferase 1 (HPRT1) genes were used (**Table 1**). The reactions were performed using Super SYBR Green qPCR masterMix 2X (Yektatajhiz, Iran) on a Rotor-Gene™ 6000 real-time PCR machine (Corbett Research, Qiagen, Germany). The initial denaturation was done at 95°C for 5 minutes following 45 cycles of denaturation at

Table 1 RT-PCR and qPCR primer sequences

Primer	Sequence	Size (bp)	Annealing	Accession
HPRT	Forward: AGG CCA GAC TTT GTT GGA TT Reverse: GCT TTT CCA CTT TCG CTG AT	119	60°C, 15 seconds	NM_012583.2
TTR	Forward: CCT GGG GGT GCT GGA GAA T Reverse: ATG GTG TAG TGG CGA TGA C	317	61°C, 20 seconds	NM_012681.2
GFAP	Forward: GGT GGA GAG GGA CAA TCT CA Reverse: CCA GCT GCT CCT GGA GTT CT	233	61°C, 20 seconds	NM_017009.2
Nestin	Forward: CCT CAA GAT GTC CCT TAG TCT G Reverse: TCC AGA AAG CCA AGA GAA GC	114	60°C, 20 seconds	NM_001308239.1
MAP2	Forward: CAA ACG TCA TTA CTT TAC AAC TTG A Reverse: CAG CTG CCT CTG TGA GTG AG	122	60°C, 20 seconds	NM_013066.1

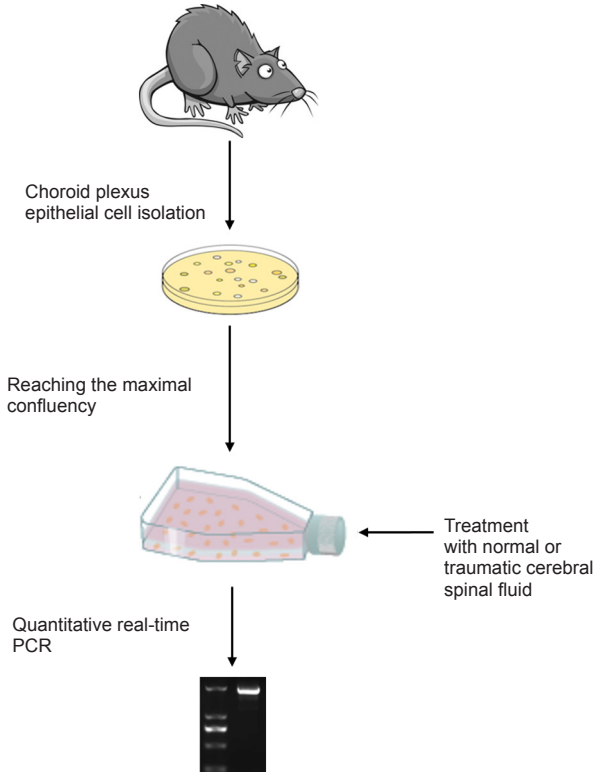

Figure 1 Schematic flowchart of experimental procedure.

95°C for 10 seconds, under primer specific conditions (**Table 1**), eventually extension was performed at 72°C for 20 seconds. Comparative quantitation was performed between selected groups using REST 2009 (Relative Expression Software Tool, Qiagen) based on Pair Wise Fixed Reallocation Randomization Test©.

Statistical analysis

All data are represented as the mean ± SEM. The comparison between groups was done by one-way analysis of variance (ANOVA) followed by a specific *post-hoc* test (Tukey's multiple comparison test). Statistical analysis was performed using REST 2009 (Relative Expression Software Tool, Qiagen, Hilden, Germany) based on Pair Wise Fixed Reallocation Randomization Test (Pfafflet al., 2002). A level of $P < 0.05$ was considered statistically significant.

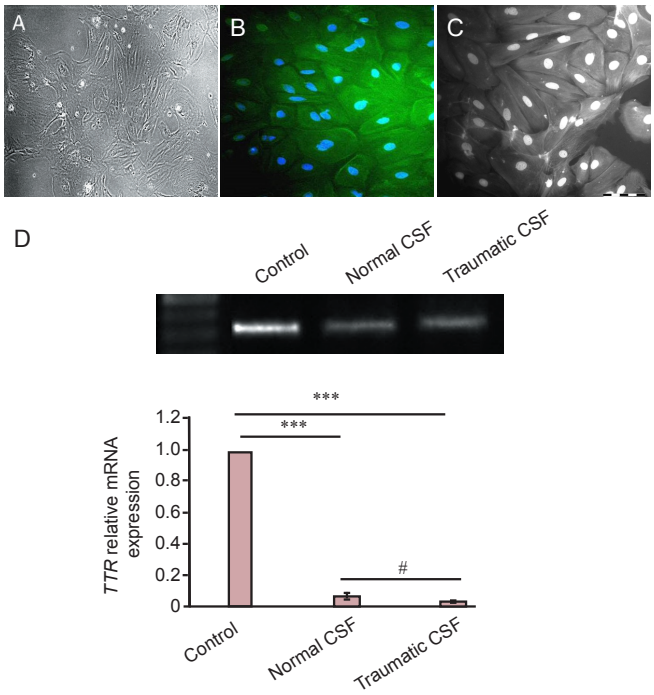

Figure 2 Cultured choroid plexus (CP) epithelial cells and the effect of normal and traumatic cerebrospinal fluid (CSF) on *TTR* mRNA expression in CP epithelial cells.

(A) Three days after culture, round cells appeared. (B, C) After reaching the maximal confluency, immunocytochemical analysis was done to ensure the expression of *TTR*, a marker for CP epithelial cells using a fluorescence microscope (Olympus, IX71, Japan). (D) mRNA expression of CP epithelial cells exposed to normal and traumatic CSF. The scale bar in C, valid for A–C, is 200 μm. The data are represented as the mean ± SEM ($n = 3$). ***$P < 0.001$, *vs.* control cells; #$P < 0.05$, *vs.* normal CSF (one-way analysis of variance followed by a specific *post-hoc* test (Tukey's multiple comparison test)).

Results

Traumatic CSF decreased expression of *TTR* in CP epithelial cells

Three days after culture of CP epithelial cells, round cells were detected in the flasks. Two weeks later, the cells covered the whole surface of the flasks. Cells were polygonal and had the appearance of epithelial cells. As shown in **Figure 2**, immunocytochemistry data showed that these cells were immunopositive for *TTR*, a marker for CP epithelial cells (Herbert et al., 1986). Furthermore, mRNA expression

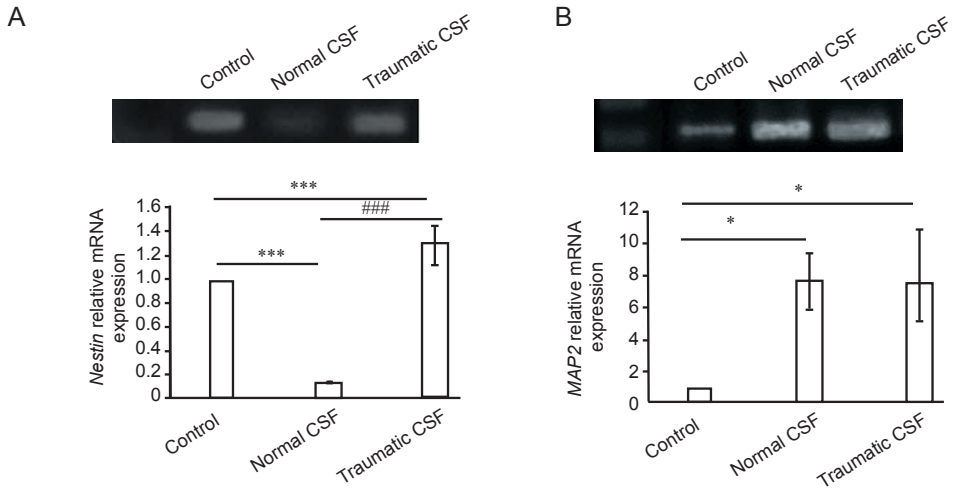

Figure 3 Effect of normal and traumatic cerebral spinal fluid (CSF) on *Nestin* (A) and microtubule-associated protein (*MAP2*; B) mRNA expression in choroid plexus epithelial cells.
The data are represented as the mean ± SEM (n = 3). *P < 0.05, ***P < 0.001, *vs.* control cells; ### P < 0.001, *vs.* normal (non-traumatic) CSF (one-way analysis of variance followed by a specific *post-hoc* test (Tukey's multiple comparison test)).

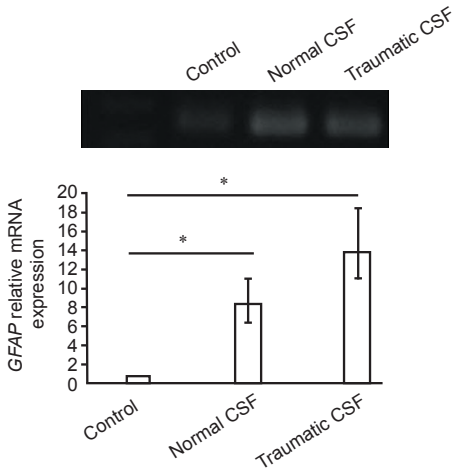

Figure 4 Effect of normal and traumatic cerebrospinal fluid (CSF) on mRNA expression of glial fibrillary acidic protein (*GFAP*) in choroid plexus epithelial cells.
The data are represented as the mean ± SEM (n = 3). *P < 0.05, *vs.* control cells (one-way analysis of variance followed by a specific *post-hoc* test (Tukey's multiple comparison test)).

of *TTR* in cells exposed to normal and traumatic CSF decreased obviously than that in the control cells (P < 0.001) (**Figure 2D**).

Effect of traumatic CSF on mRNA expression of *Nestin* and *MAP2* in CP epithelial cells

Nestin has been recognized as a marker for neural stem/progenitor cells (Suzuki et al., 2010). The expression of *Nestin* in CP epithelial cells incubated with traumatic CSF increased remarkably compared to that in control CP epithelial cells cultured with DMEM/F12. mRNA level of *Nestin* in cells exposed to normal CSF decreased significantly than that in control cells (P < 0.001). mRNA expression of *Nestin* in the presence of traumatic CSF in CP epithelial cells was 1.3-fold greater than that in the control cells (P < 0.05) and 10-fold higher than that in the cells exposed to normal CSF (P < 0.05; **Figure 3A**). The mRNA level of *MAP2* in CP epithelial cells incubated with normal and traumatic CSF significantly increased than that in the control cells (P < 0.05). mRNA expression of *MAP2* was nearly 7.7-fold (P < 0.05) and 7.5-fold (P < 0.05) greater in cells exposed to normal and traumatic CSF separately than that in the control cells (**Figure 3B**).

Traumatic and normal CSF increased mRNA expression of *GFAP* in CP epithelial cells

GFAP is a specific marker for astroglial cells (Lewis et al., 1984). As shown in **Figure 4**, mRNA expression of *GFAP* in CP epithelial cells exposed to normal and traumatic CSF increased compared to that in control cells. mRNA expression level of *GFAP* in cells exposed to normal and traumatic CSF was 8.4-fold (P < 0.05) and 13.8-fold (P < 0.05) greater than that in control cells, separately.

Discussion

Despite the fact that all CNS forming cells excepting microglia are originated from a common source located in the ventricular zone, following birth and throughout life, neurogenesis processes continue in two particular regions in adult mammalian brains: the subventricular zone lining the walls of the lateral ventricles and the subgranular zone of hippocampal dentate gyrus (Alvarez-Buylla and Garcıía-Verdugo, 2002; Gage, 2002). In addition, several lines of evidence have denoted the generation of new functional neurons from precursor cells in other parts of the brain including the cortex, amygdala, hypothalamus, striatum, and substantia nigra (Guerra et al., 2015). The present findings demonstrated that traumatic human CSF induced cultured CP epithelial cells into neural differentiation.

CSF composition probably has a significant influence on self-renewal, proliferation, and development of NSCs in the subventricular zone since the subventricular zone is locat-

ed in the CSF-filled lateral ventricle. Farivar et al. (2015) reported that CSF at proper concentrations increases the mRNA expression of *Nestin*, *MAP2* and *GFAP* which lead to the neural differentiation of mesenchymal stem cells in the Wharton's jelly of the human umbilical cord (a gelatinous connective tissue from the umbilical cord).

As a marker of neural stem and neural progenitor cells, *Nestin*-expressing cells are directly involved in regeneration and self-renewing of NSCs. Once cells became differentiated, *Nestin* expression decreases which is frequently accompanied by overexpression of *GFAP* and neurofilaments (Wiese et al., 2004). Our findings suggest that down-regulation of *Nestin* mRNA in combination with up-regulation of *GFAP* mRNA in normal CSF-treated CP epithelial cells were likely attributed to induction of cell differentiation. Up-regulation of *Nestin* mRNA by traumatic CSF in CP epithelial cells could be due to induction of stem/progenitor cells which are engaged in active proliferation. In term of CSF compositions, several reports have indicated a long list of biologically active proteins, peptides and neurotransmitters that are secreted into the CSF through various mechanisms (Johanson et al., 2008; Rodríguez et al., 2010). Biologically active compounds are secreted into CSF by the circumventricular organs including subcommissural organ, pineal body, CP and median eminence (Johanson et al., 2008). Accumulative evidence indicates that microvesicles containing signaling and proteins are released into the CSF by cells located in the ventricular walls (Chiasserini et al., 2014). CSF provides a platform for glial differentiation of neural stem cells (Kiiski et al., 2013). The protective effects of growth factors including brain-derived neurotrophic factor, nerve growth factor, and basic fibroblast growth factor on neuronal survival and differentiation have been conclusively well known. CSF consists of brain-borne and blood-borne signals and molecules targeting distinct regions within the brain that eventually leads to development, differentiation and protection of neuronal cells. Under stress condition, some factors released by CSF drive brain cells including epithelial cells toward stem/progenitor cells, which is likely considered as a potential therapy of CSF for some brain disorders. Because CSF exhibits protective effects on the brain, so it is not surprising that traumatic CSF has potential to trigger some signals in PC epithelial cells, which lead to neuronal development and differentiation. As mentioned above, CSF is a heterogeneous and highly dynamic fluid and its molecular composition changes from lateral ventricles to third and fourth ventricles as well under different physiological and pathological conditions (Johanson et al., 2008; Rodríguez et al., 2010). Pathological conditions including traumatic brain injury and neuroinflammation likely affect CSF composition. Traumatic brain injury has been shown to be associated with elevated level of monocyte chemoattractant protein-1 in CSF (Sempleet al., 2010). Hong et al. (2015) reported that activity of monocyte chemoattractant protein-1 contributes to an increase in neurogenesis and it also decreases neuroinflammation in Niemann-Pick type C disease mouse brain. Decimo et al. (2011) reported that the number of *Nestin*-positive cells in PC epithelial cells increased after spinal cord injury. Thus, it can be assumed that traumatic injury causes secretion of some molecules into the CSF, which provide brain protection and induce the proliferation and development of brain stem cells. Actually, our data revealed that under stress condition, CNS cells likely secrete some compounds into the CSF to induce CP epithelial cells to differentiate into stem/progenitor cells as confirmed by elevated expression of neuronal markers. Therefore, the composition of traumatic CSF possibly promotes the regeneration and self-renewal of injured brain tissue. However, there is a need to identify the proteins, neurotrophins and bioactive molecules that secreted into the traumatic CSF.

Taken together, CP epithelial cells contain multipotent stem-like cells, which are quite similar to other known brain regions with neurogenic features. Traumatic CSF triggers the proliferation and differentiation of CP epithelial cells into neural progenitor cells which lead to tissue regeneration. The present data provide a platform for future studies regarding targeting CP epithelial cells with therapeutic potential for a wide variety of human brain disorders.

Author contributions: *YS and Aliaghaei A conceived and designed the experiments. EH performed the experiments. Amini A, MEB, and YS analyzed the data. AS, AP, and Amini A contributed to reagents/materials/analysis tools. Aliaghaei A, FS and RP wrote the paper. All authors approved the final version of this paper.*
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