

Improving the neuronal differentiation efficiency of umbilical cord blood-derived mesenchymal stem cells cultivated under appropriate conditions

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ABSTRACT

Objective(s): Umbilical cord blood-derived mesenchymal stromal cells (UCB-MSCs) are ideally suited for use in various cell-based therapies. We investigated a novel induction protocol (NIP) to improve the neuronal differentiation of human UCB-MSCs under appropriate conditions.

Materials and Methods: This experimental study was performed in Iranian Blood Transfusion Organization (IBTO), Tehran, Iran. UCB-MSCs were cultured in DMEM medium supplemented with 10% FBS in a humidified incubator in equilibration with 5% CO₂ at 37°C. For neuronal differentiation of UCB-MSCs, DMEM was removed and replaced with pre-induction medium containing RA, bFGF, EGF, and basal medium for two days. Then, NGF, IBMX, AsA, and Neurobasal medium were used for six days for this purpose. Real-time PCR was performed to analyze the neuronal differentiation of UCB-MSCs for the first time in Iran.

Results: We found that the maximum and minimum levels of gene expression were related to GFAP and nestin, respectively. In addition, our study showed that compared to other neuronal inducers, RA might play the main role in neuronal differentiation and fate of MSCs compared to other neuronal inducers.

Conclusion: Our data showed that the combination of chemical (RA, IBMX, AsA) and growth factors (NGF, EGF, bFGF) in NIP may improve the efficiency of neuronal differentiation of UCB-MSCs and may provide a new method for easy and quick application of UCB-MSCs in regenerative medicine in the future. However, the functionality of neuron-like cells must be carefully assessed in animal experiments prior to use in clinical applications.

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Introduction

The development of stem cells for treatment of neurodegenerative diseases is currently the subject of intensive research efforts. Embryonic stem cells (ESCs), neural stem cells (NSCs), BM-derived mesenchymal stem cells (MSCs), and adipose-derived adult stem (ADAS) cells have generated differentiated neural cells both *in vitro* and *in vivo*, which can be used for substitution therapy in various neurodegenerative diseases (1-5). However, despite the differentiation capability of these cells, ethical conflicts, legal restrictions, invasive procedures and graft versus host disease (GVHD) are major challenges in the clinical application of these cells, which emphasizes the evaluation of different stem cell sources for use in various cell-based therapies (1, 6). MSCs are deemed ideal candidates for regenerative medicine (7).

In the present study, UCB-MSCs were selected due to lower immunogenic potential, lack of GVHD, higher capacity for differentiation into neural cells, easy accessibility as well as non-invasive collection after delivery compared to BM-MSCs (8). Furthermore, UCB-MSCs are more primitive than BM-MSCs and other sources. Moreover, unlike BM-MSCs, differentiation potential of UCB-MSCs does not change during frequent passages (9).

In recent years, mesenchymal-derived neuron-like cells have been the focus of attention for treatment of neurodegenerative diseases (10-13). It has been reported that MSCs could induce *in vitro* neuronal differentiation via chemical inducers, growth factors, and co-culture with neural cells (14-17).

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Nevertheless, the results of previous studies are not compatible due to the difference in MSCs isolation, culture conditions and sources.

We investigated a novel induction protocol (NIP) to improve the neuronal differentiation of human umbilical cord blood-derived mesenchymal stromal cells (UCB-MSCs) under appropriate conditions for easy and quick application of UCB-MSCs in regenerative medicine in the future.

Materials and Methods

Clinical samples

This experimental study was performed in the High Institute for Research and Education in Transfusion Medicine in Blood Transfusion Research Center, Tehran, Iran, and all procedures were approved by the local Ethics Committee at IBTO. Umbilical cord blood samples were collected after obtaining informed consent from healthy mothers (20–33 years) who had successfully passed the full-term pregnancy period. Samples were collected in special bags (Beassat, Iran) containing the citrate-phosphate dextrose-adenine anticoagulant.

Isolation of MSCs from human umbilical cord blood

Collection, isolation and expansion of human UCB-MSCs was performed as previously described (18–20). The mononuclear cells (MNCs) fraction was separated by Ficoll-Hypaque low-density [<1.077 g/ml (Cedar Lane, Canada)] gradient followed by ammonium chloride lysis of red blood cells. After twice washing by phosphate-buffered saline (PBS; Gibco, USA), the collected MNCs were re-suspended in high glucose-Dulbecco's modified eagle medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). MSCs were cultured in 25 cm² tissue culture flasks (Nunc, USA) in a humidified atmosphere of 95% air with 5% CO₂ at 37 °C.

Flowcytometric analysis

After the third passage, the cells were trypsinized (0.05% trypsin-EDTA), were twice washed with PBS and stained on ice using phycoerythrin-conjugated mouse anti-human CD44, CD45, CD105, and FITC-conjugated mouse anti-human CD34 (BD Biosciences, USA) according to the manufacturer's instructions, and were incubated in the dark for 30 min at 4 °C. To remove the unlabeled antibodies, the cells were washed with PBS containing 2% FBS (stain buffer) by centrifugation at 1300 rpm for 5 min. In the control group, PE-IgG1 and FITC-IgG1 were used. The stained cells (10000 event count) were analyzed by flowcytometry (Partec Flomax, ver 2.4e).

Neural Differentiation

The differentiation potential of cells was examined upon the fourth passage of the UCB-MSCs. For the induction of neurogenic differentiation, 20000 cells

were cultured in DMEM supplemented with 10% FBS in a humidified incubator in equilibration with 5% CO₂ at 37 °C. To induce neural differentiation of UCB-MSCs, DMEM was first removed and replaced with pre-induction medium containing a basal medium supplemented with L-glutamine, 5 μM retinoic acid (RA, Sigma), 10 ng/ml basic fibroblast growth factor (bFGF, Sigma), and 10 ng/ml epidermal growth factor (EGF, Sigma) for two days. Induction was improved after 48 hr using 10 ng/ml nerve growth factor (NGF, R&D Systems, USA), 0.5 mM 3-isobutylmethyl-xanthine (IBMX, Sigma), 100 μM ascorbic acid (AA, Sigma), and the basal medium for six days.

Osteogenic and adipogenic Differentiation

The differentiation potential of cells was examined upon the third passage of UCB-MSCs. For the induction of osteogenic differentiation, UCB-MSCs were seeded in six-well plates at a density of 10,000 cells/cm² in triplicate and incubated in osteogenic induction medium containing L-glutamine, dexamethasone, β-glycerophosphate, and ascorbic acid for 20 days. Finally, the cells were washed with PBS, fixed with paraformaldehyde, and were subject to Alizarin-Red S 2% staining to detect mineralization capacity in osteocytes 20 days after culture.

For induction of adipogenic differentiation, the third passage of UCB-MSCs was seeded in six-well plates at a density of 10,000 cells/cm² in triplicate. After two days, adipogenic induction medium containing dexamethasone, insulin, indomethacin, and IBMX was added to the cells for three weeks. Finally, the cells were fixed with paraformaldehyde and stained with Oil Red-O to detect the presence of neutral lipid vacuoles in adipocytes.

Immunocytochemistry analysis

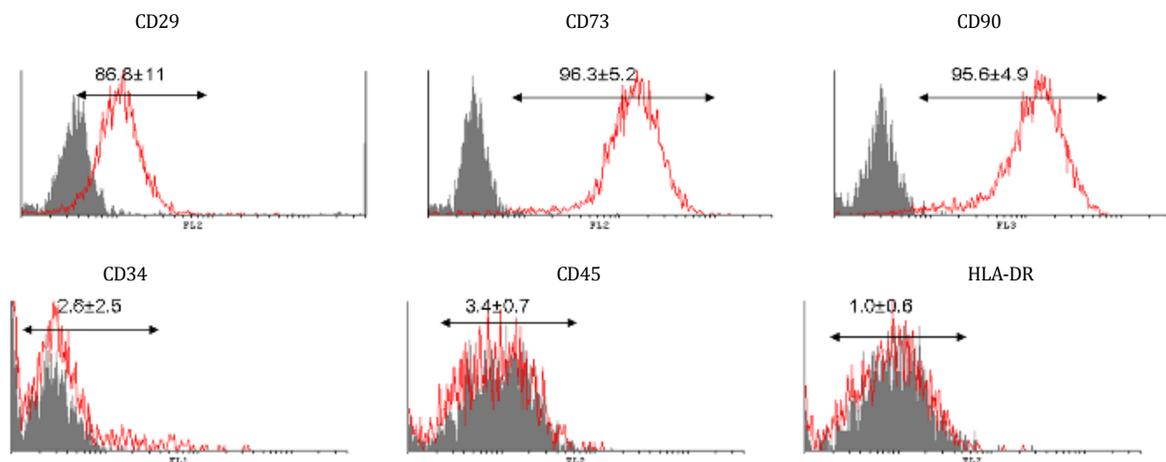
Differentiated and undifferentiated cells were fixed by incubation in 4% paraformaldehyde /PBS, were permeated with Triton X-100 (0.4%) in PBS for 5 min and processed for ICC using 5% goat serum (Sigma, USA) (23), anti-NSE primary antibody (1:100, Santa Cruz, USA), anti-β-III-tubulin primary antibody (1:50, Santa Cruz, USA), and anti-GFAP primary antibody (1:200, Abcam, USA) to confirm the expression of neural-specific proteins by ICC. The affinity purified goat Anti-Mouse IgG Rhodamine (TRITC) conjugated antibody was used as the secondary antibody (1:200, Millipore, USA), and the nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; 1:500, Roche) and visualized under a microscope.

RT-PCR and quantitative real-time PCR analysis

In brief, total RNA was isolated from undifferentiated UCB-MNCs and differentiating UCB-MSCs using an RNA isolation kit (Qiagen, USA). Synthesis of cDNA was carried out by Moloney-murine leukemia virus (M-MuLV) reverse transcriptase (RT) and random hexamer as primer based on manufacturer's instructions (Invitrogen) to confirm the

Table 1. The number of amplification cycles, sequences, and the amplicon sizes of the specific primers designed for assessing the expression of neural markers

Cycles	Amplicon size(bp)	Reverse primer	Forward primer	Markers
35	97	CAT TCT CTC TTC AGC CTT CTC	AGT TCC AGC AGC GTG ATG	MAP2
35	127	ACT CCT TAA TGA CCT CTC CAT C	GCA GAC CTT CTC CAA CCT G	GFAP
35	179	ACT CCC TTG AAT CCC TTG TG	ACC CCG TAG TCC ACT TCT TC	MBP
35	96	CCT CTT CTT CCC ATA TTT CCT G	GAA GGT GAA GGG CAA ATC TG	Nestin
35	85	GGG GTC TTT GCG GAT GTC CAC	CTT CCT TCC TGG GCA TG	β -actin

**Figure 1.** Phenotype analyses of UCB-MSCs by flowcytometry. Flowcytometry analysis showed expression of CD29 (87%), CD73 (96%), CD90 (96%), and no expression of CD34 (3%), CD45 (3%) and HLA-DR (1%)

expression of neural-specific genes by RT-PCR. PCR amplification was conducted using a standard procedure with Taq DNA polymerase with denaturation at 94°C for 15 sec, annealing at 55 °C or 60 °C for 30 sec and extension at 72 °C for 45 sec based on the primers. PCR products were separated by gel electrophoresis on 2% agarose gel in 1×tris-acetate-ethylenediamine tetraacetate buffer and visualized using SYBR safe staining, and the images were then captured using a Bio-Rad gel documentation system. Experiments were generally conducted in triplicate. Nucleotide sequences and the amplicon sizes of designed primers are listed in Table 1. cDNAs were used in a 40-cycle PCR in a Corbett 6,000 Rotor-gene analyzer (Corbett, Germany) to confirm and evaluate the expression level of neural-specific genes by quantitative real-time PCR. Quantitative real-time PCR was performed in triplicate by SYBR green real-time master mix (Takara, Japan) in Rotor-gene 6,000 system (Corbett) followed by melting curve analysis to confirm PCR specificity. The cycle threshold (Ct) was automatically calculated, and normalization was evaluated against the β -actin Ct value. Relative expression was quantified using REST 2009 (V2.0.13) software.

Statistical analysis

Two-sided paired Student's t-test was used to compare the mean values of cell percentages and numbers in wells coated with FCS as well as in uncoated wells. Two-sided paired t-test related-samples Friedman's two-way ANOVA by rank was used to analyze the flowcytometry data. All data were analyzed using SPSS software and were presented in the form of mean \pm SD. $P < 0.05$ was considered statistically significant.

Results

Fibroblastic morphology and surface markers of UCB-MSCs

The fibroblast-like phenotype was observed in cord blood after three passages *in vitro* (Figure 2A). Flowcytometry analysis of cell surface markers in UCB-MSCs (10000 events) showed CD105 (84.85 \pm 9.40, n=3) and CD44 (94.45 \pm 4.9, n=3) ($P < 0.05$) expression but not CD34 (1.80 \pm 0.35, n=3) and CD 45 (2.5 \pm 1.40, n=3) ($P < 0.05$) expression, compared to isotype control. The surface marker patterns corresponded to UCB-derived MSCs. As evidenced by flowcytometry, the isolated cells were positive for CD105 and CD44 while they were negative for CD34 and CD45 (Figure 1).

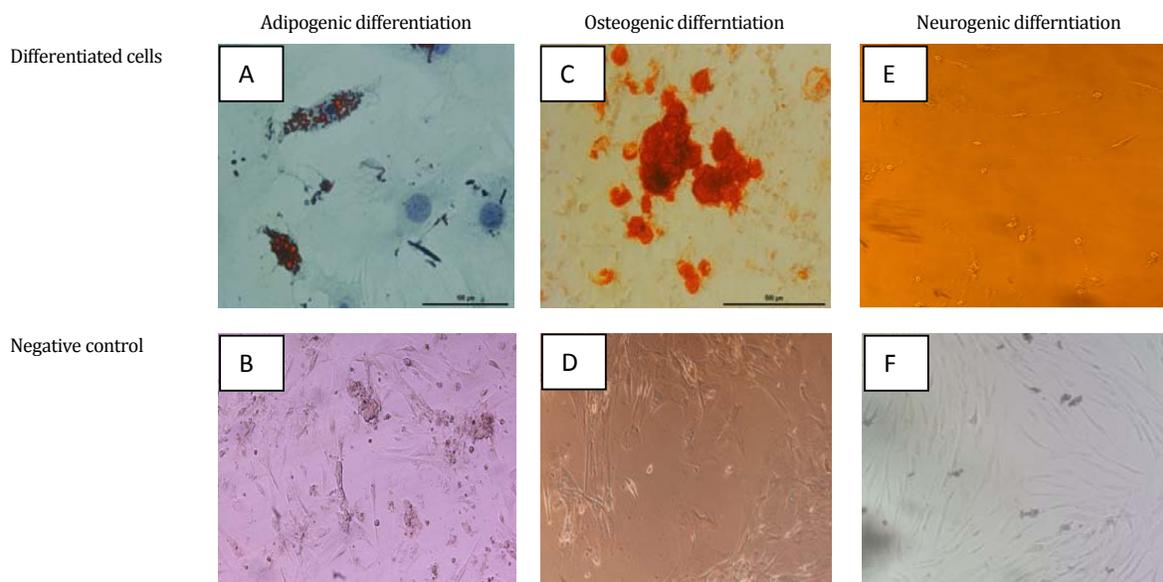


Figure 2. A, B: Adipogenic-differentiation capacity of UCB-MSCs. Adipogenic differentiated mesenchymal cells stained with Oil Red-O (A), non-differentiated mesenchymal cells – negative control (B). Magnification= x100
 C, D: Osteogenic-differentiation capacity of UCB-MSCs. Osteogenic differentiated mesenchymal stained with Alizarinred-S (C), non-differentiated mesenchymal cells – negative control (D). Magnification= x100
 E, F: Neurogenic-differentiation capacity of UCB-MSCs. Morphological neuronal differentiation of MSCs was captured after treatment (E) and before treatment (F). Magnification= x100

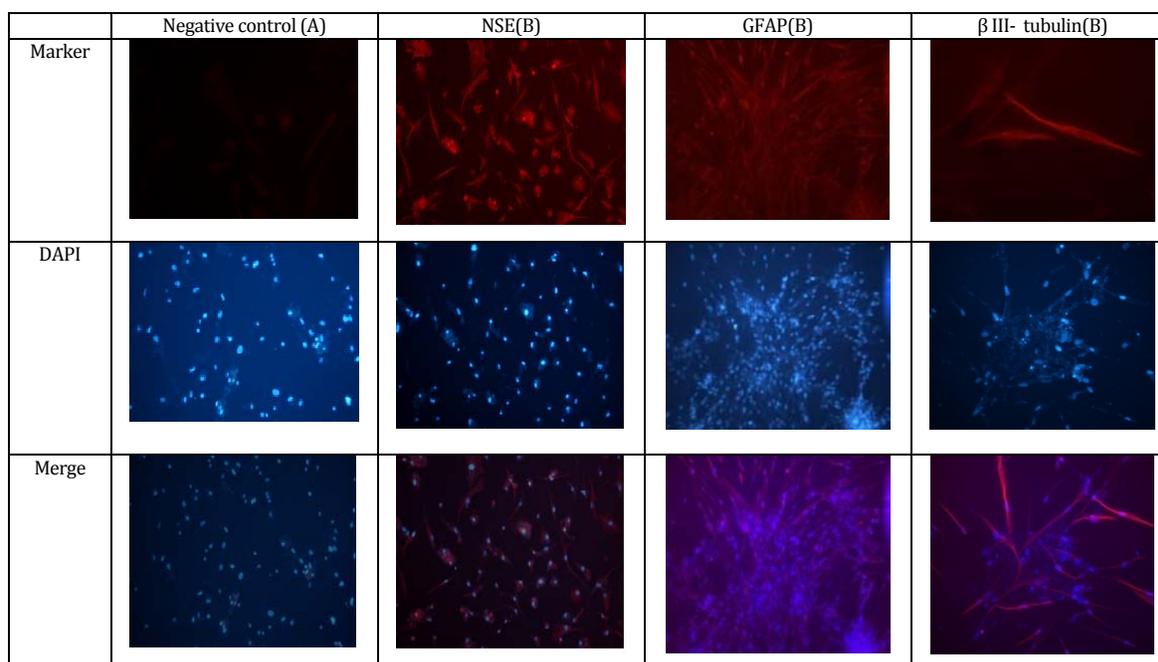


Figure 3. Immunocytochemistry analysis for expression of neuronal markers, such as NSE, GFAP and b-III-tubulin. Immunocytochemistry was performed in mesenchymal stromal cells (MSCs), which were (A) undifferentiated cells and (B) differentiated cells. All neuronal markers were detected in the cytoplasm of differentiated cells, and the nucleus was stained by DAPI. Magnification= x100

Differentiation of UCB-MSCs

Adipogenesis was evident through the formation of lipid droplets as well as transformation of MSC into round cells following oil red staining. Osteogenesis was indicated by the formation of calcified Alizarin-Red S positive deposits. Neural morphology (sign of neuronal differentiation) was observed on the second day. Some cells were stretched in one and/or two directions (Figure 2). The control samples showed no changes in shape. UCB-MSCs were differentiated into MSCs-NPCs, which were identified through morphology, RT-PCR, ICC, and quantitative real-time PCR assays

Neural-Specific Protein and Gene Studies

To evaluate the neural-specific markers at protein level, we used ICC staining to confirm neural differentiation. Comparing DAPI-stained figures with ICCs, our results showed that the induced cells expressed NSE, GFAP, and β -III-tubulin (Figure 3).

Expression evaluation of neural-specific genes using real-time PCR showed that Map2, GFAP, and MBP were upregulated in differentiated cells in comparison to control group (P -value =0.000) and nestin group was not different from the control group (P -value =0.680). In this research, the maximum and minimum levels of gene expression were related to GFAP and nestin, respectively (Figure 4).

Discussion

Recent studies have indicated the ability of MSCs to differentiate into non-mesodermal cells such as neurons and hepatocytes *in vitro* (16, 17, 24, 25). However, MSCs isolated from various origins show different differentiation potentials. At present, several methods are used to induce the differentiation of MSCs into neuron-like cells, including exposure to cytokines and their combination with chemicals, co-culture and exposure to culture medium (26, 27).

In our study, consistent with previous reports, CD105 and CD44 were positive in flowcytometry (Figure 1) but CD45 and CD34 were negative in comparison with the isotype control (28, 29). Differentiation results were assessed in the form of morphological changes, neuron markers of NSE, GFAP, and β -III-tubulin using the ICC technique consistent with other studies (Figure 3). The present study was conducted in a two-step induction protocol. We used the same protocol as Tio *et al* 2010, Wang *et al* 2007, and Levy *et al* 2003, which indicated retinoic acid (RA) and isobutyl xanthine (IBMX) as the main factors promoting neuronal differentiation of MSCs along with Neurobasal medium.

The first step was pre-induction with serum-free medium (basal medium), RA, bFGF, and EGF, and the second step included induction with NGF, IBMX, AA, and basal medium. In the present study, similar to

previous studies, the commonest growth factors as well as selection and basal medium (instead of FBS) were found to be efficient for inducing the differentiation of cells. The importance of our simple method is clear when compared with other complex and time-consuming methods (17, 21, 22). Neuron-like cells share many molecular and cellular characteristics with NSCs. In cellular level, these cells show the same morphology with formation of a spheroid body structure and stretch in one and/or two directions after *in vitro* culture (Figure 2B). In addition, in our protocol, a significant increase occurred in MAP2, MBP and especially GFAP expression by quantitative real-time PCR (Figure 4) but undifferentiated cells did not express neuron-specific genes; however, further studies are required to elucidate the neuronal proteins using western blot.

Some researchers showed that RA combined with other factors such as NGF, β -ME, BDNF, Forskolin, and IBMX is necessary for neuronal differentiation of MSCs *in vitro* (30-34). In this research, it was found that after combined treatment with a low concentration of RA, over 30% of UCB-MSCs differentiated into GFAP-expressing cells. Thus, RA can play an important role in neuronal differentiation of MSCs compared to other inducers (35, 36).

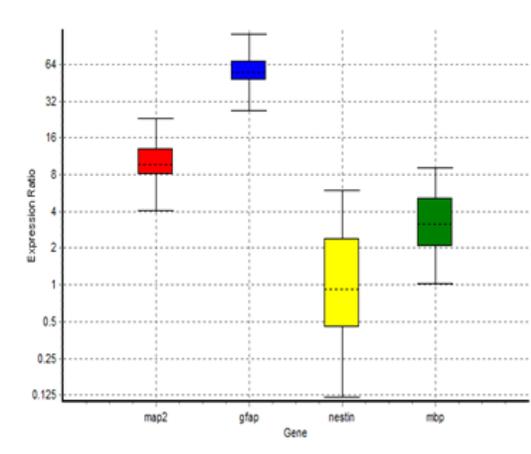


Figure 4. Neural-specific genes expression level with real-time PCR.

Map2 is up-regulated in sample group (in comparison to the control group) by a mean factor of 9.714 (S.E. range is 6.611- 16.880). Map2 sample group is different from the control group. P -value =0.000

GFAP is up-regulated in sample group (in comparison to the control group) by a mean factor of 54.569 (S.E. range is 39.909- 84.713). GFAP sample group is different from the control group. P -value =0.000

Nestin sample group is not different from the control group. P -value =0.680

MBP is up-regulated in sample group (in comparison to the control group) by a mean factor of 3.063 (S.E. range is 1.692- 6.649). MBP sample group is different from the control group. P -value =0.000.

Report produced by REST 2009 V2.0.13

Conclusion

Our results suggest that: (1) UCB-MSCs can exhibit neuronal differentiation potential under specific conditions *in vitro*, (2) The expression of GFAP, MBP, and MAP2 genes is significantly increased after serum-free medium NIP compared to the common induction protocol, (3) The maximum and minimum levels of gene expression were related to GFAP and nestin, respectively, (4) RA may play the major role in neuronal differentiation and fate of MSCs compared to other neuronal inducers, (5) if β -ME is used right from the start, neuron-like cells fail to form, (6) UCB-MSCs and neuron-like cells derived from these novel stem cells can be used in regenerative therapy of diseases such as multiple sclerosis. Our findings, therefore, form the basis for developing a better neuronal differentiation protocol from MSCs, which can be used for therapeutic applications in the future; however, the function of cells need to be further investigated prior to use in clinical neurosciences.

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