Human Tooth Germ Stem Cells Preserve Neuro-Protective Effects after Long-Term Cryo-Preservation

Mehmet E. Yalvaç[1], Mustafa Ramazanoglu[2], Murat Tekguc[3], Omer F. Bayrak[1], Aygul K. Shafigullina[4], Ilnur I. Salafutdinov[5], Natalia L. Blatt[5], Andrey P. Kiyasov[4], Fikrettin Sahin[1], András Palotás[*,6] and Albert A. Rizvanov[*,1,5,7]

[1]*Department of Genetics and BioEngineering, College of Engineering and Architecture, Yeditepe University, 26 Ağustos Campus, Kayisdagi cad., Kayisdagi, TR-34755 Istanbul, Turkey*

[2]*Department of Oral Surgery, College of Dentistry, Istanbul University, I. U. Dis Hekimligi fak., Capa, TR-34093 Istanbul, Turkey*

[3]*Department of Medical Genetics, Faculty of Medicine, Yeditepe University, 26 Ağustos Campus, Kayisdagi cad., Kayisdagi, TR-34755 Istanbul, Turkey*

[4]*Department of Anatomy, Kazan State Medical University, ul. Butlerova 49, R-420012 Kazan, Russia*

[5]*Department of Genetics, Faculty of Biology and Soil Sciences, Kazan State University, ul. Kremlevskaya 18, R-420008 Kazan, Russia*

[6]*Asklepios-Med (private practice and research center), H-6722 Szeged, Kossuth Lajos sgt. 23, Hungary*

[7]*Core research laboratory, Kazan State Medical University, ul. Butlerova 49, R-420012 Kazan, Russia*

Abstract: The use of mesenchymal stem cells (MSCs) has been shown to be promising in chronic disorders such as diabetes, Alzheimer's dementia, Parkinson's disease, spinal cord injury and brain ischemia. Recent studies revealed that human tooth germs (hTG) contain MSCs which can be easily isolated, expanded and cryo-preserved. In this report, we isolated human tooth germ stem cells (hTGSCs) with MSC characteristics from third molar tooth germs, cryo-preserved them at -80°C for 6 months, and evaluated for their surface antigens, expression of pluri-potency associated genes, differentiation capacity, karyotype, and proliferation rate. These characteristics were compared to their non-frozen counterparts. In addition, neuro-protective effects of cryo-preserved cells on neuro-blastoma SH-SY5Y cells were also assessed after exposure to stress conditions induced by hydrogen-peroxide (oxidative stress) and paclitaxel (microtubule stabilizing mitotic inhibitor). After long term cryo-preservation hTGSCs expressed surface antigens CD29, CD73, CD90, CD105, and CD166, but not CD34, CD45 or CD133, which was typical for non-frozen hTGSCs. Cryo-preserved hTGSCs were able to differentiate into osteo-, adipo- and neuro-genic cells. They also showed normal karyotype after high number of population doublings and unchanged proliferation rate. On the other hand, cryo-preserved cells demonstrated a tendency for lower level of pluri-potency associated gene expression (*nanog, oct4, sox2, klf4, c-myc*) than non-frozen hTGSCs. hTGSCs conditioned media increased survival of SH-SY5Y cells exposed to oxidative stress or paclitaxel. These findings confirm that hTGSCs preserve their major characteristics and exert neuro-protection after long-term cryo-preservation, suggesting that hTGSCs, harvested from young individuals and stored for possible use later as they grow old, might be employed in cellular therapy of age-related degenerative disorders.

Keywords: Cryo-preservation, human tooth germs, mesenchymal stem cells, neuro-blastoma, neuro-protection.

INTRODUCTION

Although embryonic stem cells (ESCs) are the most versatile stem cells in terms of differentiation and proliferation potential, their clinical use faces safety and ethical problems. In contrast, adult stem cells including hematopoietic (HSCs), neural (NSCs) and mesenchymal stem cells (MSCs), which are responsible for normal tissue cells' renewal and regeneration throughout the life, can be easily obtained and cultured. MSCs are originally isolated from bone marrow and give rise to osteo-blasts, chondro-cytes, adipo-cytes, skeletal and smooth muscle [1]. It has been shown that MSCs have great potential to help recovery after various degenerative neurological diseases such as brain ischemia, spinal cord injury and amyotrophic lateral sclerosis (ALS) [2]. Despite many reports showing differentiation of MSCs into functional neurons [3,4], it is believed that the beneficial effect of MSC-transplantation is mainly due to their secretion of neuro-protective molecules and induction of endogenous neuro-genesis rather than replacing damaged neuronal tissue [2]. There is also evidence that MSCs induce

*Address correspondence to this author at the Asklepios-Med, H-6722 Szeged, Kossuth Lajos sgt. 23, Hungary; Tel: +(36)(30)255-6225; E-mail: palotas@asklepios-med.eu; Department of Genetics, Faculty of Biology and Soil Sciences, Kazan State University, ul. Kremlevskaya 18, R-420008 Kazan, Russia; Tel: +(7)(843)231-5182; E-mail: rizvanov@gmail.com

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angio-genesis and thus help recovery of the ischemic zone in the brain [5].

Dental stem cells (DSCs), including dental pulp stem cells (DPSCs) and dental follicle stem cells (DFSCs), originate from neural crest and include both MSC and NSC properties [6]. Isolation of MSCs from dental tissue is easy, cost effective and does not raise additional safety and ethical concerns since they are obtained during regular orthodontic procedures [7]. It was demonstrated that DSCs possess neuro-protective effects on dopaminergic neurons [8] and motor neurons in spinal cord [9]. There are several advantages of using DSCs derived from 3rd molar tooth germs: (i) they start organo-genesis at around age 6 [10] and therefore cells in these teeth remain developmentally and replicatively young; (ii) they are usually obtained from teeth that are extracted because of orthodontic reasons as part of a routine treatment; and (iii) they are considered to be adult stem cells and thus, unlike ESCs, do not cause controversy. Stem cells isolated from 3rd molar tooth germs were recently shown to be pluri-potent and able to give rise to functional cells originating from three germ layers: ecto-, meso- and endo-derm [11].

One of the major issues with stem cells is their cryo-preservation, since improper storage may reduce their viability and alter their multi-potency [12]. DSCs were reported to be successfully cryo-preserved without loss of their characteristics after thawing [12-14]. The aim of our work was to further study the effect of long term cryo-preservation at low temperatures on major human tooth germ stem cell (hTGSC) characteristics. This study isolated hTGSCs from 3rd molars of young patients which were subsequently cryo-preserved at -80°C for 6 months. Our results demonstrate that long-term cryo-preservation of hTGSCs did not significantly change their proliferation rate, differentiation capacity, pluri-potency associated gene expression and surface CD marker expression. Furthermore, this report documents neuro-protective effect of hTGSCs after long term cryo-preservation for the first time.

MATERIALS AND METHODS

Isolation of hTGSCs

Human impacted 3rd molar tooth germs were surgically removed from 3 healthy patients (11-13 years of age) as part of a prophylactic treatment for orthodontic reasons. Written informed consents were obtained from the patients and their parents following approval by the Institutional Ethics Committee of Istanbul University, Turkey. The enucleated tooth germs were immediately placed in sterile physiological saline and transferred to the laboratory within 2-4 hrs. Isolation of hTGSCs was performed according to the protocol described previously by our group [15,16]. Established cell lines were maintained in growth medium containing Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM of *L*-glutamine and 1% of PSF (penicillin, streptomycin and fungizone) solution, and incubated at 37°C in a humidified atmosphere of 5% CO_2 in the incubator. Established hTGSC-lines were sub-cultured using trypsin-EDTA solution (1×) (Invitrogen, Carlsbad, CA, USA).

Cryo-Preservation and Thawing of hTGSCs

When hTGSCs reached ~70% confluency (passage #3) they were cryo-preserved in freezing medium containing 10% dimethyl-sulfoxide (Me_2SO) (*Sigma Chemical Co., StLouis, MO, USA),* 20% FBS, 2 mM of *L*-glutamine and 1% of PSF. Cells were suspended in freezing medium and placed into 2 mL cryo-vials at 1×10^6 cells/tube. The cryo-vials were placed into a freezing container (cat #5100-0001) (Nalgene, Rochester, NY, USA) filled with iso-propanol (*Sigma Chemical Co., St. Louis, MO, USA)* at room temperature and the container was placed into a -80°C freezer (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 24hrs followed by transferring the cryo-vials from the container to the freezer racks for long term storage at -80°C. After 6 months, the tubes were retrieved from their storage and immediately thawed in 37°C water-bath with gentle agitation. Cells were then carefully washed to remove the freezing medium by diluting with growth medium and centrifuging at 500×*g* for 5 min, which was followed by aspiration of supernatant and resuspending in growth medium. In order to analyze cell viability of thawed cells, a standard trypan-blue dye exclusion assay was performed: cells that do not absorb the chemical are considered to be alive. In order to compare proliferation rates of thawed cells and non-frozen cells, cells were seeded in 6-well plates (5000 cells/well, in triplicates for each day) and incubated for 4 days. Cells were harvested and counted daily for analyzing the proliferation.

Flow-Cytometry Analysis

The surface antigens of non-frozen and cryo-preserved hTGSCs were compared by flow-cytometry analysis as previously described [15]. Cells were trypsinized and incubated in PBS for 45mins with primary anti-bodies against CD14 (cat #SC-7328), CD29 (cat #BD556049), CD34 (cat #SC-51540), CD45 (cat #SC-70686), CD90 (cat #SC-53456), CD105 (cat #SC-71043), CD133 (cat #SC-65278), CD166 (cat #SC-53551) (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) and CD73 (cat #550256) (Zymed, San Francisco, CA, USA). After washing the excess primary antibodies, cells were incubated with fluorescein-iso-thio-cyanate (FITC)-conjugated secondary antibodies (cat #SC-2989) at 4°C for 45min, except for CD29 against which phyco-erythrin (PE) – red light-harvesting protein containing chromophore – conjugated monoclonal antibody was used for budgetary reasons only. The flow-cytometry analysis of the cells was carried out using Becton Dickinson FACSCalibur flow-cytometry system (Becton Dickinson, San Jose, CA, USA), with 5-10,000 events being counted for each case.

Chromosomal Analysis

Cryo-preserved hTGSCs were cultured with DMEM+ Glutamax™-1 (1×) containing 10% FBS and 1% antibiotic-antimycotic solution 100× (Invitrogen, Carlsbad, CA, USA). Cyto-genetic analysis was performed with cells at passage #10 when they reached 40-50% confluency. Cells were incubated with ProCell reagents (Genial Genetic Solutions, Wirral, UK): first with chromosome resolution additive to prevent chemical contraction and encourage chromosome elongation, and 2.45 hours later they were treated with

metaphase arresting solution for 75 min. Subsequently cells were collected by 0.25% trypsinization (Gibco-Invitrogen, Carlsbad, CA, USA), fixed with Carnoy's fixative (3:1 mixture of methanol and acetic acid) and spread onto clean glass slides. After air-drying, slides were incubated at 65°C overnight, and were stained with 5% Giemsa solution, prepared by mixing Giemsa's azur-eosin-methylene-blue solution (Merck, Darmstadt, Germany) with Gurr solution (Gibco-Invitrogen, Carlsbad, CA, USA) for 4.5mins. Metaphase images were obtained and analyzed through 100× objective lens of light microscope (Olympus America, Melville, NY, USA) incorporated by an automatic scanning system Cytovision ChromoScan (Applied Imaging, Santa Clara, CA, USA). 20 cells were examined from each cell line.

Differentiation of hTGSCs

To confirm MSC characteristics, non-frozen and cryo-preserved hTGSCs were differentiated into osteo-, neuro- and adipo-genic cells based on protocols described previously [16]. hTGSCs at passage #3 were used for differentiation experiments.

For osteo-genic differentiation, cells were counted and cultured in 6-well plates at a concentration of 3000 cell/cm^2 in growth medium. After 48hrs, growth medium was replaced with osteo-genic medium: DMEM supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 0.1mmol/L dexa-methasone, 10mmol/L β-glycerol-phosphate, 50mmol/L ascorbate (*Sigma Chemical Co., StLouis, MO, USA*). Cells were incubated in osteo-genic medium for 10 days and the medium was changed every other day. On day 14th, vonKossa staining and immuno-cytochemistry were performed to confirm osteo-genic differentiation.

For neuro-genic differentiation, cells at first step were seeded at a concentration of 3000-5000 cells/cm^2 in 6-well plates on cover-slips coated with 100 mg/mL poly-*D*-lysine (*Sigma Chemical Co., StLouis, MO, USA*) and incubated in DMEM medium supplemented with 20% FBS, 1mmol/L β-mercapto-ethanol (AppliChem, Darmstadt, Germany) and 50ng/mL basic fibroblast growth factor (b-FGF) (Promega, Madison, WI, USA) for 24hrs. At step #2, cells were incubated in NeuroBasal medium containing B-27 stem cell culture supplement (Invitrogen, Carlsbad, CA, USA), 50ng/mL b-FGF and 50 ng/mL nerve growth factor (NGF) (Promega, Madison, WI, USA) without FBS for up to 12 days. Markers such as nestin, neuron-specific β_3-tubulin (Promega, Madison, WI, USA) and the light subunit of the neuro-filament protein (NFL) were detected by immune-cytochemistry to assess the potential for neuronal differentiation.

For adipo-genic differentiation, cells were counted and cultured in 6-well plates at a concentration of 3000 cells/cm^2 with growth medium. After 24hrs, the medium was replaced with DMEM supplemented with 10% FBS, 1mmol/L dexa-methasone, 5 µg/mL insulin, 0.5mmol/L iso-butyl-methyl-xanthine and 60mmol/L indomethacin (*Sigma Chemical Co., StLouis, MO, USA*). After 2 weeks of incubation, intra-cellular lipid vesicles were observed under light microscope (Nikon TS100, Minnesota, MN, USA).

Immuno-cytochemistry Analysis

hTGSCs grown on glass cover-slips were fixed with 2% of *p*-formaldehyde and permeabilized by incubating with 0.1% Triton-X100/PBS for 5min. Non-specific binding of anti-bodies was blocked by adding 2% goat serum (diluted in PBS) for 20min. Samples were incubated with primary anti-bodies (IgG): anti-nestin (cat #AB5922) (Millipore/Chemicon, Billerica, MA, USA), anti-collagen type-I (cat #SC-80565), anti-NFL (cat #SC-2562) (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-β_3-tubulin (cat #G712A) (Promega, Madison, WI, USA) overnight at 4°C. Each sample was washed twice for 5min with PBS to remove un-bound primary anti-bodies. After washing, goat poly-clonal anti-rabbit IgG-Alexa 488 conjugate (Invitrogen, Carlsbad, CA, USA) secondary anti-bodies were added and incubated for 1hr. As a nuclear counter-stain DAPI (6'-di-amidino-2-phenyl-indole) was used (*Sigma Chemical Co., StLouis, MO, USA*). Stained cover-slips were mounted on clean glass slides using Mowiol mounting medium (Calbiochem, La Jolla, CA, USA). Prepared slides were observed under Leica TCS SP2 SE confocal microscope (Leica, Bensheim, Germany) immediately after preparation.

vonKossa Staining

After 10 days of incubation with osteo-genic medium in 6-well plates, cells were fixed with 2% of *p*-formaldehyde at 4°C for 30min. After fixation, cells were stained with vonKossa method (Bio-optica, Milano, Italy) and calcium depositions were observed with a light microscope (Nikon TS100, Minnesota, MN, USA).

Preparation of Conditioned Medium of hTGSCs and *in vitro* Neuro-Protection Assay

Cryo-preserved hTGSCs at passage #5 were cultured in T-75 flasks with growth medium. When they reached 70% confluency, conditioned medium was collected, filtered using 0.22µm filters to avoid any cell debris, and divided into 2 mL aliquots in 2mL sterile plastic tubes. Tubes were kept at -20°C until use. SH-SY5Y neuro-blastoma cells (ATCC, Manassas, VA. USA) were plated in 12-well plates at a concentration of 40,000 cells/well with either growth medium only or growth medium supplemented with 20% hTGSCs conditioned medium. Cells were treated with either 250µM of H_2O_2 (Riedel-de-Haen, Hanover, Germany) or 100µM paclitaxel (PAC) (*Sigma Chemical Co., StLouis, MO, USA*), for 24hrs. After treatment, cells were harvested and incubated with 2µg/mL propidium-iodide solution for 2mins in order to detect necrotic cells by flow-cytometry. PAC-treated cells were also analyzed for the expression of apoptotic markers annexin-V and caspase-3. Harvested cells were incubated with annexin-V FITC conjugate from human placenta (cat #A9210) (*Sigma Chemical Co., StLouis, MO, USA*) followed by washing with PBS and analyzing using flow-cytometry. Expression level of caspase-3 was determined by using real-time (RT)-PCR analysis.

RT-PCR Analysis

Total RNA from non-frozen and cryo-preserved hTGSCs was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. ESC

Table 1. Primers and Probes for Real-Time PCR

Oligo-Nucleotide	Sequence	GeneBank #
β-actin-TMprobe(human)*	CCAGCCATGTACGTTGCTATCCAGGC	NM_001101
β-actin-TM-F(human)	GCGAGAAGATGACCCAGGATC	
β-actin-TM-R(human)	CCAGTGGTACGGCCAGAGG	
h-*oct4*-TMprobe621*	TCTGCAGCTTAGCTTCAAGAACATGT	NM_002701
h-*oct4*-TM499F	CGACCATCTGCCGCTTTG	
h-*oct4*-TM664R	GCAAGGGCCGCAGCTTA	
h-*c-myc*-TMprobe1494*	TACGCAGCGCCTCCCTCCACTC	NM_002467
h-*c-myc*-TM1472F	CGTCTCCACACATCAGCACAA	
h-*c-myc*-TM1539R	TCTTGGCAGCAGGATAGTCCTT	
h-*klf4*-TMprobe1414*	CCGGTTCCTGCATGCCAGAGGA	NM_004235
h-*klf4*-TM1387F	CGCTCCATTACCAAGAGCTCAT	
h-*klf4*-TM1463R	CGATCGTCTTCCCCTCTTTG	
h-*nanog*-TMprobe453*	TGCAGAGAAGAGTGTCGCAAAAAAGG	NM_024865
h-*nanog*-TM431F	CCAAAGGCAAACAACCCACTT	
h-*nanog*-TM499R	TCTTGACCGGGACCTTGTCT	
h-*sox2*-TMprobe763*	CCGGCGGAAAACCAAGACGCT	NM_003106
h-*sox2*-TM717F	TGCGAGCGCTGCACAT	
h-*sox2*-TM809R	GCAGCGTGTACTTATCCTTCTTCA	

*TaqMan probes contain 5'FAM fluorescent dye and 3'RTQ-1 quencher.

mRNA from human embryonic stem cell line Moscow 01 (hESM01) was kindly provided by dr. Sergey L. Kiselev (Institute of Gene Biology, Russian Academy of Sciences, Moscow) [17]. cDNA synthesis was performed using random hexamer primers and moloney murine leukemia virus reverse-transcriptase (MMLV-RT) (Promega, Madison WI, USA) at 37°C for 1hr. TaqMan primers and probes (Table **1**) were designed using PrimerExpress software (Applied Biosystems, Foster City, CA, USA). RT-PCR primers and TaqMan probes were synthesized by Syntol (Moscow, Russia). Pre-mix (2.5×) for TaqMan RT-PCR was purchased from Syntol (Moscow, Russia) and used according to the manufacturer's instructions. The amount of RNA was normalized by using β-actin. Serial dilution of ESC cDNA was used for relative quantification of the expression of *oct4*, *klf4*, *sox2*, *c-myc* and *nanog* genes. Relative expression of caspase-3 mRNA was analyzed using *SYBRgreen RT-PCR method.* The PCR primers were as follows: GAPDH (sense: TAT CGT GGA AGG ACT CA, antisense: GCA GGG ATG ATG TTC TGG A), caspase-3 (sense: CCT CTT CCC CCA TTC TCA TT, anti-sense: TCG ACA TCT GTA CCA GAC CG) [18]. *cDNAs of* PAC-treated cells were mixed with primers and SYBR Premix Ex Taq (including TaKaRa Ex Taq HS, dNTP Mixture, Mg^{2+}, SYBRgreen-I) in a final volume of 20μL. GAPDH (glycer-aldehyde-3-phosphate-de-hydrogenase) gene was used as the reference house-keeping gene for normalization of the data. All RT-PCR experiments were done using iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Standard error and Student's *t*-test values were calculated using Microsoft Excel.

RESULTS

After 6 months of cryo-preservation, the viability of hTGSCs was 90% ± 2.5, and maintained a spindle-like healthy morphology just as non-frozen control hTGSCs (Fig. **1**). Proliferation rates of cryo-preserved cells were shown to

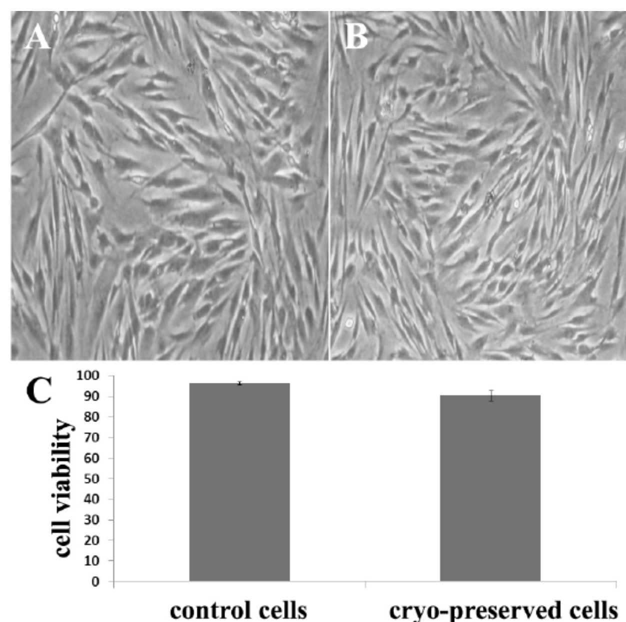

Fig. (1). Comparison of cryo-preserved hTGSCs and non-frozen control hTGSCs.
Normal spindle-like morphology was maintained in cryo-preserved (**A**) and control (**B**) cells. (**C**) cell viability of cryo-preserved hTGSCs and control hTGSCs after thawing. Values are expressed as mean ± SD (n=3).

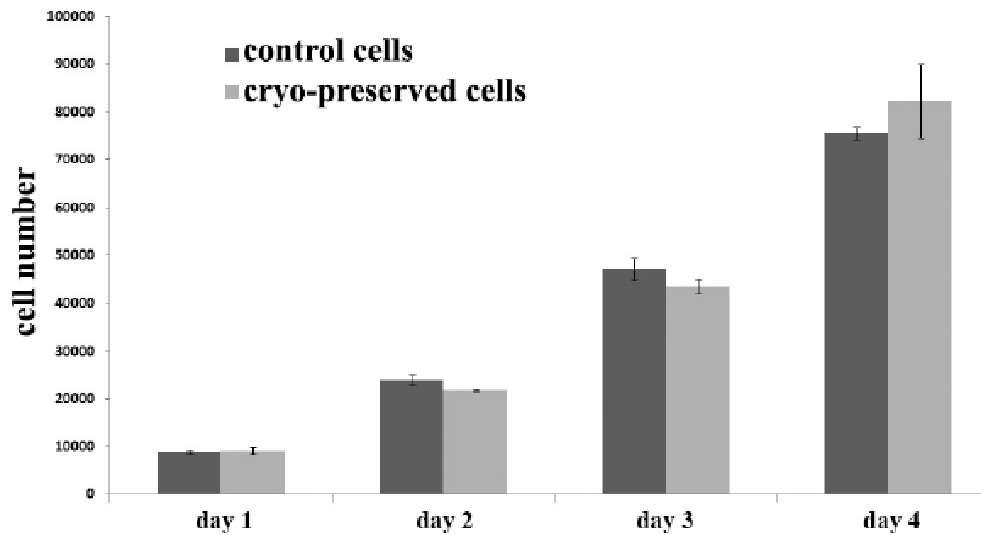

Fig. (2). Comparison of proliferation rates of cryo-preserved hTGSCs and non-frozen control hTGSCs.
Proliferation rate of cryo-preserved hTGSCs did not change after 6-month storage at -80°C.

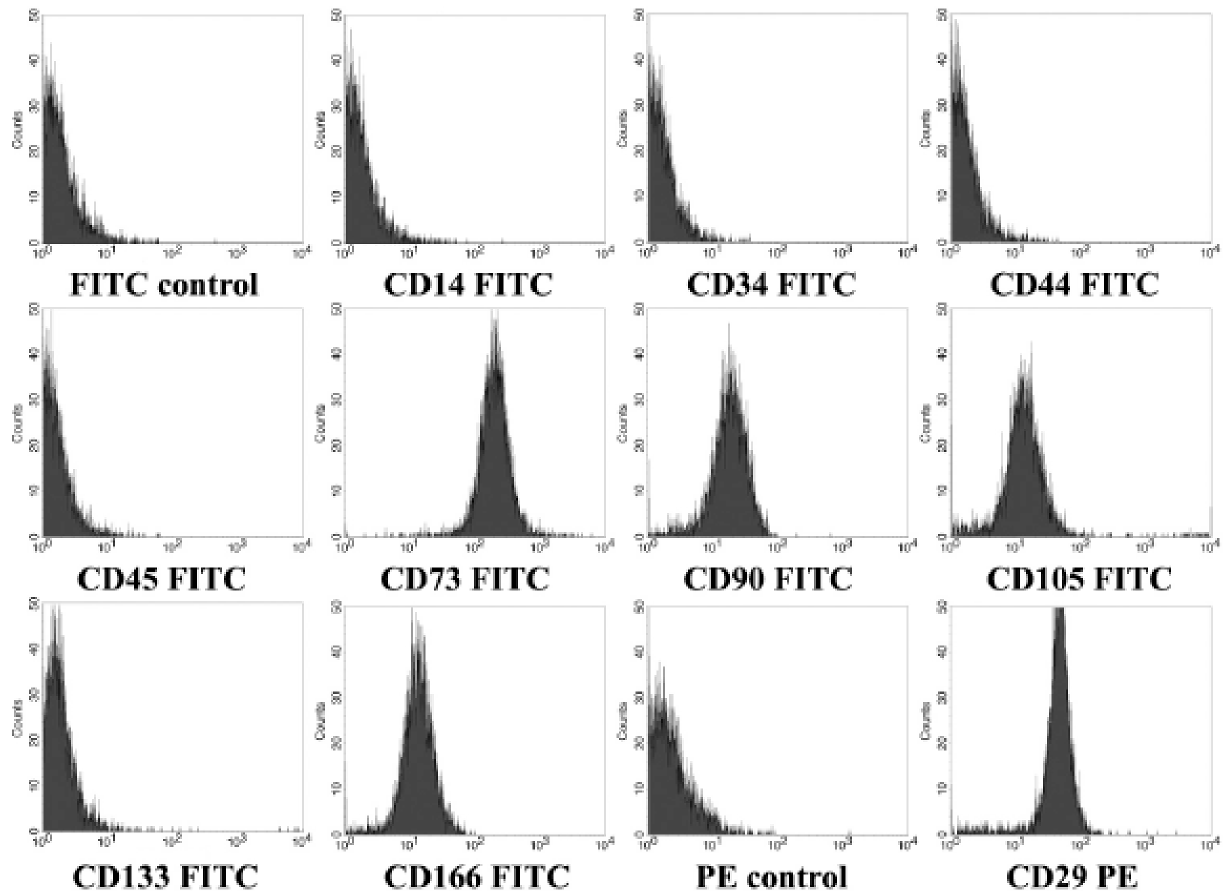

Fig. (3). Immuno-phenotypic characteristics of hTGSCs.

Flow-cytometry analyses revealed that cryo-preserved hTGSCs were positive for cell surface antigens **CD29**, **CD73**, **CD90**, **CD105** and **CD166**, but negative for hemato-poietic markers such as **CD14**, **CD34**, **CD45** and **CD133**. **FITC**: fluorescein-iso-thio-cyanate, **PE**: phyco-erythrin.

be similar to their non-frozen counter-parts (Fig. **2**). Flow-cytometry analysis showed that cryo-preserved cells express MSC markers CD29, CD73, CD90, CD105 and CD166, but not hemato-poietic markers such as CD14, CD34, CD45 and CD133 (Fig. **3**). After thawing, hTGSCs were maintained for over 10 passages and retained their normal karyotype (Fig. **4**).

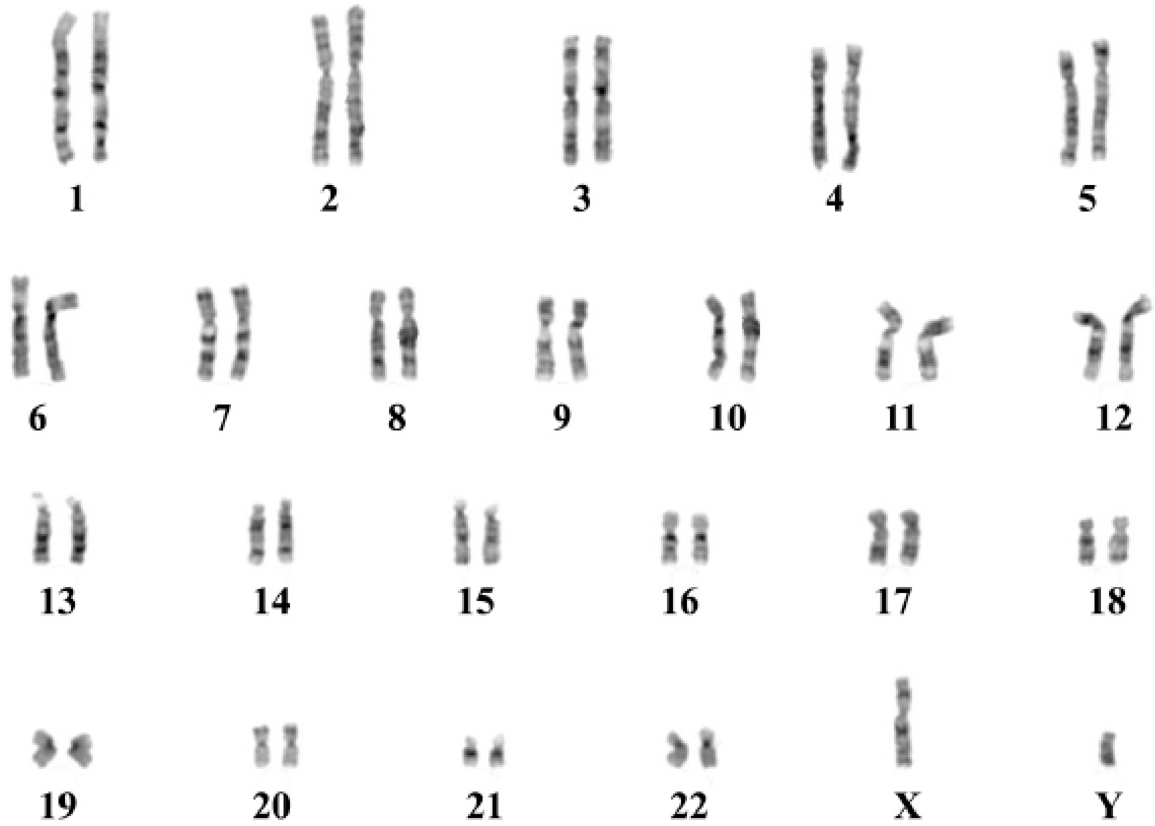

Fig. (4). Cyto-genetic examination of hTGSCs.

Cryo-preserved cells exhibited normal karyotype. Cells were stained with 5% Giemsa and observed under 100× objective of light microscope.

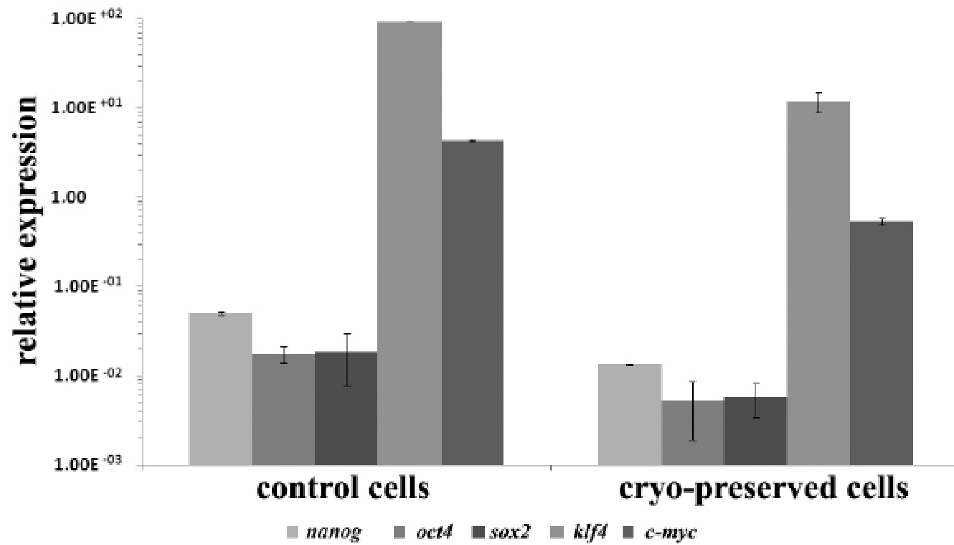

Fig. (5). Real-time PCR comparison of *nanog*, *oct4*, *sox2*, *klf4* and *c-myc* gene-expression levels in control and cryo-preserved hTGSCs.

Messenger RNA (mRNA) expression levels are represented relative to the expression of the corresponding mRNA in ESCs.

In order to analyze un-differentiated state of hTGSCs before and after cryo-preservation, we analyzed the expression of pluri-potency associated genes in hTGSCs, comparing their levels with those in ESCs. Our data showed that after cryo-preservation, expression of all genes slightly decreased, however the level of *klf4* and *c-myc* genes were still high compared to ESCs (Fig. **5**). Multi-potency of cryo-preserved hTGSCs was tested by inducing them to differentiate into adipo-, osteo- and neuro-genic cells. The results showed that cryo-preserved hTGSCs are able to differentiate into all of these cell types. Immuno-cytochemistry analysis revealed that hTGSCs were stained positive for

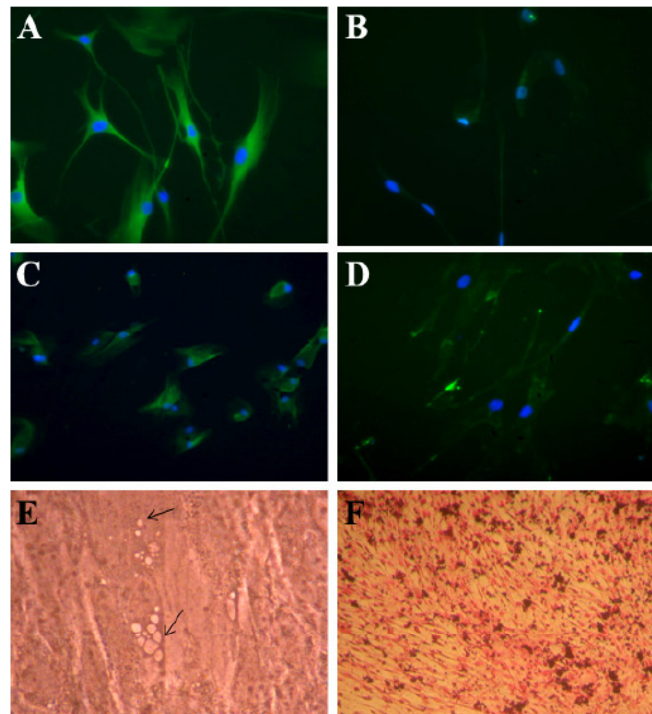

Fig. (6). Differentiation of cryo-preserved hTGSCs.

Cells were stained positive for neuro-genic markers β_3-tubulin (**A**), NFL (**B**), nestin (**C**), as well as osteo-genic marker collagen type-I (**D**). Lipid vesicles (**E**, arrows) and calcium depositions stained black (**F**) were also detected, confirming adipo- and osteo-genic differentiation, respectively.

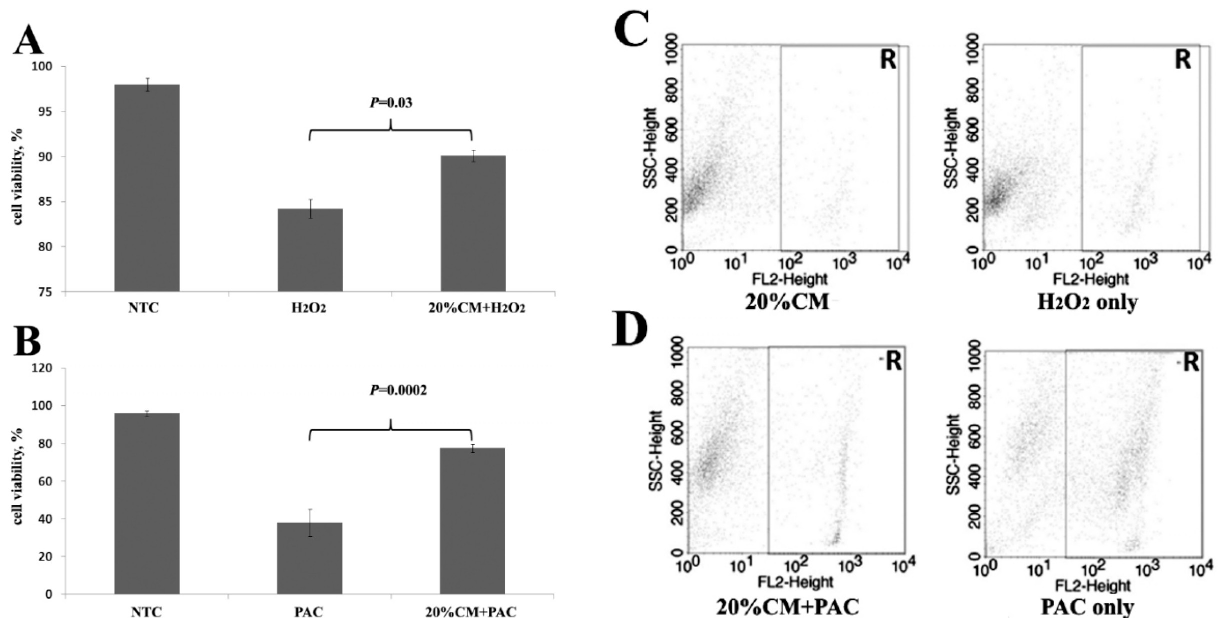

Fig. (7). Viability of SH-SY5Y cells after 24hrs of incubation with PAC (100µM) and H_2O_2 (250µM).

A-B: 20% of hTGSCs conditioned medium (CM) increased the viability of SH-SY5Y neuro-blastoma cells by 6% and 40% after treatment with PAC and H_2O_2, respectively. **C-D**: Flow-cytometry was used to detect percentage of necrotic cells (in the region labeled with R), the number of which decreased after the application of CM. NTC: non-treated control.

neuro-genic markers nestin (an intermediate filament expressed mostly in neurons), β_3-tubulin (a microtubule protein specific to neurons) and NFL (light sub-unit of a neuronal intermediate filament), as well as osteo-genic marker collagen type-I (Figs. **6A-D**). Osteo-genic differentiation was also confirmed by staining calcium depositions using vonKossa method (Fig. **6F**). After adipo-genic differentiation, cells formed classical lipid vesicles which were observed under light microscope (Fig. **6E**).

In vitro neuro-protection assay results showed that conditioned medium of hTGSCs with MSC characteristics

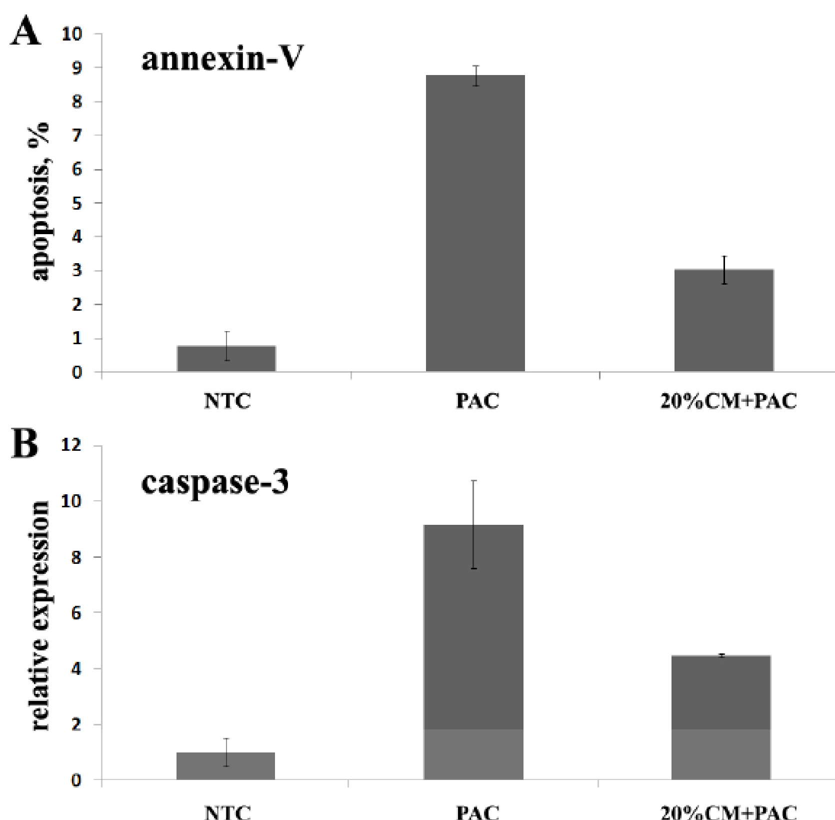

Fig. (8). Detection of apotosis in PAC-treated cells.

20% hTGSCs conditoned medium (CM) remarkably reduced the expression of apoptotic markers annexin-V and caspase-3 when compared to PAC-treated cells. NTC: non-treated control.

protects SH-SY5Y neuro-blastoma cells exposed to stress conditions induced by H_2O_2 (250µM) and PAC (100µM) for 24hrs. In the presence of 20% hTGSC conditioned medium, the cell viability increased under both stress conditions induced by H_2O_2 and PAC by 6% and 40%, respectively, when compared to standard media control (Fig. **7**). Expression of apoptotic markers annexin-V and caspase-3 in PAC + 20% hTGSC conditioned medium treated cells were decreased remarkably when compared to only PAC treated cells (Fig. **8**).

DISCUSSION

Bone marrow is a major source of non-hemato-poietic MSCs that can differentiate into osteo-, adipo-, chondro-cytes and myogenic cells [19], which makes them an attractive source for various cell therapy applications. However, isolation procedure for bone marrow MSCs carries risks of surgical trauma and bone marrow-related diseases [16]. DSCs, including DPSCs and DFSCs might be an attractive alternative source of MSCs [15]. Third molar (wisdom) tooth derived DPSCs were shown to be pluri-potent and might differentiate into various cell types from all 3 germ layers: ecto-, meso- and endo-derm [11, 20]. Another approach for the isolation of MSCs from 3rd molar tooth germs of young adults was reported by our group recently, which includes utilization of both dental pulp and dental follicle tissues for obtaining MSCs. We have extensively characterized these cells and referred to them as hTGSCs [16]. These cells might offer distinct advantage over DPSCs from fully formed teeth, including 3rd molars, since they come from an organ which is still undergoing development (*ie*. not yet completely formed), thus acting as a source of more developmentally immature stem cells with increased proliferation and differentiation properties. Several groups achieved successful cryo-preservation of DPSCs [12-14, 21, 22]. A recent study demonstrated the optimal cryo-preservation conditions for MSCs derived from dental pulps of 3rd molar teeth. Evidences were provided that MSCs can be cryo-preserved in the presence of 10% Me_2SO at -85°C for at least 6 months with high viability (96%) and without loss of functionality [12]. Despite extensive research in the field of cryo-preservation of DPSCs, the effects of cryo-preservation on hTGSCs from partially formed teeth of young adults remain poorly understood. In the present study, hTGSCs cryo-preserved at -80°C for 6 months demonstrated excellent viability and almost the same proliferation rate as non-frozen hTGSCs. Flow-cytometry analysis demonstrated that cryo-preserved hTGSCs express surface CD antigens of MSCs (CD29, CD105, CD73, CD90 and CD166), such as their non-frozen counter-parts. It was also confirmed that the differentiation potential of hTGSCs did not alter during cryo-preservation: thawed cells were able to transform into adipo-, osteo- and neuro-genic cells. On the other hand, expression levels of pluri-potency associated genes, such as *nanog*, *oct4*, *sox2*, *c-myc* and *klf4* slightly decreased after half year of cryo-preservation, which might be the negative result of stress induced by freezing and thawing procedures.

DPSCs and DFSCs originate from neural crest [10] and contain both ecto-dermal and mesenchymal components [6]. Both of these cell types are able to differentiate into neural cells [20, 23, 24]. Furthermore, a great number of studies have showed that MSCs are increasing functional recovery in cerebral ischemia by inducing regeneration of damaged tissue [25-27]. These reports showed that MSCs exert their therapeutic effect by secreting some soluble neuro-trophic factors rather that differentiating into neurons and replacing the ischemic tissue [2]. These soluble factors might play a crucial role in the induction of neuro- or angio-genesis, triggering synapse formation, reducing apoptosis and cyto-toxicity, and regulating inflammation [5]. Despite several publications demonstrating the potential of MSCs to protect neural cells by secreting neuro-trophic factors into the micro-environment, there has been a lack of information about neuro-protective effect of long-term cryo-preserved DSCs.

The importance of studying the effect of cryo-preservation of DSCs is paramount in developing future therapeutic applications, since harvesting such cells is only possible during child- and early adult-hood, while many neuro-degenerative diseases manifest in the elderly. Stem cells isolated in younger ages from 3rd molars, therefore, would need to be cryo-preserved without altering their therapeutic potential for future autologous transplantation several years or even decades later. In this study, we aimed to test the neuro-protective effect of hTGSCs after long-term cryo-preservation. Neuro-blastoma SH-SY5Y cells are commonly used for *in vitro* neuro-protection assays [28]. Our results indicate that hTGSCs produce some soluble neuro-protective molecules which increase the recovery of neuro-blastoma SH-SY5Y cells from H_2O_2- and PAC-induced stress. Further analysis revealed that hTGSC conditioned medium retard PAC-induced apoptosis, which was confirmed by the reduction in expression of apopotic markers annexin-V and caspase-3.

In conclusion, the findings of the present study demonstrate that hTGSCs can be cryo-preserved at -80°C for at least 6 months without functional loss. Surface antigen expression profile, mRNA level for key pluri-potency genes and differentiation potential was unaffected by long-term storage. Moreover, cryo-preserved hTGSCs were able to exert neuro-protective effects *in vitro*. All of these findings demonstrate that prolonged maintenance of hTGSCs at low temperatures is an inexpensive approach for preserving cells for possible downstream applications. The effects of cryo-preservation on the neuro-protective effects of hTGSCs would need to be further tested, however this report clearly revealed that these cells might be kept at -80°C for at least half a year avoiding negative effects of stress that is certain to be encountered during cryo-preservation.

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STATEMENT OF INTEREST

The authors declare that they have no conflict of interest.

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