Biomedicine & Pharmacotherapy 93 (2017) 772–778

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Original article

Herbal pre-conditioning induces proliferation and delays senescence in Wharton's Jelly Mesenchymal Stem Cells

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ARTICLE INFO

Article history:
Received 10 May 2017
Received in revised form 15 June 2017
Accepted 29 June 2017

Keywords:
MSCs
Tinospora cordifolia
Withania somnifera
Proliferation
Senescence

ABSTRACT

Background: Mesenchymal Stem Cells (MSCs) are multipotent stem cells which are being explored for various clinical applications. Isolation and *in-vitro* expansion of MSCs remain important in achieving desired cell number for the therapy. However, *in-vitro* proliferation of MSCs is often associated with senescence and early onset of apoptosis which limits its therapeutic ability and long term clinical use. *Tinospora cordifolia* and *Withania somnifera* are used widely in Ayurveda: the traditional Indian system of medicine and are reported to have rejuvenating and anti-aging potential. In the present study, we investigated the effect of *Tinospora cordifolia* and *Withania somnifera* on proliferation and senescence of wharton's jelly MSCs (WJMSCs) *in-vitro.*

Methods: WJMSCs were treated in culture medium with *Tinospora cordifolia* leaf and *Withania somnifera* root extracts to examine their effect on proliferation and senescence properties of WJMSCs. Proliferation of WJMSCs was assayed by cell count, MTT, BrdU incorporation assay, cell cycle analysis and Ki67 mRNA expression. Senescence was demonstrated using β-galactosidase senescence assay and associated mRNA markers.

Results: Culture medium supplemented with *Tinospora cordifolia* leaf and *Withania somnifera* root extracts exhibited significant increase in proliferation of WJMSCs as evidenced by cell count and MTT assay. Cell cycle analysis using propidium iodide showed increase in G2/M phase and decrease in apoptotic cells. BrdU incorporation and upregulation of proliferation marker ki67 by RT PCR showed increased DNA synthesis/proliferation in *Tinospora cordifolia* and *Withania somnifera* extract treated MSCs. Delayed senescence was confirmed by β-galactosidase senescence assay and down regulation of senescence marker p21.

Conclusion: Our results demonstrate for the first time that *Tinospora cordifolia* and *Withania somnifera* extracts support proliferation and inhibit senescence in WJMSCs making them suitable candidates as supplements for *in-vitro* expansion without affecting the cell viability indicating its non-toxic nature.

1. Introduction

The ability of Mesenchymal Stem Cells (MSCs) to differentiate into osteocytes, adipocytes, chondrocyte, osteoblasts and endothelial cells has attracted attention of the scientists working in the field of cellular therapy [1]. MSCs have shown promising results in treatment of graft-*versus*-host disease, Crohn's disease, osteogenesis imperfecta, type 1 diabetes, *etc.* [2–6]. Till date MSCs have been successfully isolated from adult tissues (bone marrow, peripheral blood, adipose tissue and dental pulp) and birth associated tissues (umbilical cord, placenta, amniotic fluid, amniotic membrane). The amount of MSCs which can be obtained from these sources vary as per the source [7]. It is important to expand MSCs *in-vitro* to achieve desired cell number for clinical therapy. However, MSCs start losing proliferative potential as passage number goes on increasing. They enter the senescence phase after long term *in-vitro* expansion. Studies show that MSCs lose their normal morphology and immunophenotype in stationary and senescent phase during long term *in vitro* expansion. Mean Telomere length decreases significantly in the 9th passage in accordance with Hayflick model of cellular aging [8].

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http://dx.doi.org/10.1016/j.biopha.2017.06.107
0753-3322/

Some cytokines, growth factors, platelet-derived growth factor (PDGF) and fibroblast growth factor have been reported to enhance proliferation of MSCs, although their molecular mechanisms are not well established and their use is also associated with practical limitations. These growth factors are cost prohibitive, uneconomical and may put cells under stress [9]. On the other hand, herbal extracts are affordable and do not induce stress as they have a battery of anti oxidants within them. Herbal extracts have shown potential in the proliferation and differentiation of MSCs. Earlier we have reported that Ayurvedic formulation 'Dhanwantharam Kashaya' showed increased proliferation and delayed senescence in WJMSCs [10]. Recent findings from studies with *Aconiti Lateralis Preparata Radix* (ALR) showed enhanced proliferation rate and increase in G2/M phase of mouse bone marrow mesenchymal stem cells [11]. Ayurveda, the traditional Indian system of medicine has explained the role of herbal drugs and therapies in the biology of regeneration [12]. *Tinospora cordifolia* and *Withania somnifera* are popular Ayurvedic Rasayanas used as general tonics. *Tinospora cordifolia* has been reported to have immunomodulatory [13], anti-oxidant [14], anti-arthritic[15], anti-osteoporotic [16] and anti-aging [17] properties whereas *Withania somnifera* has been proven to be anti-inflammatory [18–22], anti-stress [23] and neuroprotective effects [24]. The present work was undertaken to explore the effect of these two herbs (*Tinospora cordifolia* and *Withania somnifera*) on proliferation and senescence of human wharton's jelly MSCs (WJMSCs).

2. Materials and methods

2.1. Isolation and expansion of WJMSC

The present work was approved by Institutional Review Board and was carried out according to the regulations of the Research Ethics Committee of Manipal Hospital. Human umbilical cords were obtained with an informed consent, from healthy pregnant mothers who underwent uncomplicated normal delivery. The isolation and propagation of WJMSC was done using earlier protocol [10]. Briefly after removal of umbilical arteries and veins, cord were washed with PBS and cut into small pieces of 1–2 cm. These pieces were further minced to get tiny pieces of tissue which were digested with 0.25% of trypsin at 37 °C with continuous mixing for approximately half an hour. Fetal bovine serum (FBS) was added to this disintegrated tissue for neutralization of the enzymes and centrifuged at 2000 rpm for 10 min. The pellet obtained was seeded in T-25 flask supplemented with α-MEM and 10% FBS (Gibco). The cells were cultured at 37 °C in CO_2 incubator till they attained their confluence. Morphology of WJMSCs was recorded photographically at regular intervals.

2.2. Characterization of cultured WJMSC

a Flowcytometric analysis: Isolated MSCs were characterized for the expression of unique cell surface markers and were assessed by flow cytometry using varied panels of antibodies such as CD 90, CD105 (PE Tagged), CD 34, CD45 and HLADR (FITC Tagged) (BD Biosciences). Appropriate isotypes were run to determine non-specific fluorescence. Cells were harvested and fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. They were then washed with PBS containing 0.5% BSA and incubated for an hour on ice with specific antibodies dissolved in PBS. After completion of incubation, the cells were washed and resuspended in 500 μl of PBS. They were then transferred into fluorescence activated cell sorter (FACS) tubes and acquired on BD FACS Calibur flow. The data was analysed using the Cell Quest Pro software.

b Tri-lineage Differentiation: Cells were plated in 6 well dishes for trilineage differentiation. After the cells acquired 95–100% confluence, they were supplemented with chondrogenic (Stempro, A1007101), adipogenic (Stempro, A1007001) and osteogenic (Stempro, A1007201) induction cocktail as per manufacturer's instructions. Every 4th day, the cells were replenished with fresh differentiation media for 21 days. On completion of differentiation, the cells were stained with alcian blue for chondrogenesis, oil o red for adipogenesis and vonkossa for osteogenesis. The images were captured under light microscope (Nikon Eclipse TE 200-S, Chiyoda-Ku, Japan).

2.3. Molecular analysis

The total cellular RNA from MSC's was extracted using RNA easy mini kit (Qiagen) according to manufacturer's instruction. Total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using High-Capacity cDNA reverse transcription (Thermo Fisher Scientific) as per manufacturer's instruction. qPCR reaction condition and primers for β-actin (internal control), CD90 (stemness marker), Ki67 (proliferation marker) and p21 (senescence marker) were used as previously described by us [10,25]. qPCR was performed on ABI Step One Plus system using SYBR Green chemistry PCR (Applied Biosystem). All experiments were done in triplicates. The results were analysed by relative quantification, using ΔΔCt method.

2.4. Metabolic activity using MTT assay

Pure leaf extract of *Tinospora cordifolia* and root extract of *Withania somnifera* was obtained from Pharmanza Herbal Pvt. Ltd., Mumbai, India. WJMSCs at the density of 10000 cells/well were seeded in 96 well plates. Once cells got adhered (Min 12 h) they were incubated with different concentrations (5, 10, 20, 40, 50 μg) of 0.2% ethanolic extracts of *Tinospora cordifolia* and *Withania somnifera* for 48 h. After the incubation the proliferation rate was determined by MTT assay. Briefly 10 μl of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide, 5 mg/ml) was added to each well and incubated at 37° C for 4 h. Subsequently MTT solution was removed and blue formazan crystals were dissolved in 100 μl of DMSO (Dimethyl sulfoxide). The absorbance was read at 570 nm using a micro plate reader.

2.5. Cell viability by using trypan blue exclusion assay for cell toxicity assessment

The WJMSCs were plated at 2500 cells/ml and after attachment cells were treated with media containing *Tinospora cordifolia* (10 μg/ml) and *Withania somnifera* (5 μg/ml). Cells were incubated for 24 h and 48 h. After trypsinizing, 100 μl cell suspension of each condition was treated separately with 100 μl of 0.4% trypan blue. Using bright field optics, numbers of stained cells with intact plasma membranes and dead cells were determined.

2.6. Determination of cell cycle analysis by flow cytometer

The cells were treated as previously described in Section 2.5. Untreated control (0.2% ethanol) was also included. After incubation (48 h), the cells were harvested and fixed with ice-cold 70% ethanol (1 ml) at −20 at 4°C for 2 h. Prior to analysis, the cells were washed with cold PBS and re-suspended in 425 μl of PBS, 25 μl Propidium Iodide (1 mg/ml) and 50 μl RNaseA (1 mg/ml). The DNA contents were recorded by a flow cytometer (Becton Dickinson) followed by data analysis on Cell Quest software.