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Research Paper

in-Vitro Model Showing Differentiation of Wharton's Jelly Derived Mesenchymal Stem Cells (Mscs) to Neuronal Phenotype Through Supplementation of Bacopa Monneri: an in-Vitro Approach

Biotechnology

KEYWORDS : Wharton's Jelly, MSCs, Nerve cells, Differentiation, *Bacopa monneri*

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ABSTRACT

Human umbilical cord Wharton's jelly derived MSCs were induced to differentiate using an in-vitro approach. Study included exposure of MSCs to crude methanolic extract of Bacopa monneri and serum collected from healthy volunteers supplemented with Bacopa monneri as test samples. Cells were investigated for morphological changes and Nestin gene expression to prove its neuronal lineage against β-Mercaptoethanol (BME) as positive(+ve) control. Results indicated that Bacopa supplemented serum (BSS) showed greater differentiation capabilities as compared to +ve control. However, crude extract and un-supplemented serum (USS) didn't reveal neither morphological differentiation nor Nestin expression. These results lead us to hypothesize that the fortified serum with secondary metabolites in BSS play a crucial role in MSCs differentiation towards neuronal phenotype.

INTRODUCTION:

Regenerative medicine, the science of culturing human Stem cells derived from various sources to regenerate the defective or degenerated tissues and organs, is an exciting new vista of Biotechnology with a potential of improving quality of life through healthcare (Thiemann and Palladino, 2011). Several of the impressive demonstrations of regenerative medicine have used varying forms of stem cells - embryonic, adult, and most recently induced pluripotent stem cells to trigger healing in the patient (Maxson *et al.*, 2012; Liu *et al*, 2014; Hu *et al.,* 2014). The Umbilical cord stem cells can be derived from either the Umbilical Cord Blood(UCB) or the Wharton's jelly of the umbilical cord (UCWJ). The umbilical cord blood contains mononuclear cells which further differentiate into mesenchymal and hematopoetic stem cells whereas Wharton's jelly derived stem cells constituted of endodermal (from umbilical vein) and mesenchymal stem cells(MSC) derived from matrix (Hass *et al.*, 2011; Li *et al*, 2013). Mesenchymal stem cells can give rise to many kinds of connective tissue cells including those responsible for remodelling of cartilage, bone, fat, and vascular tissue as well as tendon and ligaments, muscles cells, skin cells and even nerve cells (Watt *et al.,* 2010; Ribeiro *et al.*, 2014)

Different research efforts indicates that UCWJ harbour a sub population of MSCs, which are multipotent and have the potential to give rise to majority of human cell types. Clinical expectations associated with MSCs are derived from three functional characteristics of these cells: the ability (i) to repair tissue through autocrine or paracrine mechanism, (ii) for immunomodulation, and (iii) to support cell engraftment. (Wang, *et al.*, 2004; Cardoso *et al.,* 2012,) Emerging evidence have demonstrated that adult MSCs have a broad therapeutic potential primarily due to their ability to regenerate tissue by differentiating towards multiple adult cell types under appropriate *in vivo* and *in vitro* conditions in presence of various growth factors, cytokines and specific differentiation media (Nekanti *et al.,* 2010).

Each specialized cell type is defined by its particular pattern of regulated gene expression. Cell differentiation is thus a transition of a cell from one cell type to another and it involves a switch from one pattern of gene expression to another. Differentiation is controlled by the interaction of a cell's genes with the physical and chemical environment of the cell, usually through signaling pathways or through epigenetic modulation (Cheung TH & Rando TA, 2013) The progenitor cells derived from various sources have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, and hepatic phenotypes in vitro (Mitchell *et.al.* 2003, Semenov *et al.,* 2011; Pires *et al.,* 2014). Stem cells of various lineages have been used to differentiate into cells of specific lineages for various therapies with the use of specific differentiating factors derived from culture supernantants, synthetically produced or natural products. The differentiation inducing factors have been identified and purified for the purpose of in vitro differentiation (Kawasaki *et al.*, 2008; Burnett *et al.*, 2012 Efferth *et al.*, 2012).

Bacopa monniera, a small, annual creeping herb, has been used in the Ayurvedic system of medicine for centuries as a brain tonic to enhance memory development, learning, and concentration. The triterpenoid saponins and their bacosides are responsible for *Bacopa*'s ability to enhance nerve impulse transmission. The bacosides aid in repair of damaged neurons by enhancing kinase activity, neuronal synthesis, and restoration of synaptic activity, and ultimately nerve impulse transmission (Aguiar and Borowski, 2013, Gohil and Patel 2010). The dominant component of *Bacopa monnieri* leaves is 22% bacoside A (Gubbannavar, *et al.,* 2011). The present study assesses the role of *Bacopa* metabolites on differentiation of UCWJ derived MSCs using metabolomic approach in an *in-vitro* model.

MATERIAL & METHODS

***Bacopa* supplementation:** 500 mg *Bacopa* extract was supplemented to healthy human volunteers (n=10) in two divided doses in the form of 250 mg capsules (Brahmi, of M/s Himalaya) twice a day before meal for a period of 4 weeks. Volunteers were clinically fit without any major illness in recent past with liver and renal functions within satisfactory limit. Blood samples were collected from these volunteers twice, once before supplementation as un-supplemented serum (USS) and once after 28 days of bacopa supplementation (BSS). Serum was separated after 30 min of proper clotting and stored at -20°C till analysis.

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Methanolic extraction of *Bacopa* using Soxhlet: 10gm (40 cap, Himalaya) of *Bacopa* leaf powder was extracted in 100 ml of methanol using soxhlet extraction method. The filtered methanolic extract was then poured in a petri dish and oven dried at 40°C. The residue was dissolved in DMSO in 1 mg / ml concentration. 20µl of this reconstituted extract was added to 2 ml of media containing 10^4 cells (Set-4 in Table-1) for differentiation.

Ex-vivo expansion: Umbilical Cord (UC), obtained from clinically normal pregnancies with consent from the mother, were excised and washed in a 0.1M phosphate buffer saline (pH 7.4) to remove excess blood. Blood vessels were peeled off after longitudinal resection of cord. The remaining Wharton's jelly tissues were minced into small pieces of around 1 mm^3 and placed in petri plates with low glucose DMEM, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco-BRL). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. The media were changed every 3rd day. After 7th day of incubation, proliferating cells escaped from the explants and started adhering to the plate. The non-proliferating tissue fragments were removed and the adherent fibroblast-like cells were cultured till 70% confluency. On confluency, the cells were trypsinized using 0.25% trypsin (Gibco-BRL) and passaged in to 1:3 ratio in the fresh culture medium. The cells were used for immunomagnetic separation after three passages for differentiation study.

Immunomagnetic separation for CD34-ve cells: Cells disaggregated from mesosphere were incubated with anti CD34 Ab labeled magnetic beads. CD34 -ve cell population was isolated in a separate tube following manufacturer's protocol (Stem Cell Technologies, Canada). These cells were allowed for further expansion till confluency and then splitted till passage-3(P3) for differentiation study.

Differentiation: To investigate the differentiation potential of the fibroblast-like cells, passage 3 cells were cultured under conditions appropriate for inducing the differentiation of each lineage following modified method of Woodbury et al., (2000). Cells were seeded at a density of $2x10^4$ cells/cm^2 and the media were changed every 3–4 days. Differentiation inducing factors were added on day 7 after mesospheres started appearing. Mesospheres were enzymatically disaggregated and replated in a 12 well plate shown in plate map (Table 2). Addition of differentiating factors and common factors was as detailed in Table-1. All the experiments were run in triplicate in four sets. Cells were monitored for morphological changes till day 21.

Table 1. Details of differentiating and common factor in 3 experimental sets

Expt.	Differentiating factor	Common factors
Set-1	1mM BME (+ control)	Low Glucose DMEM, 10% FCS2 ng/mL epidermal growth factor (EGF), 2 ng/mL fibroblast growth factor (FGF),
Set-2	10% Un-supplemented serum (USS) (-ve control)	
Set-3	Crude Methanolic extract (CE) of Bacopa leaf powder 1 mg/ml DMSO (Test-1)	
Set-4	4% (BSS) (Test-2)	

Table 2. Plate Map (12 well)

+control (+C) BME	-ve control USS	Test-1CE	Test-2 BSS
+control (+C) BME	-ve control USS	Test-1 CE	Test-2 BSS
+control (+C) BME	-ve control USS	Test-1 CE	Test-2 BSS

Semi-quantitative RT-PCR

Cells from each set were then harvested at 70% confluency, after visible conformational changes. About $2x10^6$ cells from each set were processed for totaI RNA extraction using TrizoI method for semi-quantitative expression of Nestin gene by RT-PCR. The PCR was performed under the following conditions : forward primer : 5'-GGCAGCGTTGGAACAGAGGTTGGA-3' and reverse primer : 5'-CTCTAAACTGGAGTGGTCAGG GCT - 3', cycling condition: denaturation (94°C), annealing (53°C) and extension (72°C) for 40 cycles. The amplicons (718bp) were separated on 1.5% agarose gel and band detected densitometrically. (Kim *et al.*, 2004)

RESULTS

Morphological changes

The information on the proliferation and differentiation of MSCs from the umbilical cord under various treatments is summarized in Table 3.

Table 3. Effect of Supplementation on proliferation and differentiation of MSCs from the umbilical cord

Growth Parameter	Details
Count of MSCs generated per cm of umbilical cord	0.25×10^5
Doubling time for MSCs (in 10% FBS-DMEM with UCBS)	60-72 Hrs
Growth of Mesosphere	3rd Passage Cells
Differentiation of MSCs into Spindle shaped, unipolar and bipolar cells, elongated, large, irregular cells with multiple projections (Neuron like)	Initiated at 7 days after induction in C+ and BSS Treatments, prominent at 14 days in BSS
1mM b-Mercapto ethanol(BME) (C+)	Few cells showing morphological Changes at day 3, character lost later on withdrawal
Un-supplemented serum (USS) (-ve control)	No morphological changes seen
Crude Methanolic extract of Bacopa leaf powder 1 mg/ml DMSO Extract (Test-1)	Occasional cells showing morphological changes At day 7, character lost later on withdrawal
10% BSS (Test-2)	Plentiful of unipolar, bipolar and multipolar neuronal cells from 4th day onwards

BSS= *Bacopa* leaf powder Supplemented serum; C+ =+VE Ccontrol; UCBS= Umbilical Cord Blood derived Serum

In the present studies on isolation and proliferation of MSCs from the umbilical cord, approximately 1x 10^5 MSCs were obtained from 4 cm of umbilical cord. The doubling time for the MSCs was less than 3 days. The 3rd passage MSCs were cultured in common growth media till the mesospheres were formed as shown in Fig. 2b. The enzymatically disaggregated mesospheres were then allowed to grow for 24 hours before induction with differentiation media as shown in Table 1. The morphology of these cells started changing within 48-96 hrs from spindle shaped bipolar cells to larger irregular or elongated cells with multiple projections (Fig. 2 d, e).

Fig.1 depicts morphological conditions in all the four sets from day1 to day 14 after addition of differentiating agents. Heparin induced and BSS group showed morphological changes on day 2-4 (d, g). However in BSS group by the end of day 14 unipolar, bipolar and multipolar neuronal cells started appearing (h, i, j). In CE there are very few cells showing morphological changes which later on lost its appearance. However in USS there is no differentiation towards neuronal lineage morphologically.

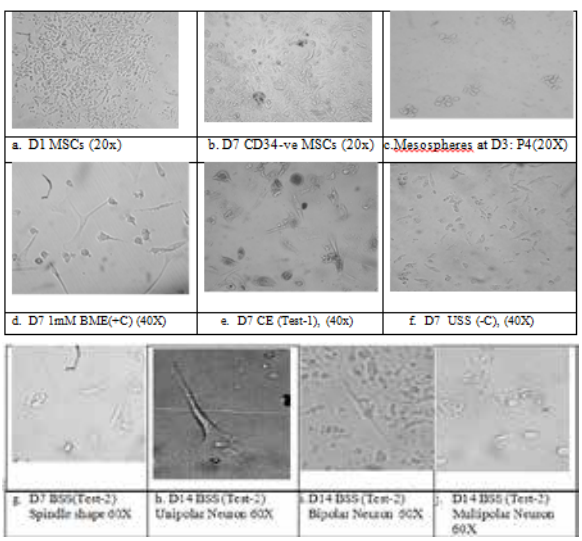

Fig.1 Impact of *Bacopa* Leaf Powder supplementation and crude extract on Morphology of MSCs undergoing differentiation. USS(-C) = Un-supplemented Serum, **BSS**= *Bacopa* leaf powder supplemented Serum, **CE**= Crude methanolic extract in DMSO**, , BME(+C)** = +ve control, **D**=Day

RT-PCR study of the growing and differentiating MSCs revealed faint expression in Heparin (+C) induced MSCs, whereas the cells exposed to supplemented serum (BSS) showed strong expression of Nestin Gene with remarkable morphological changes towards neuronal phenotype. However, cells in the un-supplemented serum and crude extract didn't show Nestin expression. These observation raises the possibility that *Bacopa* supplementation might have released secondary metabolites which induce the proliferating mesospheric cells towards neuoronal lineage.

Fig. 2. Geldoc image showing nestin expression in all sets with various differentiating agents. (NTC: No Template Control; USS: unsupplemented serum; BSS:supplemented serum; PC: Positive Control glioma tissue; CE:Crude extract and BME: β-Mercaptoethanol)

Fig. 2 reveals the expression of Nestin gene to varying extents in the different experimental conditions. It is evident from the gel image that BSS showed a stronger induction for Nestin expression than the BME induced cells. Nestin expressing glioma tissue sample was included to ensure successful PCR run. CE and USS didn't show any expression suggestive of the fact that whatever morphological changes appeared microscopically were not stable. Glioma tissue, taken as standard for Nestin gene showed the maximum presence of Nestin gene.

DISCUSSION

MSCs is a group of multipotent cells that can expand, self-replicate, and differentiate into many cell types under appropriate conditions. Strategies presently under development include transplants of these stem cells, by manipulating patient's own stem cells (autologous) or from compatible donor's (allogeneic) and the use of scaffold materials that emit biochemical signals to spur stem cells into action (Wang *et al.* 2012, Arufe *et al.*, 2011, Li *et al.*, 2012). For this reason, stem cells therapy is quickly gaining momentum in biomedical research eventually aiming at the therapeutic applications for various degenerative and traumatic conditions.

Procurement of UCWJ derived MSCs is noninvasive and can be easily obtained and processed as compared to bone marrow MSCs with greater immunological naivity. The morphological and gene expression studies revealed stable morphological changes in morphology within 1 week with concurrent expression of Nestin gene in +control and test set containing *Bacopa* supplemented serum. It has been reported earlier that secretome, a rich and complex set of molecules, plays a keyrole in co-ordinating many biological processes e.g. proliferation, differentiation, apoptosis and signaling. Its role in cognitive enhancement has been reported in our ancient literature (Singh, 2013). However the exact molecular mechanism behind its role is not clearly deciphered. In the current study it is hypothesized that supplementation of *Bacopa* in healthy individuals might have changed the biochemical constitution of the serum in the volunteers with respect to presence of secondary metabolites released into circulation following *Bacopa* metabolism. This is an unique holistic approach which simulate the in-vivo milieu using in-vitro model where the target cells are exposed to complete metabolome of the circulating blood.

Effectiveness of *Bacopa monnieri* herb alone or in formulation in cognitive improvement have been reported earlier in human and animal models possibly through regulating the expression of tryptophan hydroxylase and serotonin (Calabrese *et al.,* 2008; Charles *et al.,* 2011; Aguiar & Borowski, 2013). There are reports in favour of its antioxidant nature against decreasing toxicity due to chemical exposure in-vitro (Ali *et al.,* 2001). Antiapoptotic role of its crude extract has also been reported earlier by Anand *et al.,* 2013.

Impact of several Chinese herbs and their active ingredients on morphological changes of MSC towards neuronal cells, and rejuvenation of brain tissues from the stroke and brain injury have also been reported in earlier studies (Si et al., 2014; Su et al., 2007; Wang et al., 2007, 2013; Wu et al., 2007 & Xiang et al., 2001).

Its role as immunomodulator in diabetes suggests that chronic supplementation of this herb alone or in combination might be secreting small molecule and/or its active metabolite secreted into circulation might be responsible for its biochemical effect (Arora *et al.,* 2002). Therefore, it can be speculated that in-vitro screening of crude extract and in-vivo supplementation of this nootropic herb has different mechanism of action. Above all ascorbic acid, glutamic acid, serine, nicotinamide and nicotine present in the bacopa powder, having reported role in dopamin-

ergic and GABAminergic differentiation, might be released into serum of supplemented volunteers after metabolism (Meldrum, 2000; McEntee & Crook, 1993). Therefore, further characterization of the supplemented serum containing the metabolic by-product of *Bacopa* using various analytical methods e.g. LCMS, cytokine and growth factor profiling may add clue in identifying the factors responsible for neuronal differentiation of WJ derived stem cells *in-vitro*.

CONCLUSION

This study represents a unique in-vitro model which simulate the in-vivo environment exposed to complete metabolome specifically for edible herbal medicines which has established role as nootropic or nutraceuticals in alternative and complimentary regenerative medicine. Role of *Bacopa monnieri* as differentiation inducer in MSCs is validated using this in-vitro model and confirmed by gene expression study. Time lapse gene expression study may provide sequential switching on and off of genes responsible for the transitions to neuronal cells possibly through epigenetic modulation. The study opens up avenues to undertake further investigation to get better insight into the molecular mechanism of neuronal differentiation which may be useful in management of managing neurodegenerative or ischemic brain injury.

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