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Evaluation of Culture Morphology of Neuronally Trans-differentiated Wharton's Jelly Derived Mesenchymal Stem Cells

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ABSTRACT

Background: The prospect of mesenchymal stem cells (MSCs) as an adult stem cell source for neuronal tissue regeneration via their ability to differentiate into neurons has generated considerable excitement in regenerative cell therapy.

Methods: In this study, we isolated ovine Wharton's jelly derived MSCs and expanded *in vitro* in adherent culture. After the characterisation of MSCs using specific markers, we analysed the culture morphology of MSCs differentiated into neurons by a two-step chemical-based induction protocols involving a pre-induction step and a direct one step chemical-based induction protocol. Morphological changes after induction were evaluated.

Result: In both the methods, after neuronal induction, the cells displayed phenotypic characteristic of neurons and comparatively less cytotoxicity was observed in the direct induction method. This study confirmed the possibility of generating neuron like cells from ovine WJ-MSCs and thereby exploring the potential of MSCs as therapeutic tool for treating neurological disorders in Veterinary Medicine.

Key words: Cytotoxicity, MSCs, Neurons, RT-PCR, Wharton's jelly.

INTRODUCTION

Mesenchymal stem cells (MSCs) are one of the most easily accessible stem cells that can be obtained from various tissues. They have been widely tested in therapeutics due to their easy accessibility, availability from ethically acceptable tissues such as bone marrow aspirates and fat tissues and their efficacy in various diseases and tissue damage regeneration (Han *et al*., 2019). Umbilical cord Wharton's jelly derived mesenchymal stem cells (WJ- MSCs) have emerged as remarkable cells for cell based regenerative therapeutics based on the back of its own proven prominent characteristics. Their superiority with regard to ease of isolation, abundance, proliferative capacity, low immunogenicity, absence of tumourogenicity has been well documented (Kalaszczynska and Ferdyn, 2015; Gauthaman *et al*., 2012; El Omar *et al*., 2014). Ovine WJ- MSCs have been isolated and characterised (Eswari *et al*., 2016) and their ability to differentiate into cells of mesodermal origin is also well established (Dias *et al*., 2016; Zhao *et al*., 2019). However, their ability to differentiate into cells of non-mesodermal origin such as neurons using different induction protocols has not been reported. In the present study, we aim to study the culture characteristics of ovine WJ-MSCs that have undergone neural differentiation by two different chemical based neural induction protocols.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Ethical Committee for Stem Cell Research and Therapy, Tamil Nadu Veterinary and Animal Sciences University and all the procedures were performed according to ethical committee approval. The study was carried out in the Department of Veterinary Physiology, Madras Veterinary College in the year 2020. All the chemicals were purchased from Invitrogen (USA) and all the plasticware are from the Nunc unless otherwise stated.

Umbilical cords from fetus in the gravid uterus were collected from the Chennai Corporation slaughter house, Perambur and transferred aseptically to the stem cell laboratory for processing. After giving a thorough wash in sterile Phosphate buffered saline (PBS) fortified with antibiotics, the umbilical cord was cut opened longitudinally and WJ pieces were separated. The WJ pieces were cut into small pieces of one mm^3 size and the explants were seeded in 24 well culture plates (Nunc) in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% foetal bovine serum and 1% antibiotics. Once the confluence was attained the cells were subcultured using TrypLE™ Express solution. The isolated cells were characterised by expression study of MSC specific markers gene expression.

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The MSC specific marker genes CD 44, CD73 and CD 90 expression were analysed by reverse transcriptase polymerase chain reaction (RT-PCR) as per the standard protocol. To carry out the analysis, total RNA was extracted from MSCs at passage 5 using Qiagen RNeasy kit (Qiagen, USA). The RNA was then reverse transcribed using iScript cDNA synthesis kit (Biorad, USA). The details of primers used are provided in Table 1. The thermocycling conditions consist of an initial denaturation step at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 54°C to 58°C for 30 sec and 72°C for 60 sec with final extension at 72°C for 10 min. The PCR reactions were performed for the specific genes and the PCR products were visualized with ethidium bromide in 2% agarose gel.

For neuronal induction, two different chemical methods were used. In method I, the 3rd passage WJ MSCs was subjected to neuronal induction as per Woodbury *et al.* (2000) with slight modifications. Briefly, the WJ MSCs were subjected to pre-induction in complete medium containing 2 mM β-Mercaptoethanol (β-ME) for 24 hours. This was followed by washing with PBS and incubation in induction media containing 2% dimethyl sulphoxide (DMSO) (Sigma), 200 µM Butylated Hydroxy Anisole (BHA) (Sigma) and 40 ng/ml basic fibroblast growth factor (bFGF) (Sigma) in DMEM. Neural induction media was replenished every 48 hours and the culture was maintained up to seven days.

In the second method, the cells were directly subjected to induction medium consisting of 10 µM forskolin (Sigma) and 100 µM isobutyl methylxanthine (IBMX) (Sigma) in DMEM. Media was changed every 48 hours and the culture was maintained up to seven days.

In both induction methods, the culture morphology of WJ-MSCs and the differentiated neuron like cells were observed under inverted phase contrast microscope (TS-100 Nikon, Japan).

Cell cytotoxicity was analysed using trypan blue exclusion test. To quantify cytotoxicity of the two chemical methods, 1×10^6 P3 cells were subjected to neuronal induction using both protocols. Putatively differentiated cells from the two methods, which have been in induction media for three days were used for the cytotoxicity study. Briefly, 50 µl of cell suspension was mixed with 50 µl of 0.4% trypan blue dye solution and mixed well by pipetting. The mixture was incubated for one minute and cells were counted with haemocytometer to calculate the percentage of viable cells. The viable cell yield was calculated taking into account, the dilution factor and the hemocytometer factor.

RESULTS AND DISCUSSION

In vitro culture and expansion of ovine WJ MSCs

In this study, the ovine WJ-MSCs isolated by explant culture method and the cells were expanded *in vitro* and maintained up to 6th passage. The cells with fibroblastoid morphology, started sprouting from the WJ explant on the third day itself after the attachment. The cells migrated throughout the culture dish and 80-90% confluency was observed on the day 7 of culture. The cells of primary culture exhibited typical spindle shaped morphology with elongated ends. Cells aggregated into colonies and showed positive reaction on staining with alkaline phosphatase (Fig 1A-D).

RT-PCR

The RT-PCR analysis of cultured cells at third passage confirmed the expression of MSC specific markers CD44, CD73, CD90 and negative expression of CD34-the hematopoietic marker (Fig 2).

Morphological characterisation of differentiated cells

Cells of third passage (P3) were adherent, arranged in the form of monolayer in culture dish. They exhibited spindle shaped fibroblast morphology. In the pre-induction method, after 24 hrs of incubation, the cells lost their fibroblastoid morphology and cell shrinkage was also observed. On day 3 in neural induction medium, cells exhibited further shrinkage and neurite like outgrowth (Fig 3 A-C).

In the second direct induction method using forskolin and Ibmx, morphological changes were observed as early as five hours of incubation. Bipolar or multipolar neurite like outgrowth and other secondary network forming extensions were observed with the aid of phase contrast microscopy (Fig 4 A-D).

Comparison of cytotoxicity of two methods revealed that direct induction protocol had less cytotoxicity compared to that of modified Woodbury *et al.* (2000) method (Fig 5).

Neural stem cells (NSC) based therapy has been proposed to hold great potential in treating neurodegenerative diseases. However, the clinical translation of NSCs is severely hampered by some of the innate limitations that NSCs have like poor accessibility and the need for immune suppression during transplantation (Ahmedy *et al.,* 2015).

Table 1: Primer sequences for RT-PCR.

Genes	Primer sequences (5′-3′)	Product size (bp)	Ta (C)	Accession no
CD 34	CCTTAGTTCCAGCGTCTAC	591	54	AB021662
	ACCAGTGCTCTTCCAGAA			
CD 44	ACATCCTCACATCCAACAC	245	56	NM001206523
	GCCACTGCTCATCTCATC			
CD 73	GAGAACCTGGCTGCTGTGT	411	54	BC114093
	CCGACCTTCAACTGCTGGAT			
CD 90	GACACAGGAAGCCACAAG	302	56	BC104530
	CTCGCCATCAGGTCTCTA			

These limitations can be overcome by generating NSCs from other sources like MSCs which has got immunomodulatory potential. The MSCs from WJ has shown potential in *in vitro* neuronal transdifferentiation (Kruminis *et al*., 2020; Balasubramanian *et al.,* 2013). Satheesan *et al*. (2020) reported successful transdifferentiation of ovine WJ-MSCs into neuronal phenotype using neuronal conditioned medium harvested from ovine fetal brain tissue culture without supplementing any induction chemicals.

In the present study, ovine WJ-MSCs were transdifferentiated into neuron like cells using chemical induction methods. The obtained results indicated that in both methods, majority of the differentiated cells displayed the phenotype of neural stem and progenitor cells. The differentiated cells were discerned from normal cells on the basis of factors like refractile appearance, presence of multipolar or bipolar neurite like projections and presence of secondary extensions. This characteristic morphology was

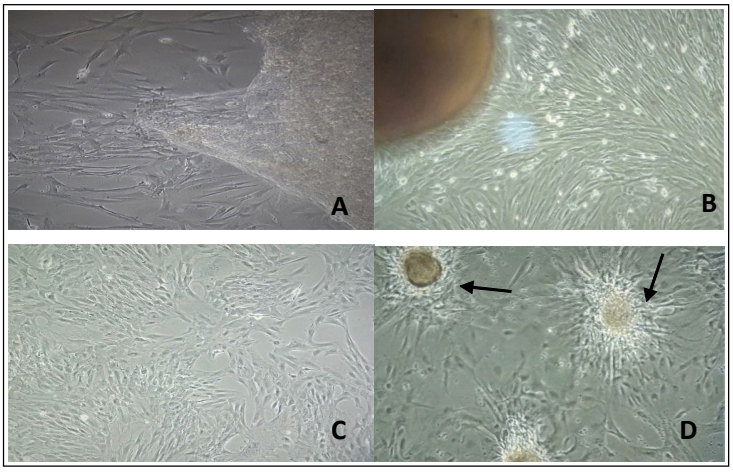
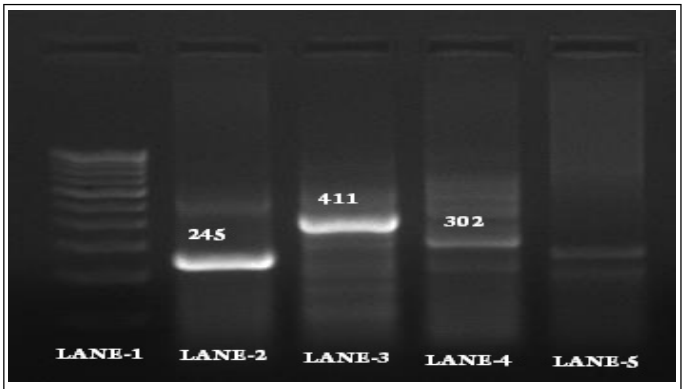
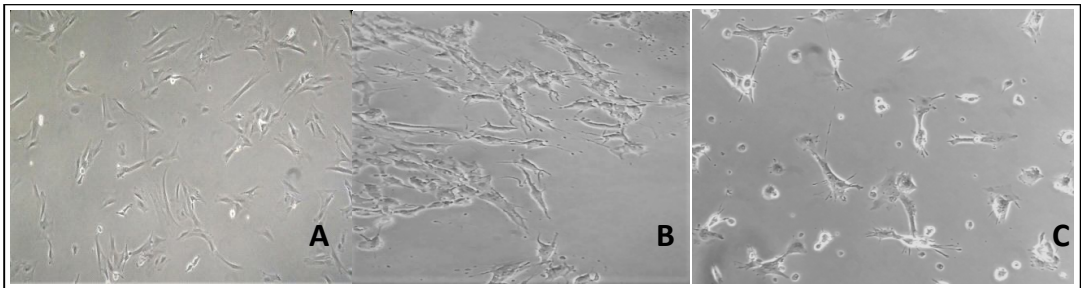


Fig 1: (A) Cells migrating out of explant tissue in primary culture (200X); (B) Ovine WJ-MSCs showing 80-90% confluence in primary culture (200X); (C) Confluent third passage (P3) cells in culture; (D) Aggregates of P3cells forming colony (arrow) in culture.



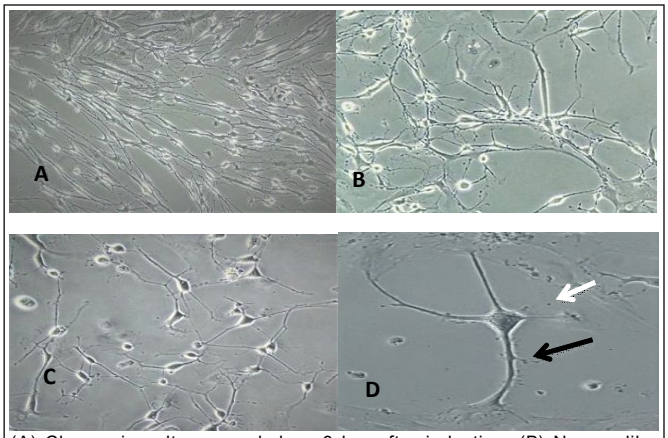
Lane 1 - DNA marker 100 bp; Lane 2- CD44; Lane 3 - CD73; Lane 4 - CD90; Lane 5 - CD34 negative marker.

Fig 2: RT PCR analysis of mesenchymal specific marker gene expression in ovine WJ MSCs.



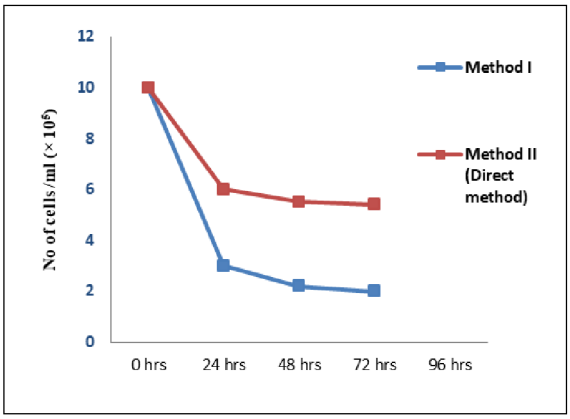
(A) P3 cells with normal spindle shaped morphology (200X); (B) After 24 hrs in pre induction treatment with β-ME(200X); (C) Morphological changes after 72 hrs in neural induction medium containing DMSO, BHA and bFGF (400X).

Fig 3: Neural differentiation and morphological changes in ovine WJ MSCs.



(A) Change in culture morphology 3 hrs after induction; (B) Neuron like morphology was observed 5 hrs after induction (200X); (C) Induced cells with multiple neurite out growth forming connections with neighbouring cells (200X); (D) Multipolar neuron like morphology after 24 hrs in culture with cell body (white arrow), processes resembling dendrites and a process resembling an axon (black arrow); (400X).

Fig 4: Neural differentiation and morphological changes in method II.



Viable cells/ml	24 hrs	48 hrs	72 hrs
Method I	311666.7±6009.252	230833.3±7406.829	220833.3±3333.33
Method II	611666.7±15898.99	563333.33±8333.33	531666.7±4409.586

Fig 5: (A) Time course of viable cells from cultures incubated in the two induction media. Cells were counted at the indicated times. (B) Data represent means±SEM of three independent experiments.

in accordance with Lija *et al.* (2019), who succeeded in isolating and culturing neural stem cells from ovine fetal cerebral cortex. Similar results with regard to culture morphology were observed in neuronally transdifferentiated human WJ-MSCs, adipocyte derived MSCs and bone marrow derived MSCs (Kruminis *et al.,* 2020; Zheng *et al.*, 2017).

In the first method, pre-induction using β-ME lead to changes like shrinkage of cells and rounded cell bodies. Incubation in induction media containing DMSO, BHA and bFGF lead to phenotypic changes featuring neuron like morphology like branching processes and growth of cone like terminal structures. The second method using forskolin and IBMX, yielded cells with axon and neurite like extensions and formed connections with adjacent cells which resembled synapses. This was similar to the culture morphology of transdifferentiated neuronal phenotype of ovine WJ-MSCs using neuronal condition medium (Lija *et al.*, 2020). Moreover, comparatively less cytotoxicity was observed in the second method of chemical induction using forskolin and IBMX.

CONCLUSION

The MSCs can be successfully isolated from ovine Wharton's jelly and can be considered as an important source of MSCs. The results suggest that ovine WJ-MSCs have the ability to trans-differentiate into putative neuron like cells upon chemical based neural induction. Further refinement in differentiation protocol, characterisation of differentiated cells and developing protocol for safe transplantation will aid to promote therapeutic application of these differentiated cells in veterinary regenerative medicine.

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