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Transdifferentiation of canine mesenchymal stem cells into neuron-like cells by induction with β-mercaptoethanol

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Spinal cord injury is a concern for both humans and domestic animals. Therapeutic approach has been tried with direct delivery of neurotrophic factors, stem cells as well as a combination of both to treat such conditions (Jung *et al.* 2009). Despite progress in the treatment of spinal cord injury, recovery from the spinal cord with normal motors and sensory activities remain a challenge (Wright *et al.* 2012). The cell based therapy had been attempted with embryonic spinal cord stem cells, schwann cells, olfactory unsheathing glia, and bone marrow stromal cells (Willam *et al.* 2011, Bhat *et al*. 2018). Among the cell types, mesenchymal stem cells (MSCs) are showing great promises in therapeutic application owing to their multipotency, immunomodulatory properties, migratory behavior and minimum ethical concern. MSCs have plastic adherence properties and can be expanded *ex vivo;* differentiated into mesoderm origin including chondrocytes, adipocytes, and osteocytes as well as ectoderm origin cell-like neurons (Woodbury *et al.* 2000, Sanchez-Ramos *et al.* 2000, Yang *et al.* 2008). The neuron-like cells derived from bone marrow-derived MSCs might be an efficient source of cells for transplantation for the treatment of neurological diseases (Edamura *et al.* 2012).

Transdifferentiation of MSCs into neurons was initially demonstrated by Woodberry *et al* (2000) using chemical agent β-mercaptoethanol (BME), followed by several modifications such as supplementation of neurotrophins (Sanchez-Ramos 2000, Hayase *et al.* 2009, Kim *et al.* 2014), and only with neurotrophin factor in the absence of specialized pre-induction chemicals (Mili *et al.* 2018). However, neurotrophin mediated directed differentiation of stem cells into neuron or neuron-like cells *in vitro* would take a longer duration. In this context, BME based method is less laborious and can pre-differentiate MSCs into neurons like cells in a reasonably short time. We hypothesize that supplementation of β-mercaptoethanol in the culture medium could induce the transdifferentiation of canine MSCs towards neuron-like cells. Hence, the present study was aimed to transdifferentiate canine bone marrow-derived MSCs into neurons/ neuronal like-cells *in vitro*.

The study was conducted with the approval of the Institutional Animal Ethics committee of the ICAR-Indian Veterinary Research Institute, Izatnagar, India. The bone marrow was aspirated aseptically with an 18 G bone marrow biopsy needle from the iliac crest of healthy dogs below one year of age. The dogs were anaesthetized with a combination of xylazine (1 mg/kg) to ketamine (10 mg/kg) at the ratio of 1 : 2. The canine MSCs were isolated from the bone marrow and characterized in our previous studies (Das *et al.* 2017, Mili *et al.* 2018). The canine MSCs of the third passage were induced to transdifferentiate *in vitro* towards neuronal-like cells as per the method described by Woodbury *et al.* (2000) with minor modification. The MSCs were maintained in the MSC culture medium to become confluent. Thereafter, the MSC medium was replaced with a pre-induction medium consisting of DMEM-LG (Gibco) supplemented with 20% fetal bovine serum (FBS) and 1 mM BME (Sigma Aldrich, USA). The pre-induction medium was removed after 24 hours and the culture plates were washed with PBS. Cells were then incubated for another 6 days with a serum-free neuronal induction medium composed of DMEM-LG and 4 mM BME. After this incubation period, the cells were characterized by immunocytochemistry and Real Time-PCR (RT-PCR). The cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton-X-100 for 20 min and immunocytochemistry was carried out as per the procedure described earlier (Das *et al.* 2017). Primary antibodies β-tubulin III, MAP-2, and Nestin of Santa Cruz Biotechnology, USA were used at 1 : 100 dilutions and the Alexa Fluor 594 conjugated secondary antibody at 1 : 800 dilutions.

The total RNA was extracted by Trizol reagent

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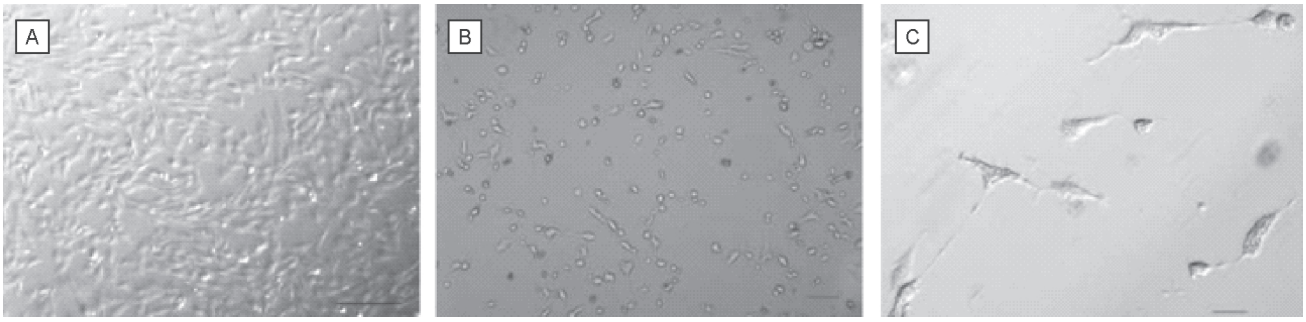

Fig. 1. Morphological changes in the canine bone marrow derived MSCs during the transdifferentiation process. (A) Confluent MSCs of 3rd passage (10×); (B) After 24 h pre-induction with 1 mM BME (10×); (C) After 6 day induction with 4 mM BME (20×).

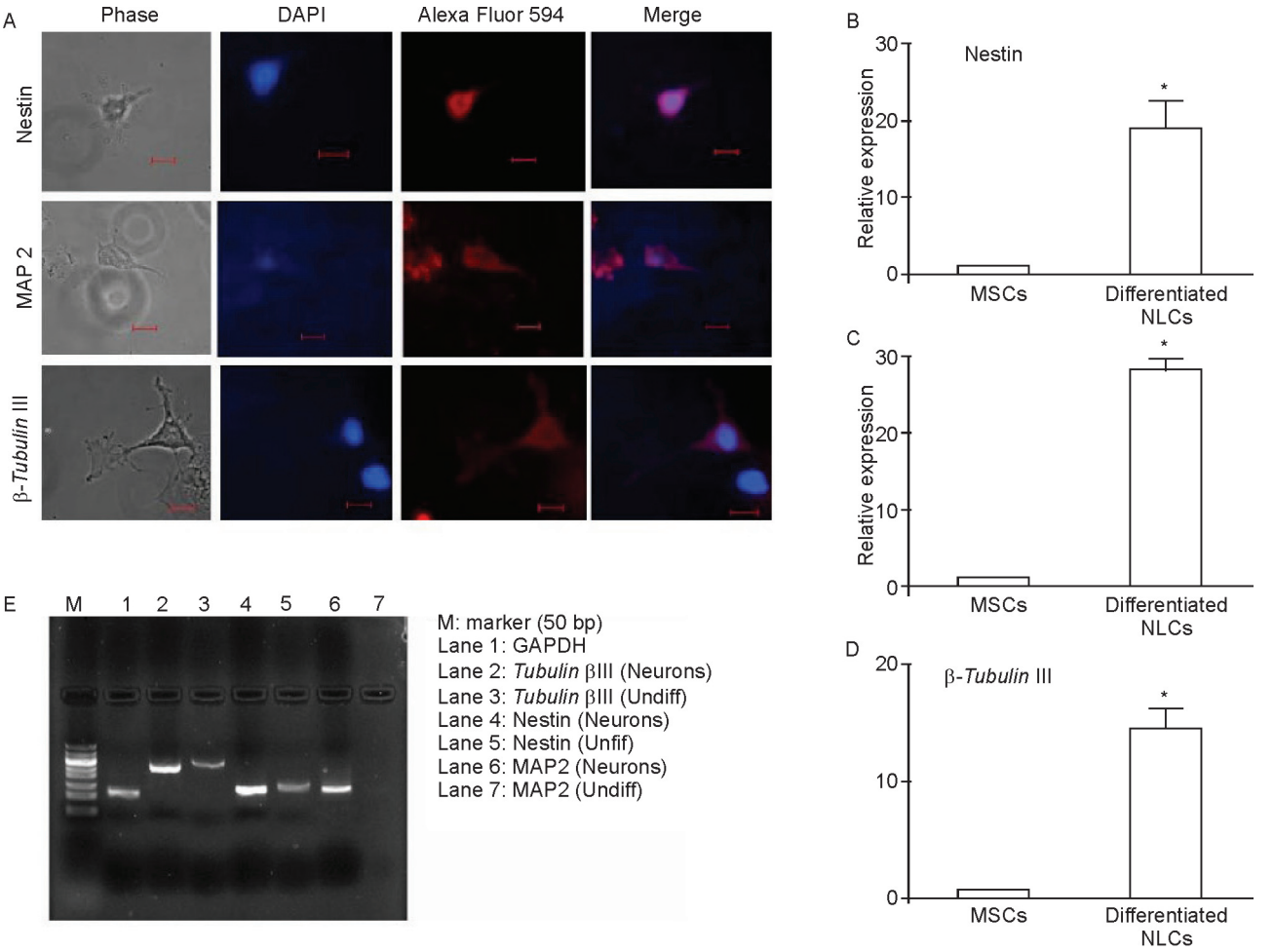

Fig. 2. Characterization of the neuron-like cells. (A) Immunolocalization neuronal specific markers (Scale bar 20 uM); (B, C and D) Relative mRNA expressions of the neuron specific genes; (E) Size fractionized RT-PCR amplicons.

(Invitrogen, USA) from the induced cells and undifferentiated MSCs (control). The c-DNA was synthesized from total RNA using c-DNA synthesis kit (Bio-Rad, USA) and the comparative threshold cycles (Ct) was measured using Eva Green SuperMix (Bio-Rad, USA) as per the manufacturer's instructions. The primer sequences were β-tubulin III-F: AGCCAAGTTCTGGGAAGTCA/ R: CCCACTCTGACCAAAGATGAA (Wilcox *et al.* 2011), MAP-2-F: GAAGTTCAGGCCCACTCTCC/R: CCTGTTGCTGTGGTTTTCCG (XM_005640598.1), Nestin-F: GCCACCGAGAAGTTCCA/ R: GGACATCTTGAGGTGTGCTA (XM_547531.4), and GAPDH- F: CCATCTTCCAGGAGCGAGAT/R:TTCTCCATGGTGGTGAAGAC (Vieira *et al.* 2010). RT-PCR thermal conditions included an initial period of enzyme activation at 95°C for 30 sec, denaturation at 95°C for 5 sec, annealing at 60°C (GAPDH), 57°C (β-tubulin III), and 58°C (Nestin and MAP-2) for 10 sec. The transcript levels of all genes were quantified using the relative quantification method based on comparative threshold cycles values (Ct). The Ct values were analyzed by the method as proposed by Pfaffl *et al.* (2001) with the abundance of the housekeeping gene

GAPDH. The RT-PCR amplicon was size fractionated on 2% Agarose TBE gel containing 0.5 ug/ml LabSafe^{TM} nucleic acid stain (G-Bioscience, USA) in gel electrophoresis. The experimental results were expressed as mean±SD. The simple "t" test was performed to compare the relative expression between transdifferentiated cells and undifferentiated cells using a prism Graphpad version 5.0. $P<0.05$ implies statistically significance.

The isolated cells exhibited morphologically homogeneous fibroblastic populations, formed a confluent monolayer over the culture plates (Fig. 1A). Sequential changes were observed in the appearance of MSCs from typical fibroblastic like morphology to round shaped cells after 24 h of pre-induction with 1 mM BME (Fig. 1B). After another six days of induction with 4 mM BME, neuronal cell-like branching was noticed from the edges of the incubated cells (Fig. 1C). These cells were characterized by immunocytochemistry. The transdifferentiated cells were positive for neuronal markers MAP-2, Nestin, and â-Tubulin III (Fig. 2A). The expressions of these markers were further confirmed by RT-PCR. The relative mRNA expression of MAP-2, Nestin, and β-Tubulin III were significantly higher ($P<0.05$) in comparison to undifferentiated cells (Fig. 2 B, C, and D). The gel electrophoresis of the PCR products confirmed the expression of those genes in the transdifferentiated cells (Fig. 2E). Our results revealed that the BME induction resulted in significant changes in the morphology of canine MSCs. It was observed that after 7 days of neuronal induction, around 40–60% of cells showed the neuron like phenotype. Previously, it was noticed that after 7 days of induction of rat MSCs, neuronal-like phenotypes accounted for above 80% of the total population which also expressed the neuronal cells markers (Jiang *et al.* 2010). Induction of BME in mesenchymal stem cells causes changes in their morphology from fibroblastic to the cells with neuronal phenotypes as evident by the previously reported literature on bone marrow MSCs of other species (Woodbury *et al.* 2000, Sanchez-Ramos *et al.* 2000, Yang *et al.* 2008). Neuronal differentiation potentiality of the MSCs of humans, rats, and mice has been reported (Woodbury *et al.* 2000, Sanchez-Ramos *et al.* 2000, Deng *et al.* 2006). The β-tubulin III has been reported as a potential transdifferentiation inducer of canine MSCs, and Nestin and MAP-2 are expressed in neurons (Chung *et al.* 2013; Heo *et al.* 2013). From the experiment, it can be inferred that induction with BME resulted in the transdifferentiation of canine MSCs into neuron-like cells

SUMMARY

The objective of this study was to check whether β-mercaptoethanol in a culture medium can induce the neuronal differentiation of canine MSCs. The canine bone-marrow derived MSCs were first pre-inducted with 1 mM BME for 24 hrs followed by induction in a serum-free medium supplemented with 4 mM BME without FBS for another 6 days. Morphological changes in MSCs from spindle-shaped to neuron-like branching from the edges of the cells were noticed at the end of induction. These neuron-like cells were found positive for the immunophenotypic expression of different neural cell markers β-tubulin III, MAP-2 and Nestin. In RT-PCR analysis, it was also evident that the relative expressions of these representative genes were significantly higher in the differentiated cells. On the basis of our observations, it can be summarized that the BME induction of canine MSCs resulted in morphological changes that resembled neuron-like cells which were found to express the representative neuronal markers. Therefore, inducing canine MSCs with BME resulted in the generation of neuron-like cells that might be utilized for the prospective therapeutic applications in veterinary medicine.

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