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DIFFERENTIATION OF CANINE BONE MARROW MESENCHYMAL STEM CELLS INTO ISLET–LIKE CELLS

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ABSTRACT

The present study was aimed to study *in vitro* differentiation of canine bone marrow mesenchymal stem cells (BMSCs) to islet-like cells of pancreas. BMSCs were isolated from healthy dogs and cultured in laboratory. BMSCs after 3rd passage (at 80% confluence) were induced to differentiate into islet-like cells by using 2-step induction methods. Characterization of differentiated cells of BMSC into islet-like cells was done by observing cell morphology, expression of canine insulin gene by reverse transcriptase PCR (RT-PCR) and quantitative PCR (qPCR), detection of nestin and insulin proteins by immunocytochemistry and quantification of insulin level in culture media. Typical grape-like clusters of cells were observed under bright field microscope. Expression of insulin gene and protein was observed in differentiated cells. Insulin level in culture media of differentiated cells was significantly high. It was concluded that canine BMSCs could effectively be differentiated into islet-like cells. These differentiated cells could play an important role in diabetic therapy of dogs.

Key words: BMSCs, differentiation, dogs, islet-like cells

INTRODUCTION

Among the metabolic and endocrine diseases, diabetes mellitus is an emerging disease in animals. Canine diabetes mellitus is a common endocrine pathology. Dogs, especially the middle-aged ones, mainly suffer from insulin dependent diabetes and show some similarities with human latent autoimmune diabetes of adults (LADA) (Cernea *et al*., 2009). At present, the management of diabetes in dogs involves injection of human recombinant insulin. However, the therapy with insulin cannot mimic natural state (Cook *et al*., 2011). The injected insulin can lead to glycemic instability because of differential tissue insulin sensitivity and hyperinsulinemia which is associated with high incidence of coronary artery disease (Reaven, 2012).

The stem cells have the potential to differentiate into different cell types in body, therefore the unique regenerative potential of these cells renders their use indispensable in the area of therapeutics. In veterinary sciences, stem cells are used for the treatment of chronic spinal injuries, chronic wounds, osteoarthritis and tendon injuries in canines and equines. Mesenchymal stem cells (MSCs) are greatly used in clinical practice because of their ease to isolate and extensive expansion rate and differentiation potential (Flynn and Preistly, 2014). MSCs secret a number of biologically active molecules that exert beneficial effects on other cells (Caplan and Dennis, 2006). MSCs paracrine effect can be tropic, immune modulatory, anti-scarring

and chemo-attractant. MSCs have great ability to modulate immune response and release various growth factors in response to inflammation (Ma *et al.*, 2014). Autologous MSCs could improve metabolic control, measured by enhanced insulin secretion, ameliorate insulin sensitivity and increase islet numbers in pancreas in diabetic rats (Si *et al.*, 2012). MSCs possess specific immunomodulatory properties that appear capable of disabling immune dysregulation that leads to β-cell destruction in type1 diabetes (Mabed and Shahin, 2012). In stem cell educator therapy immune modulation by cord blood derived multipotent stem cells reverse autoimmunity and promote regeneration of islet of β-cells (Zhao *et al*., 2012). Rat bone marrow MSCs have successfully been differentiated into functional islet-like cells (Chelluri *et al*., 2011). Therefore, the present study was undertaken to generate islet cells capable of producing insulin by differentiation from canine bone marrow mesenchymal stem cells which may serve as an ideal candidate for cell-based therapy in treatment of type 1 diabetes.

MATERIALS AND METHODS

BMSC isolation and culture

The present study was conducted with the permission from the Institutes Ethical Committee under institute project IVRI/MED/12-15/008. BMSCs were obtained from young healthy dogs brought to Referral Veterinary Polyclinic, IVRI, Bareilly (India) with the consent of their owners. The bone marrow was collected from iliac crest of dogs and mononuclear cells were isolated with a Ficoll density gradient method (Takemitsu *et al.*, 2012). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 15% fetal bovine serum (FBS) in 24 well tissue culture plate (Nunc). Well characterized 3rd passage plastic adherent mesenchymal stem cells (MSCs) were used for *in vitro* trans-differentiation (Takemitsu *et al.*, 2012).

In vitro trans-differentiation

Canine MSCs after 3rd passage (80% confluence) were induced to differentiate into islet-like cells by using 2-step induction methods (Chen *et al.*, 2004). MSCs were pre-induced with L-DMEM supplemented with nicotinamide (10 mmol L^{-1}), β-mercaptoethanol (2.5 mmol L^{-1}) and FBS (5%) for 24 h to differentiate into neural stem cells specific marker [nestin] (pre-marker for islet-cell differentiation) positive cells. These cells were re-induced with nicotinamide (10 mmol L^{-1}), β-mercaptoethanol (2.5 mmol L^{-1}) in serum free H-DMEM for 10 h to differentiate into islet-like cells. Cells induced without nicotinamide and β-mercaptoethanol served as controls.

Characterization of differentiated cells

Nestin-positive cells and islet-like cells were observed under inverted phase contrast microscope (Zeiss Axio Vert A1, Carl Zeiss, Lab. India, Mumbai) for their morphological changes.

Expression of canine insulin gene by RT-PCR

Total RNA was isolated from pre- and post-treated MSCs using trizol reagent (Invitrogen, USA) as per the Manufacturer's instructions. The integrity of total RNA was checked on 1% agarose gel using 1X tris borate EDTA (TBE) as electrophoresis buffer. The bands of 28s and 18s RNA showed intact RNA. The purity and concentration of total RNA was checked using Nanodrop (Thermo-scientific, 2000C). The optical density (260:280) of isolated RNA samples was >1.9 indicating that the samples were free from protein contamination. The concentrations of RNA samples were in the range of 700-2000 ng μL^{-1}. A constant amount of 1 μg total RNA was reverse transcribed using cDNA synthesis kit (ThermoFisher Scientific, Mumbai, India). RT-PCR was performed using the Master Mix (Thermo scientific, India). The pancreatic biopsy samples, collected from a dog, were used as positive control and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as internal control. The reaction mixture consisted of PCR master mix (2X) 12.5 μL, forward primer 0.5 μL, reverse primer 0.5 μL and cDNA 0.5 μL to a final volume of 25 μL. The protocol followed was initial denaturation

at 95°C for 3 min, denaturation at 95°C for 30 sec., annealing at 54°C for 45 sec. and extension for 1 min at 72°C for 40 cycles. The confirmation of amplification of specific RT-PCR amplicon was done by using 1.5% agarose gel electrophoresis. The primer sequences used for RT-PCR were INS F5'GGGGAGCGCGGCTTCTTCTA3' (179bp; Genbank Accession No. NM_001130093.1) and INS R 5'TAG A GGGAGCAGATGCTGGTGC3'.

Localization of nestin and insulin by immunocytochemistry

Pre-islet like cell differentiation marker such as nestin and insulin in differentiated islet-like cells were detected with immunocytochemical techniques using appropriate antibodies (Chen *et al.*, 2004). After discarding media in culture plate, the cells were fixed using 4% paraformaldehyde. Then cells were incubated with sodium citrate buffer (10 mM sodium citrate, pH 6.0, 0.05% tween-20) for epitope retrieval, rinsed and blocked with 5% BSA for 40 min at 37ºC. The cells were then incubated with polyclonal goat nestin antibody (1:200) and polyclonal rabbit anti-insulin antibody (1:200) (Santa Cruz, USA), respectively. Primary antibodies were detected using FITC-conjugated anti-goat antibodies (1:400) and PE conjugated anti-rabbit insulin antibodies (1:400). Counter-staining was done using 4'6-diamidino-2-phenylindole (DAPI) (0.4 μg mL^{-1} in PBS) to stain nuclei of the cells. Undifferentiated MSCs were taken as negative controls.

Quantification of insulin in culture media

The concentration of immune-reactive insulin in media secreted from differentiated cells 48 h after treatment were determined by canine specific enzyme immunoassay kit (Crystal Chem Inc.).

RESULTS AND DISCUSSION

Canine mesenchymal stem cells after 3rd passage (80% confluence) (Fig. 1) were induced to differentiate into islet-like cells using 2-step induction methods. Round mononuclear cells on 1st day of seeding were changed to spindle-shaped and fibrocyte-like cells on 14th day with 90% confluence. MSCs were transformed into elongated neuron-like cells (Fig. 2) and on further induction, these cells changed into oval or round cells and appeared like clusters of grapes (Fig. 3). Insulin mRNA expression in differentiated islet-like cells after 48 h of differentiation and in undifferentiated MSCs was assessed by RT-PCR. Insulin mRNA from healthy pancreatic tissue of a dog, collected by pancreatic biopsy, was taken as positive control. Insulin mRNA transcription could be detected in differentiated islet-like cells similar to the positive control (Fig. 4).

Fig. 1: Spindle shaped mesenchymal stem cells isolated from canine bone marrow after third passage having 80% confluence [scale bar 100 μm]

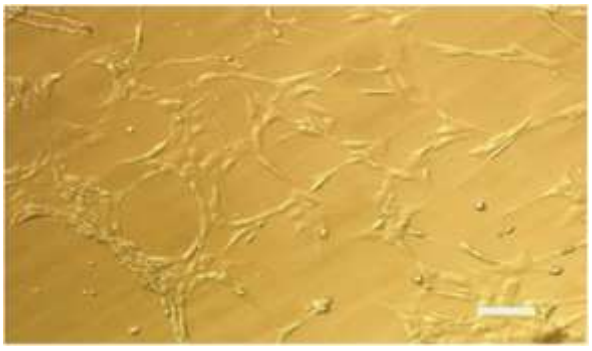

Fig. 2: Pre-differentiated neuron-like cells after passaging mesenchymal stem cells (3rd passage) in L-DMEM medium supplemented with nicotinamide (10 mmol L^{-1}) and β-mercaptoethanol (2.5 mmol L^{-1}), FBS (15%) for 24 h [scale bar 100 μm]

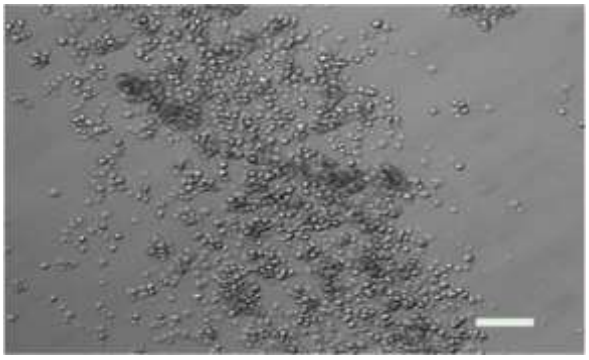

Fig. 3: Differentiated islet-like cells (bunches of grapes) after pre-differentiated neuron-like cells with H-DMEM serum-free medium supplemented with nicotinamide (10 mmol L^{-1}) and β-mercaptoethanol (2.5 mmol L^{-1}) for 10 h [scale bar 100 µm]

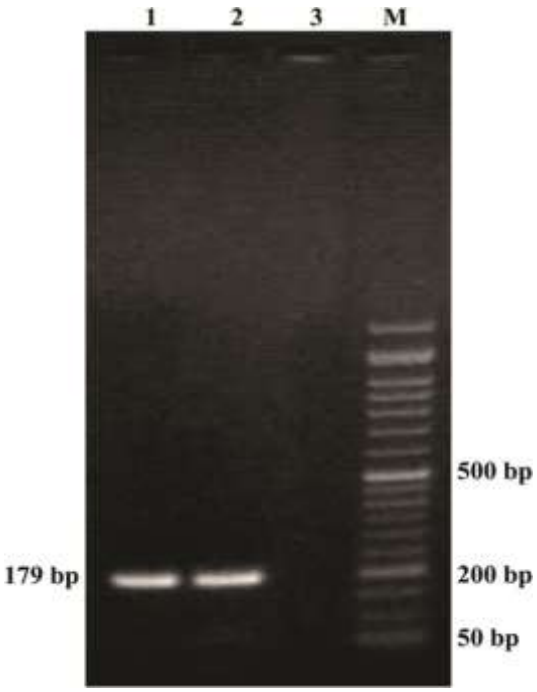

Fig. 4: mRNA expression profile of insulin gene of islet like cells; Lane M: Marker 50 bp DNA ladder, Lane 1: Islet-like cells (179 bp); Lane 2: Canine pancreatic tissue (+ve control); Lane 3: Canine bone marrow derived MSCs (-ve control)

Immunocytochemistry was performed to detect nestin and insulin expressions during various differentiation pathways of MSCs. The protein expression of nestin, which was considered to be an important pre-differentiation marker for islet-cells, was tested 24 h after induction in 1st differentiation medium. These pre-differentiated cells demonstrated nestin positivity (Fig. 5) whereas undifferentiated MSCs didn't exhibit any nestin positivity. Immunocytochemistry was carried out to to detect the expression of insulin in differentiated islet-like cells 24 h after induction in 2nd differentiation medium. These cells showed insulin positivity (Fig. 6) while undifferentiated MSCs and pre-differentiated islet-like cells didn't express insulin positivity.

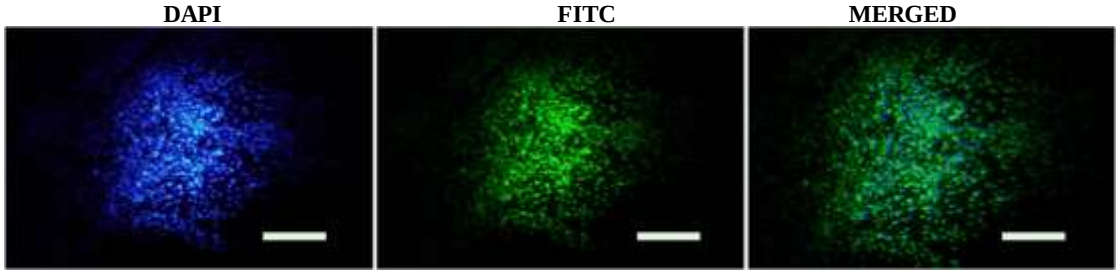

Fig. 5: Immunocytochemistry revealed nestin positivity in pre-differentiated islet-like cells

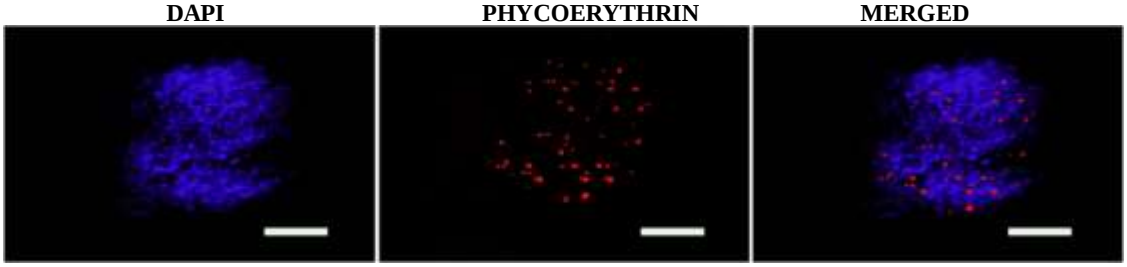

Fig. 6: Immunocytochemistry revealed insulin positivity in differentiated islet-like cells [scale bar 100 µm]

The insulin hormone concentration in culture supernatant was quantified by enzyme linked immune sorbent assay (ELISA) by using canine specific insulin ELISA kit. The insulin level in culture media of differentiated islet-like cells after 48 h of differentiation was significantly ($P_{0.05}$) high (1.84±0.37 ng mL^{-1}). The insulin level in culture medium of undifferentiated MSCs was meager (0.13±0.1 ng mL^{-1}).

MSCs, because of great multiplication potency and low immunogenicity, might be used for allogenic transplantation for cure of type1 diabetes. MSCs are multipotent and can differentiate not only to cells of mesodermal lineage like chondrocytes, adipocytes and osteocytes (Sheng, 2015) but also have the potential to trans-differentiate into pancreatic β-cells in rat and mice. *In vitro* trans-differentiation of MSCs into pancreatic β-cells is a very promising approach for the treatment of diabetes mellitus. Rat bone marrow derived MSC scould effectively be trans-differentiated into pancreatic β-cells by using high glucose and nicotinamide in culture medium (Zhang *et al.*, 2009). Nevertheless, the studies on differentiation of canine bone marrow MSCs into insulin secreting cells are very scarce. The only work available with respect to trans-differentiation of canine bone marrow derived MSCs into insulin producing cells was by transfection methods for *Pdx1*, *Beta2* and *Mafa* (Takemitsu *et al*., 2013). In our study, the protocol for trans-differentiation of canine BMSCs into insulin producing cells by chemical methods was optimized. Morphologically these cells are similar to rat islet-cells like bunches of grapes. Islet-cells expressed insulin both at mRNA and protein levels. Nestin positive cells are regarded as progenitors for pancreatic islets that resides within pancreatic islets and help in islet cell neogenesis (Zulewski *et al*., 2001). Nestin positivity in pre-islet cells observed in present study indicated that these cells can be differentiated *in vitro* into the cells that express pancreatic endocrine markers. Nicotinamide induces differentiation and maturation. High glucose promotes complete maturation of *Pdx1*, thus aiding in cell differentiation (Cao *et al*., 2004). Nicotinamide improves recovery from diabetes by stimulatory effect on islet cell neogenesis (Kim and Lee, 2016). Nicotinamide and β-mercaptoethanol act through notch signaling pathway by suppression of intracellular notch, thus promote pancreatic β-cell differentiation (Chelluri *et al*., 2011). To conclude, the study indicated that canine BMSCs could effectively trans-differentiate into islet-like cells which could express insulin gene and excrete insulin into culture media of islet cells. The results suggest that trans-differentiated islet-like cells could be used for type 1 diabetes therapy.

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Ethical Statement: The present study was conducted in compliance with the institutional ethical guidelines and after obtaining approval from the concerned bodies. Further, authors have no potential conflict of interest.

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