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RESEARCH ARTICLE

Isolation, expansion and Characterization of Canine Adipose derived Mesenchymal Stem cells (cADMSCs) from Omental tissue

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Abstract

Mesenchymal stem cells from omental fat were harvested from twelve adult clinically healthy dogs for isolation, expansion and characterization of canine Adipose derived Mesenchymal Stem cells (cADMSCs) *in-vitro*. Collected tissue samples were weighed and digested using collagenase enzyme to isolate cADMSCs. Cell yield and viability of the cells were calculated by using trypan blue exclusion test. Viable cells were seeded at a density of one million cells per T-25 culture flask and incubated in DMEM-HG, containing 10 per cent FBS, at 37°C under 5 per cent CO_2 tension. In the present study, the amount of cADMSCs collected in SVF was 3.46 X 10^6 cells per gram of omental adipose tissue. Flow cytometry analysis revealed that only 5 per cent cells were positive for CD34. Cells harvested at passage 3 were maintained in differentiation medium for an optimal time period of 21 days and their differentiation into chondrocytes and was observed by histochemical staining. Differentiated cells were positive for Toluidine blue with distinct cell morphology. These findings suggested that cADMSCs as a reliable source of adult stem cells which can differentiate into mesodermal lineages.

INTRODUCTION

In Veterinary medicine, stem cell based therapies gained more and more interest for the treatment of various musculo-skeletal disorders (Smith and Webbon, 2005). Adult stem cells, like all stem cells, can make identical copies of themselves for long periods (self-renewal) and can give rise to mature cell types that have characteristic morphologies and specialized functions (plasticity).

Mesenchymal stem cells have the ability to differentiate into varieties of connective tissues such as cartilage, bone, tendon, adipose tissue and muscle. By virtue of their cellular differentiation potential (Caplan and Bruder, 2001) and trophic effects (Caplan and Dennis, 2006) they are very much considered for clinical applications in canine (Kraus and Kirker, 2006).

Grzesiak *et al*. (2011) suggested that in ovine, buffalo, goat and human, fat tissues were easy obtainable source and had higher number of stem cell/progenitor cells. Adipose tissue was abundant, accessible and easy to

obtain from the body and also there is an increasing interest in adipose-derived stem cells (ADSCs) for tissue engineering (Ren *et al*., 2012 and Bourin *et al*., 2013).

Although some studies have been done to demonstrate the *in-vivo* potential of canine bone marrow–derived MSCs for tissue regeneration, immunogenecity, and gene delivery (Yamada *et al.,* 2004; de Kok et al., 2005) and there are little evidences on *in-vitro* characterization of cADMSCs from omental fat. Hence, the present study is designed to explore the isolation, expansion and charaterization of cADMSCs *in-vitro.*

Materials and Methods

Harvesting of Omental fat

The present experiment and protocol was approved by the Institutional Ethical Committee for Stem Cell Research, Tamil Nadu Veterinary and Animal Sciences University, Chennai. Omental tissue for the study was collected from 12 dogs of both sexes in the age group of 1 - 2 years, clinically healthy which were brought to the Small Animal Operation theatre for elective ovario-hysterectomy unrelated to this study after obtaining written consent from the animal owners. The animals were premedicated with atropine and xylazine at the dose rate of 0.02 mg per kg and 1 mg per kg respectively. Anaesthesia was induced using ketamine and diazepam at the dose of 5 mg per kg and 0.2 mg per kg respectively. The anaesthesia was maintained using inhalant anaesthetic, isoflurane. During surgery, a portion of omental fat tissue was identified and ligated at its base and then excised off using scissors.

The harvested omental fat was collected in a sterile 50 ml tube (Tarson's®) containing sterile phosphate buffered saline (PBS) with added antibiotic and antimycotic solution (100 units per ml of Penicillin, 100 grams per ml of Streptomycin, GIBCO®). The harvested omental fat sample was transported in sterile transport container.

Isolation of cADMSCs

Isolation was done by enzymatic digestion of Omental tissue using Collagenase type-I (Sigma, India) as reported by Neupane *et al.* (2008) in a sterile environment at Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College.

The tissue was digested using 0.075 per cent Collagenase type-I (Sigma®) for 30 minutes at 37°C in magnetic stirrer. Enzymatic activity was neutralized using Dulbecco's modified Eagle's medium-high glucose (DMEM-HG, GIBCO®) containing 10 per cent fetal bovine serum (FBS, GIBCO®). The infranatant was centrifuged at 1500 rpm for 10 minutes to pellet the cells. The resultant cell pellet was resuspended and washed two times in DMEM-HG with 10 per cent fetal bovine serum (FBS) at 1500 rpm for 5 minutes. The final pellet was resuspended in 1 ml of DMEM-HG with 10 per cent FBS. The cell viability and total cell density were determined by 0.1 per cent Trypan blue exclusion test (Viera *et al.,* 2010 and Stocchero and Stocchero, 2011).

Expansion of cADMSCs

Viable cells were seeded at a density of one million cells per T-25 culture flask and incubated in DMEM-HG, containing 10 per cent FBS, at 37°C under 5 per cent CO_2 tension. The spent medium was replaced with fresh medium after 72 hours and subsequent medium change was done on alternate days and plated at a density of 75,000 cells per T-25 culture flask. The cultures were cultured upto 3 passages to achieve a density of 70 to 80 per cent (Viera *et al.,* 2010 and Kisiel *et al.,* 2012).

Characterization of cADMSCs using CD markers

In the present study, characterization of ADMSCs at the level of P1 and P2 was done as per the protocol (Liu *et al.,* 2010 and Polisetti *et al.,* 2010) using CD34 primary antibodies. Flow cytometric analysis was performed on Becton, Dickinson FACS using a 488nm-argon-ion laser and 632nm red LASER for excitation. In the histogram, P1 is the unstained population and P2 is the stained population *(i.e)* positive percentage of cells for the specific marker (CD34).

Chondrogenic differentiation

The subcultured cells of passage 3 (P3) were plated at a density of 3×104 cells/ well in a 12 well plate with 1.5 ml of HG-DMEM and was allowed to get confluent. Then the medium was replaced by same volume of differentiating medium composing of basal medium (Chondrocyte differentiation Basal Medium (GIBCO®, Cat. No. A10069-01 and HiAdipoXL Basal Medium Cat No. AL521, HiMedia® (Part-A) supplemented with chondrocytic growth factors (Cat. No. A10064-01, GIBCO®) in the ratio of 10:1 and cells were incubated at 37°C and 5% CO_2 with change of medium in every three days.

After 21 days of induction, chondrogenic differentiation was confirmed by Toluidine blue staining. For staining, the cultures were fixed with 4% formaldehyde for 20 minutes at room temperature and then subjected to 0.04% Toluidine blue solution for 10 minutes to detect the presence of proteoglycans in the culture.

Result and Discussion

Weight of the sample and yield of cADMSCs

In the present study, the amount of cADMSCs collected in SVF was 3.46 X 10^6 cells per gram of omental adipose tissue revealing omentum as a rich source of ADMSCs over other sources such as submental (3600 CFU/g), arm (3800 CFU/g), pre-axillary (7100 CFU/g), gynaecomasta (5700 CFU/g), abdomen (5500 CFU/g), flank (5300 CFU/g), thigh (4300 CFU/g), knee (7100 CFU/g) (Zhang *et al*., 2012) and liposuctioned fat (3600 to 10700 CFU/g) (Black *et al*., 2007; Schäffler *et al*., 2007; Neupane *et al*., 2008 and Stocchero and Stocchero, 2011). The variation in weight of adipose tissue sample and yield of stem cells could be attributed to variations in donor particulars diversity and isolation procedure.

Sample No.	Weight (gms)	Yield (X 10^6 cells)
1	1.30	7.43
2	6.15	14.74
3	5.20	20.00
4	6.42	16.00
5	5.47	34.00
6	3.28	4.28
7	1.70	7.46
8	2.79	8.47
9	5.78	12.69
10	4.54	8.42
11	5.27	14.87
12	4.98	21.50
Mean ± S.E.	4.08 ± 0.34	14.13 ± 0.46

Table 1 Weight of Omental fat and yield of Stromal vascular fraction

Morphology of cADMSCs

Both serpiginous and fibroblast-like cells were observed in the primary culture (P0) in the present study (Fig.1). Both symmetric and asymmetric division of these cells was evident at low density (Tang *et al.,* 2001; Zulewski *et al.,* 2001).

Confluence of cADMSCs

In the present study, a confluence of 60 to 70 per cent was observed after nine days of incubation at 37°C under 5 per cent CO_2 tension. The cells were seeded at a density of 1 x 10^5 cells per T-25 culture flask. A double-fold increase was noticed after 7 days post-incubation. However, Neupane *et al.* (2008) reported that 5-6 days to reach confluence in subcutaneous fat and 11-12 days for the cells from omental fat.

Characterization of cADMSCs using CD marker

In the present study, flow cytometry analysis revealed that only 5 per cent cells were negative for CD34. Generally, the MSCs were negative for CD34 and haemopoietic stem cells were positive for CD34 (Vieira *et al.,* 2010; Martinello *et al.,* 2011 and Baer and Geiger, 2012). The predominant population of ADMSCs were positive for CD34 in the present study as they were located in the perivascular areas of the omental fat. Whereas, bone marrow mesenchymal stem cells are predominantly CD34 negative following isolation.

Differentiation of cADMSCs

After twenty one days of culture in chondrogenic differentiation medium, the cells were stained using Toluidine blue which showed typical metachromasia of cartilage and the cartilage differentiation was demonstrated by mucopolysaccharide rich ECM (Fig-2) as reported by Vieira *et al. (*2010) and Zuk (2013). The chondrocytic differentiation could be due to the genetic expression of Remax 2 (Chang *et al.,* 2013). The ADMSCs of ometal origin readily differentiated into chondrocytes revealing that the cADMSCs of omental origin are mesenchymal and could be used for clinical trials.

Diekman and Guilak (2012) stated that the viability, differentiation pathways were decreased when the cells were harvested from morbid, geriatric and obese animals. In the present study, omental fat was harvested from healthy, young animals reported for elective ovario-hysterectomy.

List of Figures and Legends

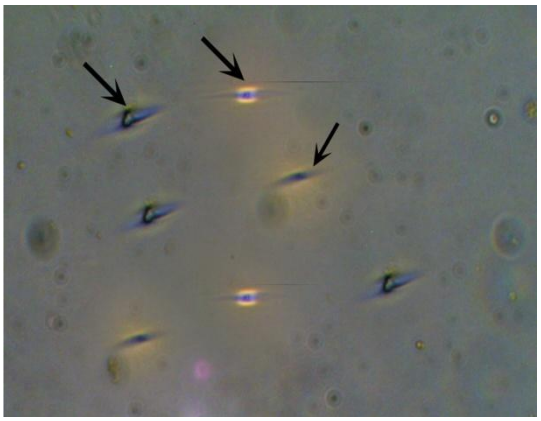

Fig-1 Photomicrograph showing Morphology of cADMSCs at P0 x 200

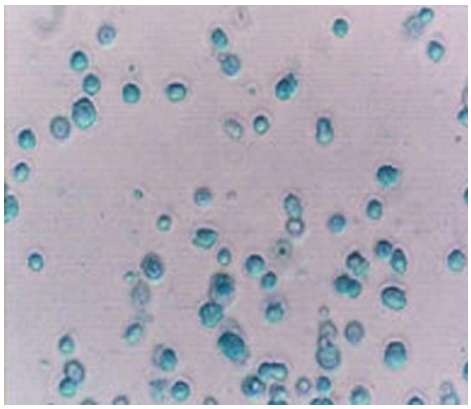

Figure-2 Photomicrograph of adult cADMSCs showing chondrogenic differentiation at P4 level (arrow) Toluidine Blue X200

Conclusion

Adult mesenchymal stem cells are considered as ideal option for cell based therapy. Yield of mesenchymal stem cells from the omental tissue is comparatively higher and the cells readily take-up chondrogenic differentiation revealing that ADMSCs of ometal origin was an alternate source of mesenchymal stem cells as piece of ometum could be collected from dogs subjected for ovario-hysterectomy. The advantages are the cells can be collected from young dogs and can be used as fresh isolates without cryopreservation.

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