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Evaluation of enriched canine hematopoietic progenitor cell colony forming potential

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Abstract
Hematopoietic stem cells (HSCs) are the well characterized adult stem cell that has been accepted for autologous cell therapy after minimal manipulation. The clinical use of HSCs to treat a variety of human and animal disorders has made them a key building block in the foundation of regenerative medicine. They represent a potential source of adult stem cells for transplantation in Veterinary Medicine. In the present study, Canine peripheral blood from apparently healthy animals were subjected for progenitor cells separation using an enrichment protocol and subjected for colony formation cell assay. The standardized simple protocol for progenitor cell enrichment and CFC assay would be applicable in clinical autologous cell therapy and can be used to analyze effects of drugs and biomolecules that induce erythropoiesis in canines as a model applicable in analysis of cell based clinical therapy.

Keywords: Hematopoietic Progenitor Cells (HPCs)-enrichment- *in vitro* - colony formation assay -analysis

Introduction
Hematopoietic stem cells (HSCs) represent the first identified and most well characterized adult stem cell (Denham *et al*. 2005) [3]. The clinical use of HSCs to treat a variety of human disorders and diseases has made them a key building block in the foundation of regenerative medicine. (George and Yuben 2013 and Smith 2010) [4, 13]. The pool of adult HSCs is required to support the hematopoietic system over the lifespan of human and animals are generated by the mesoderm during embryogenesis (Holyoake *et al*. 1999) [7]. The prevailing consensus about the cellular origin of primitive HSCs in vertebrates is that they are produced by a mesodermal precursor cell, called the hemangioblast, which gives rise to both hematopoietic and endothelial lineages (Choi *et al*. 1998) [1]. In the present study, Canine peripheral blood samples were used for analysis of the status of cell populations in dogs used for transfusion.

Materials and methods
The research work has been carried out as per the approval of the Institutional ethical committee for stem cell research and therapy N0.05/ICSCRT/2017 dated 11.12.2017 of Chairman, ICSCRT and Dean, Faculty of Basic Sciences, Madras Veterinary College, Chennai-7.

Isolation of canine peripheral blood mono nuclear cells
Blood samples obtained from apparently healthy animals from Clinics, Madras Veterinary College were processed (Boyum 1968) [2] with minor modifications. Blood samples were diluted with PBS in 1:2 ratio in 15ml centrifuge tubes. Diluted blood samples were laid very slowly in 1:2 ratio on lymphocytes separation medium (LSM).The cells were centrifuged at 400xg for 30 minutes at room temperature. The mononuclear cells (lymphocytes and monocytes) appeared as a hazy layer was carefully aspirated by a sterile pipette into another sterile tube. The cells were washed with PBS in 2 per cent FBS buffer solution at 1500 rpm for ten minutes. The supernatant was discarded and the pellet was resuspended with 1ml of RBC lysis buffer for 2-3 minutes over ice. It was centrifuged at 2500 rpm for ten minutes. Supernatant was discarded and the pellet was reconstituted in 1ml of culture medium.

Enrichment of progenitor cells
Enrichment of cells was done as per the manufacturers' instruction of Easy Sep™

(Stem cell technologies) with minor modifications to suit our needs. Enriched progenitor cells were counted in hemocytometer and tested for livability by Fluorescent di acetate staining.

Characterization of progenitor cells *in vitro* hematopoietic colony forming cell (CFC) assay

Methocult™ with cytokines (Stem Cell technologies) was thawed overnight under refrigeration, followed by shaking of bottle vigorously for one to two minutes. The bottle was allowed to stand for 15-20 minutes until bubbles rose to the top. Two aliquots of 3ml of methocult were used for duplicate studies. Hematopoietic suspension cells were diluted in culture media to prepare $1x10^6$cells/ml. 10X cell concentration was prepared for plating. About 0.3ml of diluted cells were added to a 3ml of methocult™ and vortexed for 4 seconds.1.1ml cell of content was dispensed into two 35 mm petridishes. Fresh sterile water was added to another uncovered petridish to maintain the humidity. Then all three petridishes were placed in another larger petridish and incubated at 37℃ in CO_2 incubator undisturbed for 14 days.

Counting of *in vitro* colonies

Colonies were counted at day 7 and cultures returned to the incubator until day 14, when colonies were counted again. The number of colonies derived from committed progenitors was obtained by subtracting the number of colonies scored at the day 14 from the number scored at day 7. The colonies present at day14 were scored and processed for secondary culture.

Replating of cells from primary *in vitro* colonies

Day 14 colonies harvested from methylcellulose cultures were resuspended in 200µl of culture media and cells were counted and replated in secondary methylcellulose cultures. Secondary colonies were scored at day 7 and 14 and processed for tertiary culture.

Results and Discussion

Animal model play an important role in evaluation of disease and clinical trials for research work (McCulloch *et al* 2005) [9]. Dog is a source of many well characterized homologues of human genetic disease which make them an ideal animal model to evaluate gene therapy protocols (Vats *et al*. 2005) [14]. Hematopoietic progenitor cells (HSCs) can self-renew and give rise to all the cells of the blood and the immune system. HPCs have self-renewal capacity and generate lineage-restricted multipotential progenitor cells that in turn give rise to mature cells (Kennedy *et al*. 1997) [8]. Hematopoietic progenitor cells (HSCs) are a rare population of cells residing in the bone marrow (BM) and continuously replenish all mature blood cells throughout the life span (Nayan, *et al.* 2010) [10]. The Colony Forming Unit (CFU) assay is most commonly used to detect multipotential and lineage-restricted progenitors of the erythroid, granulocytic and macrophage lineages (Golde, D.W., and M. J. Cline *et al*. 1978).

Methylcellulose based media is commonly used to promote the optimal growth and differentiation of hematopoietic progenitor cells (Nissen-Druey *et al* 2005) [11]. Hematopoietic stem cells are an extremely rare population in the marrow and so the identification of hematopoietic stem cells in the marrow is still challenging (Pereira *et al* 2007) [12]. The functional assays, including *in vitro* colony-forming assay and *in vivo* serial dilution transplantation assay, are used to determine stem cell activity. A recent study indicates that the combination of several cell-surface markers can be used to identify hematopoietic stem cells (Wognum *et al*. 2010) [15].

In the present study, isolation of CD34 positive cells were carried out using a rapid easy sepmat protocol. Canine PBMCs were fractionated according to density and were then separated by CD34 positive selection and subjected to CFU assays. The results showed that the initial number of colonies formed between days 7–14 from 3–5 significantly decreases while the size of colonies were increased.

Cells from primary cultures generated on both day 7 and day 14 were replated. Secondary culture day 14 colonies still contained the cells with day 7 colonies. (Fig1). However, the size of colonies and clonogenic ability of the cells decreased progressively from primary to tertiary culture. Later, the colonies were disappeared and showed diffused morphology.

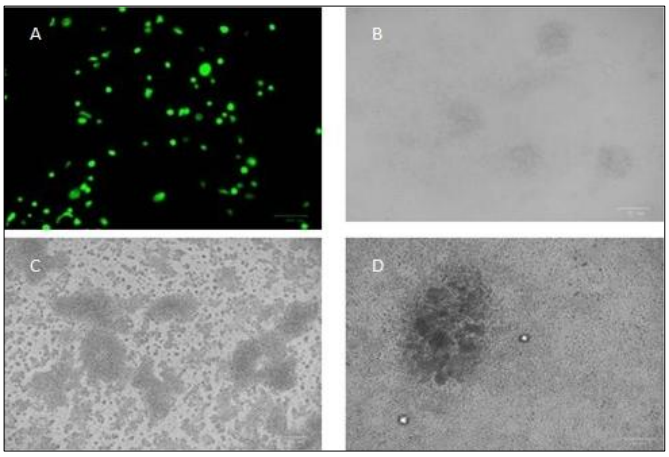

Fig 1: Canine blood derived CD34 positive cells *in vitro* process-CFU assay

A. Fluorescent di acetate stained CD34 positive cells (100µm scale bar)
B. Blast like colonies in methocult colony assays 3rd to 5th day of colonies (87µm scale bar)
C. Blast like colonies in methocult colony assays 7th to 10th day of colonies (100µm scale bar)
D. 14th day representative colony showing expansion (100µm scale bar)

Table 1: Details of the colony formed in *In vitro* CFU assay (N= 8)

S. No	Colonies in Primary cultures		Colonies in Secondary cultures		Colonies in Tertiary cultures	
	Number	Size (µm)	Number	Size (µm)	Number	Size (µm)
7th Day	19 -22±0.03	7.7-19.9±0.21	7-10±0.19	5.4-8.2±0,32	3-5±0.1	4.2 -2.6 ± 0.24
14th Day	9-12±0.17	20.2-42.5±0.21	5-8±0.24	4.5- 2 3±0.25	2-4±0.19	3.2.-1.8±0.19

* The values represented with mean±SE

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