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Culture Methods for Isolation of Canine Hair Follicle Stem Cells (CHFSC)

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ABSTRACT

Adult stem cell models are important tools interacting in tissues, replacing animal experimental studies for understanding the basis of physiology and therapeutics. In this study, we aimed to isolate and culture stem cells from canine hair follicular tissue *in vitro* by three methods viz, explants cuture, microdissection and filtrate culture methods. Dispase II enzymatical digestion gave higher cell yield ($3.08 \pm 0.47 \times 10^6$) than trypsin/EDTA enzymatical digestion of canine skin samples. Filtrate culture method gave high cell yield among three different culture methods. In microdissection method, colony forming unit (CFU) and colony size of cells from isthmus fragment were 14.71 ± 1.57 in numbers and 11.21 ± 2.45 mm^2 respectively which was significantly higher than other fragments of hair follicle ($P<0.01$). We concluded that the dispase II enzyme digestion and filtrate culture method was the best method to isolate cHFSCs.

Key words: Stem cells, Canine, Hair follicle, Micro-dissection, CFU, Yield

Hair follicle is the only organ in the mammalian body which contains intrinsic stem cells and undergoes cyclic transformations (from periods of organ regeneration to rapid growth) for its entire long-life time. Liu *et al.* (2003) described that the putative epithelial stem cells in the hair follicle bulge were thought to play pivotal role in the homeostasis, aging and carcinogenesis of the cutaneous epithelium.

Schmidt-Ullrich and Paus (2005) suggested that the hair follicle was functioning as the ''bone marrow of the skin'' and continuously remodeled its cutaneous microenvironment including skin innervation and vasculature. The epidermis contained two subpopulations of progenitor/stem cells: basal keratinocytes and cells residing in the bulge region of the human hair follicles. These cells ensured the maintenance of adult skin homeostasis and hair regeneration (Drewa, 2008).

Gho *et al.* (2004) and Hung *et al.* (2015) isolated Hair Follicle Stem Cells from both human scalp biopsies and plucked hairs by using dispase (20U/ml) in defined serum free medium as a digestion enzyme at 37°C, in CO_2 incubator for 30 minutes and hair follicle explants were cultured in defined serum free keratinocyte medium (dSFK) with 500 µg/ml penicillin and 0.25 µg/ml streptomycin, expressed positive for CK19, approximately one in fifth of the follicular cells in 14th day culture and these positive cells were commonly found as cell clusters. cultured Hair follicle stem cells were cultured from human scalp skin pieces, digested in 12.5mg/ml dispase for 24 hours at 4°C and cell suspension was made by treating the plucked hairs twice in 0.25 per cent trypsin/EDTA solution Yu *et al.* (2006). Gilanchi *et al.* (2014) performed explant culture for mouse hair follicle stem cell culture.

Rochat *et al.* (1994) and Maleki *et al.* (2014) in human Kobayashi *et al.* (2010) in canine dissected the hair follicle from the skin tissue and then carefully cut into four fragments: the bulb was designated as P1; the lower intermediate fragment as P2; the upper intermediate fragment as P3 and uppermost fragment containing sebaceous glands as P4 and then incubated them in collagenase – dispase for a short period to facilitate the dissociation of dermal sheath and epithelial core. It was then teased with needle and fragments were put into trypsin to obtain single cell suspension. The cells were seeded on 3T3 feeder cell layer and attained confluency in 11th day culture.

The cultured hair follicle stem cell colonies from rat vibrissa were well formed around their central explants within a few days and appeared small, round, 4-7µm in size and frequently detached within few days. Commonly the cells were morphologically variable and more compactly arranged (Renolds and Jahoda ,1991). The average size of the P3/isthmus colonies were 1.6-fold larger than that of P1/ bulb colonies which were small and irregularly shaped. P3 colonies were predominantly composed of uniform compact cells with smooth and circular perimeters (Ã3mm^2), suggested that they were derived from "holoclones". Keratinocytes of P3 fragment and isthmus region were passaged for 7.7±0.7 generations (Kobayashi *et al*., 2010).

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In keratinocyte culture, the bulge keratinocytes yielded larger colonies than those from other skin sites in mice (Tumbar *et al.*, 2004; Morris *et al.*, 2004). Blanpain *et al.* (2004) cultured FACS isolated follicular cells and their populations formed appreciable numbers of tightly packed, large colonies (Ã20 mm^2; Ã10^4 cells) containing cells of small size and relatively undifferentiated morphology. They were referred as holoclones, such colonies were clonally derived from single stem cells (Barrandon and Green, 1987). This present work was aimed to study the efficacy of these culture methods in isolation of cHFSCs from canine hair follicle.

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 and was processed in the Department of Veterinary Anatomy, in collaboration with Centre for Stem Cell Research and Regenerative Medicine, Madras Veterinary College, Chennai-07. Canine skin samples were collected in Normal saline (50ml) with 20µl Povidone Iodine then changed into DPBS with antibiotic-antimycotic solution. Collected samples were transferred to petriFig.s and washed with 2 per cent chlorhexidine for three minutes and with 70 per cent isopropyl alcohol for one minute as per Hibbard *et al*. (2002). Excessive subcutaneous fat was removed by scraping the dermal aspect of the skin and thoroughly washed with Dulbecco's phosphate-buffered saline (DPBS) with Antibiotic-antimycotic solution. Samples should be processed within 12 hours of collection for efficient cell yield.

Hair follicles were separated from canine skin by digesting the tissue in Dispase II and also with with trypsin/EDTA enzyme as per Kobayashi, *et al*. (2009). After digestion, skin pieces were placed in petriFig.s with DPBS and the epidermis was carefully separated from the dermis. Hair follicles were separated from dermis and inter follicular area of the epidermis and rinsed with DPBS. Canine hair follicle explant culture method was done as per Hung *et al.* (2015). Hair follicle explants were incubated with William's E medium or DMEM supplemented with 10 per cent fetal bovine serum, and 10ng/ml of epidermal growth factor, 5ng/ml of insulin growth factor, two per cent ABAM and one per cent amphotericin B in collagen coated culture Fig.s in a humidified atmosphere at 37°C with 5 per cent CO_2 for 14 days to obtain the cHFSC colonies.

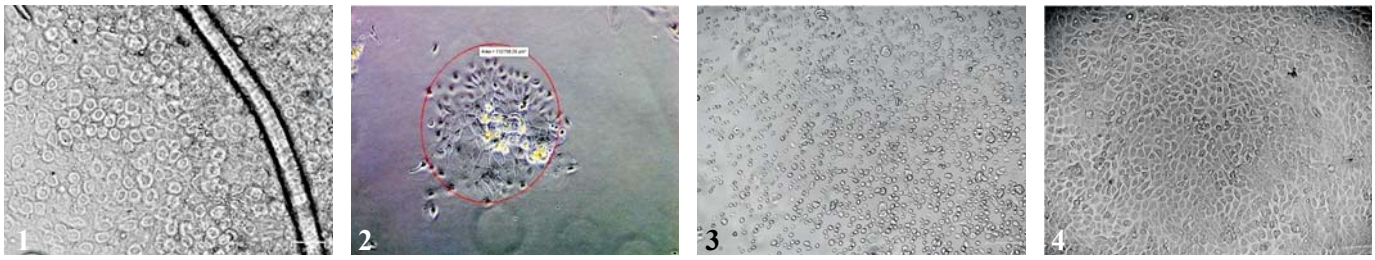
Canine hair follicles were microdissected into three parts: upper, middle and lower fragments using stereo zoom microscope. The upper fragments contained the infundibulum part of the hair follicle, middle fragments contained the isthmus portion (bulge) of the hair follicle and the lower part contained bulbar and supra bulbar portion of the hair follicle and digested with trypsin/EDTA and incubated in the above media for 14 days. Colony forming units (CFU) and colony size were measured in each fragment culture. The statistical significance of differences was determined by independent student's t test using SPSS software. $P<0.05$ was considered significant.

In filtrate culture method, to prepare single cell suspension, hair follicles were placed into 0.25 per cent trypsin/EDTA solution for digestion at 37°C in CO_2 incubator for 15-20 minutes. Trypsin/EDTA was neutralized by adding serum and follicular cells were dissociated into single cell by gentle pipetting repeatedly. Isolated follicular cells were filtered through 100µm cell strainer and centrifuged at 5000rpm for three minutes. Cell pellet was resuspended and cell counting was done by using trypan blue dye exclution test (Bhatt, 2011). Isolated follicular single cell suspension was cultured in the growth medium on collagen coated culture dishes in a humidified atmosphere at 37°C with five per cent CO_2. Culture medium was changed every three to four days. Primary culture was maintained until 1seven to eight days to obtain the colonies. The statistical significance of differences was determined by One-way ANOVA using SPSS software. $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

In this study, canine hair follicles were separated from dermis by digesting the skin samples in dispase II enzyme which had concentration of 2.4U/ml in DMEM as a working solution. The digestion was completed within two hours in the CO_2 incubator or overnight in 4°C while Kobayashi *et al*. (2009) used dispase II at the concentration of 1500U/ml which was varied in this study. A similar combination of enzymes had been used by Gho *et al*. (2004) and Yu *et al*. (2006) in human scalp tissue to separate hair follicles. However, the concentration of dispase II enzyme was 20U/ml and 12.5mg/ml respectively and the digestion time was extended upto 24 hours at 4°C. In the present study, efficacy of the two enzymes was determined by the effective isolation of viable hair follicle stem cells from canine hairs. Out of the two enzymes, Dispase II digestion produced significant number of cell yield and viable cHFSCs *i.e* $2.76 \pm 0.26 \times 10^6$ and $1.59 \pm 0.16 \times 10^6$ than trypsin/EDTA digestion.

Explant culture method: The Outgrown cells from explant were observed round in shape for first few days of culture which was similar to the findings of Renolds and Jahoda, (1991) in rat HFSCs. The outgrown cells from hair follicle explant culture were round and slabstone-



Figs. 1-4. 1. Photomicrograph of canine hair follicle explant culture on 14 day showing outgrowing cells with less nuclear: cytoplasmic ratio scale bar 31 µm; **2.** Photomicrograph of microdissected hair follicle cultured cells derived from F2- isthmus fragment showing colony forming unit (circle) on day seven × 100; **3.** Photomicrograph showing heterogenous cell population in single cell suspension on day one of primary culture scale bar = 100 µm; **4.** Photomicrograph of cHFSCs showing 90-100 percent confluency after seven days of primary culture scale bar = 100 µm.

shaped appearance with high refraction Zhang *et al*. (2006); Hung *et al.* (2015). Cell migration was observed from explant culture on day 3 and on 5th day of culture cell number was increased and confluent at day 14 and displayed cobble-stone morphology and were closely packed as described by Hilmi *et al*. (2013) in human and He *et al*. (2016) in goat (Fig. 1).

Microdissection method: In this method, the attachment of F1 and F2 fragments of hair follicles was observed on the third day of culture and cells in the culture Fig.s were found to be outgrown from F1 and F2 fragments on day 3-4. However the outgrowth of cells from F3 fragment was not appreciable. The outgrown cells (cHFSCs) were commonly round in shape in first few days. But, after forming the colony, the cells possessed flat, cobblestone shaped appearance and cells were closely packed (Fig. 2). Colonies were predominantly formed from F1 and F2 fragments. The cHFSCs from F2 fragments were observed to form circular colonies with large cells in the middle and smaller cells distributed in the periphery. F1 fragments formed irregular colonies. The mean diameter of colonies derived from F2 fragment was 11.21 ± 2.45 mm^2 while that of colonies derived from F1 fragment was 1.80 ± 0.30 mm^2. The size of colonies derived from F2 fragment was significantly larger than that of colonies derived from F1 and F3 fragments (P Â0.01). Colony forming unit (CFU) was assessed by counting the colonies which were formed by both F1 and F2 hair fragments. cHFSCs from F2 fragment formed approximately 14.71 ± 1.57 number of CFU while cHFSCs from F1fragment formed 6.67 ± 0.88 number of CFU.

Thus, the number of colonies formed by F2 hair fragment was 2.2-fold higher than that of the F1 and F3 hair fragments. The cHFSC colonies from middle fragment of hair follicle were 3.7 folds larger in size than the bulb colonies. This finding was comparatively higher than the previous reports of Kobayashi *et al*. (2010) who measured that the average size of the isthmus fragment colonies were 1.6-fold larger than that of bulb fragment colonies.

Fitrate culture method: In line with previous studies (Zhang *et al*., 2006) in human, Kobayashi *et al*. (2009) in canine and He et al. (2016) in goat) the cHFSC in filtrate culture showed heterogenous population on first day of culture (Fig. 3) and adhesion of cells on day two of culture and proliferation started from third day and colonies were noticed from fourth day onwards. Cultured cHFSC were flat in shape with short cytoplasmic processes which contained round nucleus with two or more dense nucleoli and had a less nucleus: cytoplasm ratio in early days of primary culture. The nucleus and cytoplasmic ratio was increased in cells which were located in the middle of the colony on day seven of the culture (Fig. 4). 90-100 percentage confluency was observed at this stage and were used for further passages. Sieber-Blum et al. (2004) showed that the cultured mouse HFSCs had stellate morphology with long processes after 72 hours of culture. By two weeks of culture, this type of colony consists of thousands of cells and constitutes 83.0 ± 2.7 per cent of all colonies and the remaining 17 per cent of colonies were small, made up of flattened cells. cHFSCs were subcultured upto passage 3 level. Cell yield and viable cell count were performed in all passages.

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