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Isolation and Characterization of Mesenchymal Stem Cells from Canine Ovarian Surface Epithelium

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ABSTRACT

Background: Few studies have confirmed the presence of ovarian tissue stem cells indicating the capacity for differentiation. Based on this fact, it was hypothesized that mesenchymal stem cells (MSC) were found in ovarian surface epithelium (OSE) of canines that could easily be isolated.

Methods: Both left and right ovaries were minced and digested using collagenase to obtain a stromal vascular fraction (SVF). MSCs were characterized using RT-PCR. To ascertain the trilineage differentiation potential, MSCs were stained with respective stain for osteocytes, chondrocytes and adipocytes.

Result: We observed elongated, spindle-shaped and fibroblast like appearance of cells after 72 h of initial culture. Expression of MSC specific surface markers were observed through RT-PCR. Using Stem Pro® differentiation medium, OSE were differentiated into osteogenic, chondrogenic and adipogenic lineages and were found to be potential source for isolation, characterization and differentiation of MSCs. Canine (OSE) is easily accessible, multipotent and has high plasticity, holding promise for applications in regenerative medicine.

Key words: Canine ovarian surface epithelium, Mesenchymal stem cells, Ovariectomy, Stromal vascular fraction, Trilineage differentiation.

INTRODUCTION

The ovary in mammals is surrounded by ovarian surface epithelium (OSE), a single layer of epithelial cells. These cells are originated from the peritoneal mesothelial cells. The mesothelial cells goes differentiation and convert mesenchymal cells into epithelial cell type by remodeling of extracellular matrix (Salamanca *et al.,* 2004). Ovarian surface epithelium is attached poorly to the underlying stromal layer and can easily be detached by mechanical means. Ovarian surface epithelium plays an active role in follicular rupture, oocyte release, subsequent ovarian remodeling, and repair of follicle walls. It covers only a certain area in a functional ovary and gets disrupted by regular ovulatory processes whereas, during anovulatory cycles and in non-functional ovaries, the entire surface is covered with ovarian surface epithelium (Auersperg *et al.,* 2001). In dog, stem cell kinetics are more similar to those of humans than are those of mice or other laboratory animals, making the dog highly relevant for cell biology studies (Horn *et al.,* 2004). The most important reason that enforce us to study on canine ovarian tissue is that these tissues are frequently available in veterinary clinics during ovary-salpingo-hysterectomy surgery and the ovaries are often discarded as biological waste.

MATERIALS AND METHODS

Ovarian tissue processing and culture of mesenchymal stem cells

The study was conducted at Stem cell laboratory, Department of Veterinary Clinical Complex (VCC) and Department of Veterinary Gynaecology and Obstetrics, Faculty of Veterinary and Animal sciences, West Bengal University of Animal and Fishery Sciences, 37, KB Sarani, Kolkata, West Bengal, India during June 2019 to February 2020. Both ovaries of healthy female dog were collected aseptically during ovario-hysterectomy procedure at VCC of West Bengal University of Animal and Fishery Sciences, Kolkata, India. Ovaries were collected in a falcon tube and washed repeatedly with DPBS (Sigma, USA) to remove

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blood cells. It was then minced (1-2 mm in size) with surgical scalpel inside the biosafety cabinet. The sample tissues were digested with 500 µg/ml collagenase II for 60 min at 37°C temperature. The ovarian cells were then filtered through 70 µm cell strainer and grown in T75 flasks in a humidified incubator at 37°C and 5% CO_2. Cells were grown in DMEM-LG medium for 7-8 days before passaging and every 48 h intervals cells were refeed with fresh medium. Microscopic observations were performed regularly to ensure cell confluency.

Characterization of MSCs

Expression of stem cell surface protein analysis using RT-PCR

Total RNA was isolated using TRIzol reagent (Ambion, USA) and then subjected to cDNA synthesis using reverse transcriptase as per the manufacturer's protocol (Thermo Fisher Scientific, USA). PCR was prepared in twenty microliters of reaction mixture containing 2 µl of cDNA, 200 µM of dNTPs 0.5 µM of forward and reverse primers and 1.25 U of Taq polymerase. Mesenchymal stem cell specific gene PCR was performed for CD73, CD44, CD34, CD90, CD45 and CD105 while GAPDH was selected as internal control. PCR for oct4, nanog and sox2 was done for evaluation of pluripotency markers expression. PCR profile was set in the following order; 95°C for 5 min, 95°C for 30 s, 58°C for 30 s, 72°C for 30 s followed by 72°C for 5 min. The PCR products were resolved in 2.0% (w/v) agarose gel electrophoresis and images were captured in a gel documentation system (Bio-Rad, USA).

Trilineage differentiation

Mesenchymal stem cells (MSCs) were grown in StemPro medium for differentiation into osteogenic, chondrogenic and adipogenic lineages (Thermo Fisher Scientific, USA). Cells were cultured for 14 to 21 days and regularly observed under microscope for any morphological changes. Alizarin red S (Sigma, USA) was used for staining of osteogenic differentiated cells which detect calcified extracellular matrix deposits (Zajdel *et al.* 2017). Culture medium was removed and cells were washed with phosphate buffer, followed by fixation with 4% (w/v) formaldehyde solution. Then, the cells were washed with distilled water and stained with 0.5% alizarin red s (pH 4.1- 4.3). After incubation for 8-10 min at room temperature and cells were observed under phase contrast microscope. For chondrogenic differentiation, cells were incubated with alcian blue (1%) staining solution for 30 min. Then, cells were washed with 0.1 N HCl followed by rinsing with sterile distilled water and observed under phase contrast microscope (Ogawa *et al.* 2004). Similarly, oil red o (Sigma, USA) staining was done to determine the lipid droplet formation for the determination of adipogenesis (Neupane *et al.* 2008). After fixation, cells were incubated in staining solution for 30 min. The cells were then washed with sterile distilled water to remove excess stain and observed under phase contrast microscope.

RESULTS AND DISCUSSION

Isolation and propagation of canine ovarian mesenchymal stem cells

Canine ovary beyond its primary properties *i.e.* (production of ova) is considered as waste after optional ovariohysterectomy in the veterinary hospitals. Herein we provide evidence that MSCs can be isolated from canine ovarian surface epithelium. Literatures confirm that mesenchymal stem cells are present in various part of our body like adipose tissue, cord blood, fallopian tube, peripheral blood, and fetal lung and liver, amniotic membrane *etc.* and those all play a vital role in tissue repair (Erices *et al.,* 2000; Romanov, 2003; Johnson *et al.* 2004; Dominici *et al.,* 2006; Stimpfel *et al.,* 2014; Mandal *et al.,* 2021). However, the presence of mesenchymal stem cells in ovarian tissue is poorly understood and hence, we conducted the study

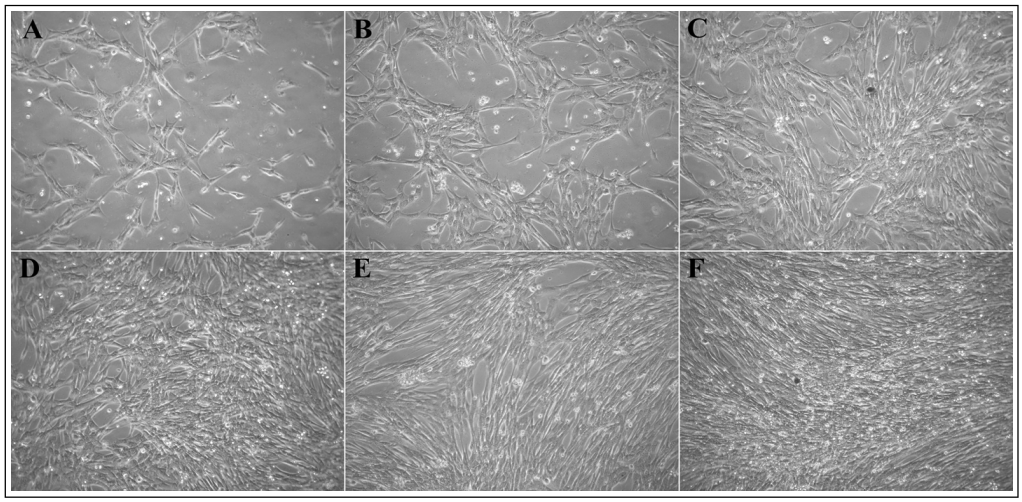

Fig 1: Canine Ovarian surface epithelium derived primary culture. A-C. Cells from passage zero were captured at day 2, 4 and 6. D-F. 70-80% confluent monolayer cells from passage one to passage three.

on canine to confirm its existence and differentiation ability. Ovarian tissues were collected aseptically from operation theatre and brought to the stem cell laboratory for MSCs culture. Under appropriate growth condition, cells reached approximately 20%, 40% and 80% confluency after 2, 4 and 6 days of initial culture. Fibroblast like adherent spindle shaped cells growing in a monolayer were observed, a typical morphology of MSCs (Fig 1 A-C).

These cells were passaged every six day's interval to keep them viable and to grow for longer period of time. Passage 1, 2 and 3 cells were very similar in shape, size and other phenotypical characteristics (Fig 1D-F). The results of this study showed that cells present in canine ovarian surface epithelium morphologically similar to fibroblasts. After 72 h of initial culture cells had elongated and spindle-shaped structure (Fig 1), which are specialized phenotypic features of mesenchymal stem cells (Haasters *et al.* 2009).

Analysis of surface marker expression for characterization of ovarian MSCs

RT-PCR analysis have given significant information for MSCs characterization. The result revealed that the MSCs specific positive markers CD44, CD73 and CD105 were expressed well compare to CD90 (Fig 2). Pluripotency marker nanog expressed remarkably in the cultured cells while the expression of another two marker sox2 and oct4, is slightly lower (Fig 2). Several reports showed that MSCs can be isolated from various types of tissues and organs (Wang *et al.* 2004; Uezumi *et al.* 2006), but some differences exist between them. Different studies have shown diverse level of gene expression and differentiation potential for MSCs depending on their tissue source (Kern *et al.* 2006; Wagner *et al.* 2007). However, when the most important MSCs markers were examined, such as CD73, CD105, CD44, CD90 all were expressed in respective of tissue sources. In our study also expression of specific markers like CD73, CD44, CD105 and CD90 has confirmed the presence of MSCs in canine ovarian surface epithelium. Negative expression of cell surface markers CD34 and CD45 further established the presence of MSCs in ovarian tissue (Fig 2). The outcome of this study is very similar with the previous reports (Yaneselli *et al.* 2018; Hill *et al.* 2018).

As shown in RT-PCR result, positive expression of pluripotency markers; nanog, oct4 and sox2 (Fig 2) had ensured the ability of the stem cells to undergo self-renewal and may give rise to all types of cells of a living body depending on the types of induction. Stemness of cells can be supported through the detection of the above mentioned pluripotency associated transcription factors (Kerr *et al.* 2008).

Differentiation of ovarian MSCs into osteogenic, chondrogenesis and adipogenic lineages

Osteogenic induced cells has specified notable information about the gradual changes in cells morphology. After 21 days of incubation,unique polygonal shaped, crystalline structures were found during microscopic observation (Fig 3A). Deposited calcium crystals in extracellular matrix were stained through alizarin red s which produced reddish-brown coloration of those calcified bodies (Fig 3B). This has given significant indication of successful osteogenic differentiation.

During chondrogenesis, micromass formation in cultured cells was clearly observed and after 14 days of induction larger size micromasses were observed in respective induced cells (Fig 3C). After staining with alcian blue, those micromasses were turned into bluish-green (Fig 3D) which indicated the presence of sulphated proteoglycans in the matrix of the cell. This data confirmed the successful chondrogenesis in this *in vitro* experimental study.

Similarly, adipogenic differentiated cells were characterized by the formation of lipid droplets in cytoplasm of flat, large and wide cells. In our study, presence of a few

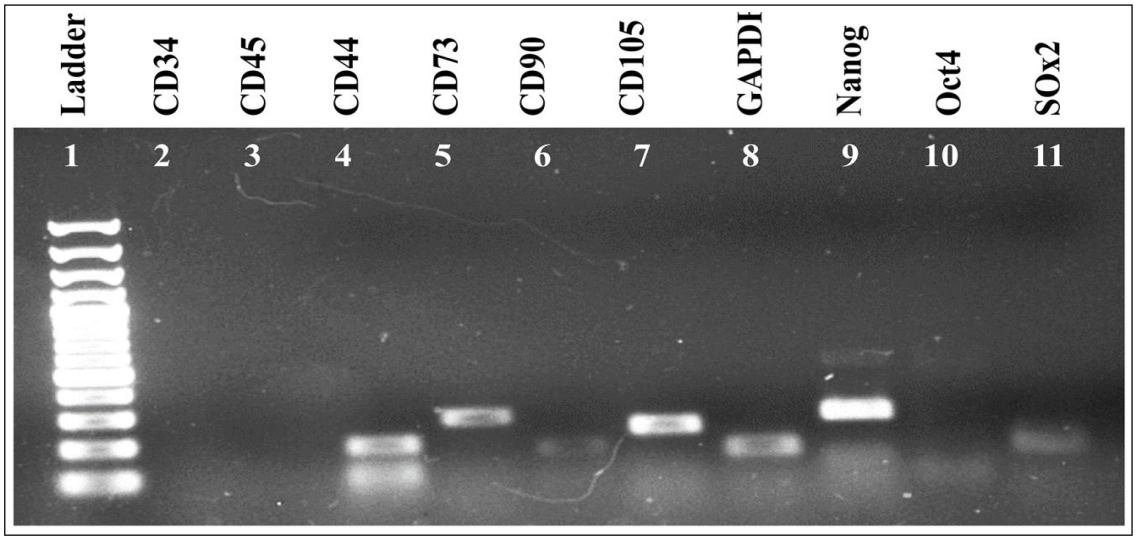

Fig 2: RT-PCR analysis of MSCs specific positive markers CD44, CD73, CD90, CD105 have been shown in lane 4-7. Lane-2-3 MSCs specific negative markers; CD34 and CD45. Lane-8 GAPDH used as internal control. Nanog, Oct4 and Sox2; pluripotent marker have been shown in lane 9-11.

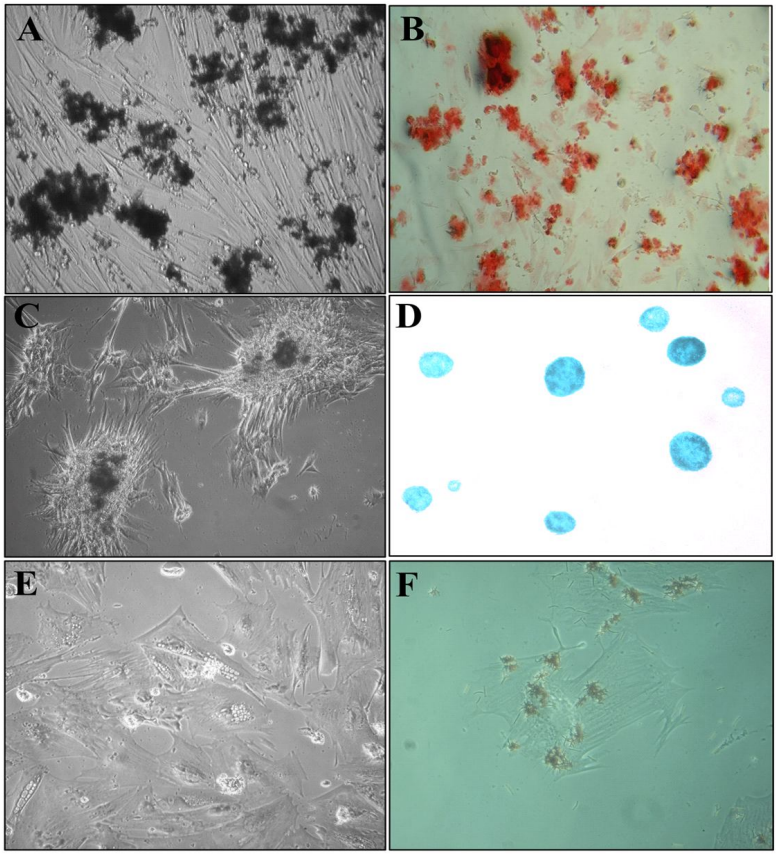

Fig 3: Differentiation of ovarian derived MSCs. A. Small black crystalline structures were observed at 21 days after osteogenic differentiation. B. Deposited crystals were stained with alizarin red S stain which showed reddish-brown coloration. C. Micromass formation in cultured cells was clearly observed after 14 days of chondrogenic induction. D. Alcian blue staining for aggrecan bodies during Chondrogenic differentiation showed bluish-green coloration. E. Presence of a few glossy, spheroid lipid droplets was observed after 21 days of adipogenic induction. F. With oil red o staining, deep brown colouration of those lipid bodies were observed.

glossy, spheroid lipid droplets was observed after 21 days of adipogenic induction (Fig 3E). Through oil red o staining, deep brown colouration of those lipid bodies were observed which confirmed the occurrence of adipogenesis (Fig 3F).

The results of our study also indicated that cells in the ovarian surface epithelium are capable of differentiating into osteogenic, chondrogenic and adipogenic cell lineages, that is one of the main characteristic feature of MSCs (Johnson *et al*. 2004). Calcium deposition has provided a proof for osteogenic differentiation. Aggrecan mediates chondrocyte-chondrocyte interaction and formation of micromass detected by staining confirmed the chondrogenic differentiation. Again, detection of lipid droplets in adipogenic differentiated cells further proven presence of MSCs in canine ovarian surface epithelium (Fig 3). Our result corresponded to the analytical studies of Johnson *et al*. (2004), Dominici *et al*. (2006) and Stimpfel *et al*. (2014).

CONCLUSION

We observed *in vitro* cultured cells from ovarian surface epithelium have plastic adherence property, trilineage differentiation potential and expressing MSC specific surface markers. These properties confirmed that the ovarian surface epithelium of canines contain mesenchymal stem cells.

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Authors' contributions

Ajeet Kumar Jha and Anirban Mandal conducted lab work, prepared manuscript, analyzed and interpreted all the data. Kalyani Ray and Shyamal Kanti Guha guided throughout the study and conducted surgical work. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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