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Enrichment and Short Term Culture of the Ovine Gonocyte

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Abstract: The aim of this study was to investigate the effects of two types of ovine testis cells population as feeder cell on *in vitro* culture of the enriched ovine gonocytes. The feeder cell populations were prepared from 5-6 months old ovine testis. The 1-2 months old neonatal rams were used to isolate germ cells through a two step enzymatic digestion followed by differential plating for SSCs enrichment. Isolated and enriched cells were characterized by using PLZF and VASA antibody. During the 1st week of culture, gonocyte formed pairs and chains of type A spermatogonia. After 1 week, colonies started to increase in size. About 2 weeks later, more colonies in type II feeder group kept undifferentiated and looks more effective regarding colony formation of spermatogonia compared with type I feeder group in the ovine spermatogonial stem cell culture system.

Key words: Ovine, gonocyte, cell culture, immunostaining, spermatogonia, Australia

INTRODUCTION

Spermatogenesis is a complex, highly organized process that originates from Spermatogonial Stem Cells (SSCs). Spermatogonial stem cells have the potential to self-renew. The regulation of the SSCs self-renewal process is not yet completely understood. SSCs is the only cell type in the adult body that mitotically divides and passes its genetic material on to the next generation, making it an attractive target for genetic manipulation. Development of SSCs culture system will make it possible to genetically manipulate SSCs. Then, the genetically manipulated SSCs could be used for direct germline transmission of modified genomes and expansion of technology to produce genetically modified livestocks (Oatley and Brinster, 2006; Kanatsu-Shinohara *et al*., 2008).

To study the culture of SSCs, enough populations of pure SSCs must be isolated. In the bovine neonatal and peripubertal testes before the onset of spermatogenic differentiation, the seminiferous tubules contain a relatively larger proportion of spermatogonial stem cells (Izadyar *et al*., 2002; Herrid *et al*., 2007). Therefore, it has been expected that the testes from young rams are likely to be more suitable for the isolation of SSCs. The previous study has described that the testes from 1-2 months old ram was most suitable material and the differential plating was appropriate enrichment strategy for ovine gonocytes or SSCs isolation (Borjigin *et al*., 2010). In studies on spermatogonial stem cell isolation, purification and culture, the availability of markers that can conclusively establish the identity of the spermatogonia is essential. Application of c-Kit marker for spermatogonia isolation resulted in the selection of more differentiated spermatogonia than spermatogonial stem cells (Shinohara and Brinster, 2000). In contrast, isolation of mouse spermatogonia on the basis of α-6 and β-1 integrin markers resulted in an enriched population of spermatogonial stem cells (Shinohara *et al*., 1999). Until 2006, the SSCs could only be identified by colony formation after testis germ cell transplantation (Kubota and Brinster, 2006). As type A spermatogonia marker for bovine, DBA has been reported not to bind to any type of cell in prepubertal ovine testis (Rodriguez-Sosa *et al*., 2006). PGP 9.5 has been used to identify type A spermatogonia in ovine testis (Rodriguez-Sosa *et al*., 2006; Herrid *et al*., 2009). In 2010, Promyelocytic Leukaemia Zinc-Finger (PLZF) was used as a specific marker for gonocytes and spermatogonial stem cells in ram testis (Borjigin *et al*., 2010). Various methods have been employed to enrich spermatogonia in rodents and large animals. Vit. A-deficient mice (McLean *et al*., 2002) and rats (Van Pelt *et al*., 1996) and artificially induced cryptorchid animals (Shinohara *et al*., 2000) can provide a relatively enriched source of spermatogonia. Enrichment of SSCs can rely on the relative surface properties of different types of testicular cells. For example, the somatic cells adhere more strongly to a plastic surface than other types of germ cells (Herrid *et al*., 2006; Luo *et al*., 2006). The process of removing other types of testicular cells from a suspension of mixed cells via incubation in plastic containers coated

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with extracellular matrix substances is called differential plating and this technic become potentially more practical to enrich spermatogonia from testes of the large animals. Within the testis, the renewal mechanism of SSCs is still not completely understood. One important factor which enhances SSCs renewal is Glial cell line Derived Neurotrophic Factor (GDNF) (Meng *et al*., 2000). GDNF has been associated with SSCs renewal *in vivo* (Yomogida *et al*., 2003) and *in vitro* situations (Kubota *et al*., 2004; Nagano *et al*., 2003). In the seminiferous epithelium, sertoli cells secrete GDNF and the receptors for this growth factor, GFR-alfa 1 and c-ret (Oatley *et al*., 2004; Tadokoro *et al*., 2002).

Based on above observations we used 1-2 months old ram to isolate and enrich gonocytes and studied the short-term culture of ovine spermatogonial stem cells by adopting differential plating technic and using PLZF as ram gonocytes or undifferentiated type A spermatogonia marker. The researchers investigated the effects of two type of feeder cells on primary development of SSCs and hypothesized that type II feeder cells contain more sertoli cells and will stimulate the proliferation and increase the number of ovine SSCs *in vitro*.

MATERIALS AND METHODS

Animals: Merino rams were selected to provide testis samples. The neonatal period (1-2 months old) and prepubertal period (5-6 months old). Three animals were used for each age group. Animals were handled and treated according to the guidelines of the Animal Ethics Committee at Inner Mongolia University, Hohhot.

Collection of testis samples: Rams were castrated under general anaesthesia (0.1 mg kg^{-1} xylazine followed by 3 mg kg^{-1} ketamine). Antibiotics and an analgesic/anti-inflammatory were administered postoperatively. After castration, the testes were washed in Dulbecco's Phosphate-buffered Saline (DPBS; Sigma-aldrich). The tunica albuginea, epididymides and excess connective tissue were removed and testis weight was recorded.

Enzymatic isolation of testis germ cells and enrichment of gonocytes by differential plating: A two-step enzymatic isolation and differential plating procedure was used to obtain individual testicular cells and enrich gonocytes as described by Borjigin *et al*. (2010). Briefly, testis tissues were placed in a tea strainer and ground with a 5 mL syringe plunger. The remaining tubule section was incubated with collagenase (1 mg mL^{-1}; type IV; Sigma-Aldrich) in a shaking water bath at 37°C. During this time, the tissue samples were frequently monitored under microscopy and the reaction was stopped when individual tubules were observed then treated with trypsin (2.5 mg mL^{-1}; Gibco-BRL) in PBS for 5-10 min at 37°C. DNAse I (7 mg mL^{-1}; Sigma, St Louis, MO, USA) in DMEM was added 1 min after trypsin treatment. An equal volume of heat-inactivated fetal bovine serum (FCS; Invitrogen) was used to inactivate the trypsin digestion. The resultant cell suspension was then filtered though a cell strainer and centrifuged at 1500 g for 5 min at room temperature. The pellets were resuspended in 10 mL DMEM containing 5% FBS at a density of 40-80×10^6 cells mL^{-1}. Cell viability was assessed by Trypan blue exclusion.

For differential plating, fifty million cells were added per cell culture flasks (125 cm^2; Nunc, Roskilde, Denmark), resulting in a final density of 0.4×10^6 cm^{-2}. Following 2 h incubation at 37°C. The cell suspension was centrifuged at 400 g for 5 min at room temperature and resuspended in 10 mL DMEM/F12+5% FBS. Thirty million cells were added to a new cell culture flasks (75 cm^2; Nunc) that were treated with 0.2% gelatin in PBS for at least 1 h at 37°C and then incubated overnight in DMEM+5% (16 h). Thus, the final incubation density was 0.4×10^6 cm^{-2}. The collection process was the same for the 2 h enrichment.

Double fluorescent staining of the isolated or enriched cells smears: Smears were prepared from freshly isolated cells or enriched cells and dried on a warm stage at 37°C. The slides then were cooled at room temperature for 30 min and wrapped in foil and stored at -80°C until staining. Prior to being stained, slides were brought to room temperature before being unwrapped to prevent condensation. Smears were fixed in Bouin's solution for 2 min, rinsed thoroughly with TBST then incubated with a mixture of mouse anti-PLZF (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-VASA (1:10; Abcam, Cambridge, UK) for 30 min. After incubation, slides were rinsed in TBST and a mixture of chicken anti-rabbit Alexa fluor 488 (1:200; Invitrogen) and goat anti-mouse Alexa fluor 594 (1:200; Invitrogen) in 0.5% Bovine Serum Albumin (BSA) in TBS was applied for 30 min before slides were again rinsed in TBS and mounted in Prolong gold (Invitrogen).

Prepare feeder cell: Type I feeder cells were prepared from two-step enzymatic isolation of 5-6 months old ovine testis cells. Type II feeder cells were obtained from attached cells of the 2 h differential plating. Feeder cells were plated in flasks coated with 0.2% gelatin in a DMEM/F12 (Invitrogen) supplemented with 20% FBS (Gibco) and expanded over two to seven passages. Cells were then cryopreserved for future use or plated in dishes coated with gelatin (for subsequent passages) at 0.4-1.0×10^6 cells per 35 mm dish. The cells were treated with mitomycin-C (10 μg mL^{-1}; Sigma-Aldrich) for 2-3 h and washed twice with PBS then cultured in DMEM/F12+10% FCS.

***In vitro* culture of the ovine gonocytes:** Enriched ovine gonocytes were seeded in prepared culture dishes with type I or II feeder cells in DMEM/F12+10% FCS at a concentration of 1000 cells mL^{-1} and then cultured at 37°C in a humidified atmosphere with 6% CO_2. The media were changed every 2 days. The cell culture lasted 15 days depending on the experiment.

Immunocytochemistry and double fluorescent staining of the cultured cells: After 15 day's culture, cells were fixed in Bouin's solution for 2 min, a standard protocol (Herrid *et al*., 2007) was followed for each antibody. Antigen retrieval was performed on cells by heating in 0.01 M citrate buffer (pH 6) for 10 min on the high setting in a microwave and then cooling for 30 min. Cells were then permeabilised for 5 min in Tris-buffered Saline (TBS) with 0.01% Triton X-100 (Sigma-aldrich) and then rinsed twice in TBS (pH 7.5). Cells were treated with 0.6% (v/v) H_2O_2 (Merck, Darmstadt, Germany) for 10 min to inhibit endogenous peroxidase and were subsequently rinsed in TBS. In order to block non-specific adhesion sites, cells were incubated in TBS containing 0.05% Tween 20 (TBST) for 30 min before antibody incubation. Cells were then incubated with mouse anti-PLZF for 30 min. Cells were then rinsed thoroughly three times with TBST for 5 min each time and incubated with EnVision (DakoCytomation; Dako) for 30 min at room temperature. Specific staining was visualized using diaminobenzidine kits (Vector Laboratories, Burlingame, CA, USA). Mouse immunoglobins (Sigma-aldrich) instead of primary antibodies as negative controls with replacement of the secondary antibody with buffer regarded as a further negative control. All incubations were performed in a moist chamber. For fluorescent staining, cells were fixed in Bouin's solution for 2 min, rinsed thoroughly with TBST and then stained with PLZF and VASA. The antibody concentration and procedures were the same as for isolated or enriched cells double fluorescent staining.

RESULTS AND DISCUSSION

Enrichment of the gonocytes population by differential plating: Different cell types in the initial isolation samples and supernatants collected from differential plating were analyzed by fluorescence double staining with PLZF and VASA (Fig. 1). During the process of enrichment, the

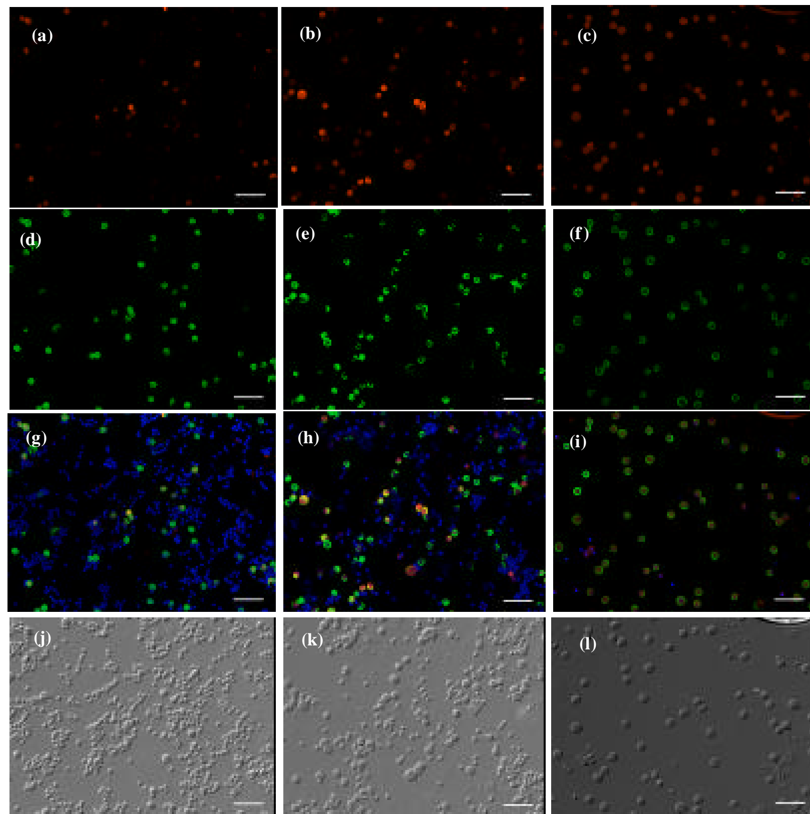

Fig. 1: Evaluation of the efficiency of enriching gonocytes by fluorescence double staining of PLZF and VASA (a: Initial isolated cells, b: 2 h enriched cells, c: 2+16 h enriched cells). Red indicates PLZF-positive cells, green indicates VASA-positive cells (d-f). Blue indicate 4'-6'-diamidino-2 phenylindole staining of the nuclei. Merged picture of the PLZF and VASA (g-i). Phase contrast micrographs of the stained cells (j-l). Scale bar = 50 μm

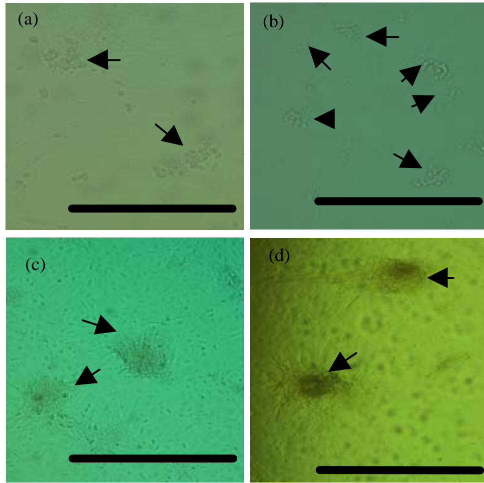

Fig. 2: Ovine SSCs culture on type I and II feeder at day 7 and 15th. Phase contrast micrographs; a) type I feeder culture at day 7th; b) type II feeder culture at day 7th; c) type I feeder culture at day 15th and d) type II feeder culture at day 15th. Arrows indicate ovine SSCs colonies. a, b) Scale bar = 200 μm; c, d) Scale bar = 500 μm

proportion of VASA and PLZF double positive cells increased significantly and final enrichment got 80% pure gonocytes.

Culture of ovine SSCs on the two kind of feeder cells: The researchers cultured enriched ovine gonocytes on type I or II feeder cells in DMEM/F12+10% FBS media for a short term. Gonocytes attached to the dish after 1-2 days and began to grow at 3-4 days. SSCs colonies appeared around a week. Type A spermatogonia were present in the form of small clones and later on colonies. Small clones comprised of pairs (Apr) or chains (Aal) of type A spermatogonia (3-64 cells, Fig. 2a, b). The colony number per 35 mm disk in sertoli feeder group (37±2.3) were significantly more than that in stromal feeder group (20±2.6). Spermatogonial colonies aggregated into clumps of spermatogonia and cell proliferation appeared plocoid. At day 10th of culture spermatogonial colonies increased in size and became round bird nest-like shape. Cells look more compactly in central area. Small size colonies look more transparent and during increasing size, SSCs colonies became darker and some colonies onwards morphologically resembled the radial colonies (Fig. 2c, d). About 2 weeks later, more colonies in type II feeder group kept undifferentiated and shown more health morphology.

Immunocytochemistry and double fluorescent staining of the cultured cells: After 14 days culture, SSCs colonies and chains of spermatogonia appeared to be strong PLZF-positive but feeder cell is negative to PLZF staining (Fig. 3). The researchers used trypsin to digest mechanical separated SSCs colonies and made smears then did immunocytochemistry and double flourescence staining. Immunocytochemistry results show that about 30% cells derived from type II feeder group appeared PLZF-positive but only <10% cells derived from type I feeder group appeared PLZF-positive. The results shown that type II feeder cells were more suitable for ovine SSCs culture than type I feeder cells. Double fluorescence staining results shown that PLZF-VASA double positive cells were detected from SSCs colonies in both groups but VASA and Vimentin double staining nearly did not show Vimentin-positive cells whereas most of the cells were VASA-positive (Fig. 4).

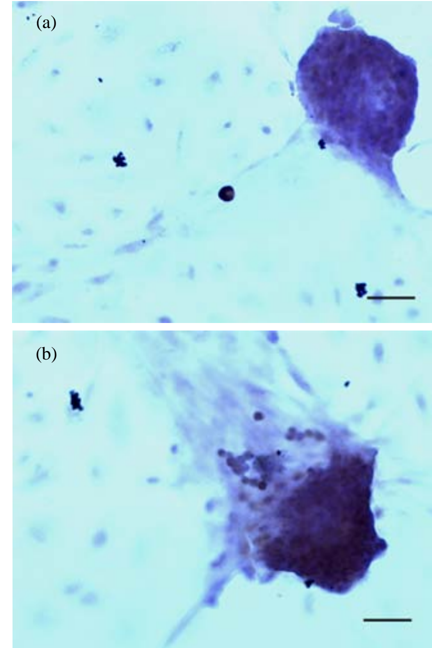

Fig. 3: PLZF staining of the ovine spermatogonia colonies on two different feeders; a) type I feeder and b) type II feeder. Scale bar = 50 μm

A panel of antibodies was used to determine the number and proportion of spermatogonia and somatic cells isolated enzymatically from tubules and then validated by reference to fixed tubule sections. Antibodies against DBA and PGP 9.5 were used to label spermatogonia whereas antibodies against Vimentin and GATA-4 were used to identify sertoli cells and leydig cells (Wrobel, 2000). The expression of DBA lectin has been identified in bovine and porcine gonocytes and type A spermatogonia (Wrobel *et al*., 1995; Izadyar *et al*., 2002; Herrid *et al*., 2007) and has been used in enriching SSCs by MACS (Herrid *et al*., 2009) and FACS (Izadyar *et al*., 2002; Herrid *et al*., 2007, 2009). In addition, the binding of DBA to bovine type A spermatogonia has been used to investigate the fate of transferred cells in mouse testis

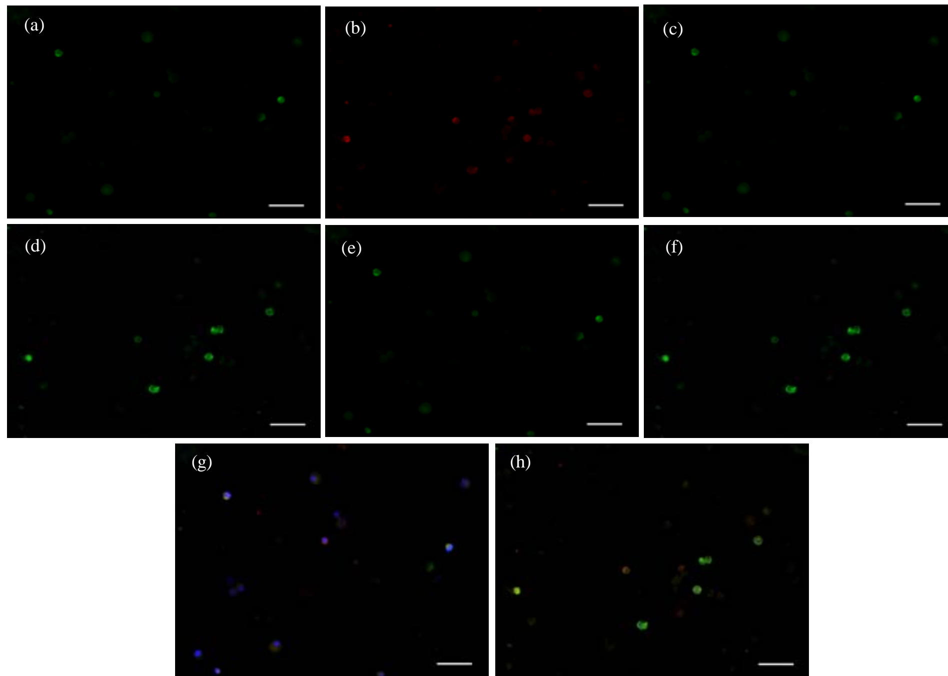

Fig. 4: PLZF and VASA double flourescence staining of the cells from ovine spermatogonia colonies from type I feeder (a) and type II feeder (b). Red indicates PLZF-positive cells (a, b), green indicates VASA-positive cells (c, d), blue indicate 4'-6'-diamidino-2-phenylindole staining of the nuclei (e, f). Merged picture of the PLZF and VASA double staining (g, h) Scale bar = 50 μm

after xenotransplantation because murine testicular cells do not express DBA (Zhang *et al*., 2008). One study attempted to test DBA expression in prepubertal ovine testis cells but got inconclusive results (Rodriguez-Sosa *et al*., 2006). The previous results (Borjigin *et al*., 2010) confirmed that DBA lectin does not react with any type of ovine testicular cell at any stage of development. Therefore, it will be useful to identify a unique marker (s) for ovine type A spermatogonia for identification purposes if xenotransplantation is used to establish a functional assay system for ovine SSCs.

With the development of the testis, total germ cell numbers increase progressively due to the increased number of more advanced germ cells. Therefore, isolation of tissues from neonatal ram testes is expected to yield relatively higher numbers of gonocytes or SSCs due to the lack of an advanced germ cell population in the seminiferous tubules. Differential plating of testis cells is a simple method to enrich spermatogonia by removing the majority of somatic cells and incubation periods of 2-4 h (Dirami *et al*., 1999; Hofmann *et al*., 2005) or overnight (Izadyar *et al*., 2002) are commonly used in different species to deplete testicular somatic cells. In the experiments, the researchers carried out 2 h quick differential plating and following overnight (about 16 h) differential plating on the gelatin coated flask to maximize the number of somatic cells attaching to the culture dish. Results of isolation efficiency showed that most PLZF-VASA double positive cells in the tubular sections were recovered in the cell suspensions from neonatal testes and final enrichment got >80% pure gonocyte.

The present results provide a detailed description of the culture and development of neonatal ovine gonocytes on two types of testis cells population. During 1st couple days spermatogonia started to proliferate and formed pairs and chains of type A spermatogonia. Clone formation was observed and colonies of spermatogonia were found to appear from day 5th onwards. Then colonies were increased as clusters of type A spermatogonia of at least 64 cells, this result is similar to bovine spermatogonia culture (Aponte *et al*., 2006). Many spermatogonia showed morphological signs of apoptosis and resulted in an initial decrease in spermatogonial numbers. After day 7th, the decline in spermatogonial numbers slowed down. Then the colonies started to form mixed aggregates of loosely connected spermatogonia and most advanced germ cell. These colonies continued to grow and condense and become round bird nest-like

shape. The colony numbers declined during the culture, very likely because some of the initial colonies disintegrated as the percentage of small colonies decreased from day 10-15. However, the remaining surviving colonies increased in size. Regarding the morphology and cell differentiation stage of SSCs colonies, type II feeder cells were more suitable to serve as feeder layer than type I feeder cells in ovine gonocyte culture system. The present study rendered a primary method to culture and harvest ovine spermatogonial colonies in a basic culture. This will provide a basis for further studies such as the effects of specific growth factors secreted by the testis somatic cells likely favor self-renewal of SSCs during *in vitro* cultures.

CONCLUSION

Isolation efficiency showed that final enrichment got >80% pure gonocyte. Type II feeder cells were more suitable to serve as feeder layer than type I feeder cells in ovine gonocyte culture system.

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