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In-vitro propagation of pre-pubertal bovine Spermatogonial Stem Cell in prepation for transplantation

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ABSTRACT

Backgroud: We investigated the expression of stem cell markers in *in -vitro* cultivated speramatogonial stem cells (SSCs) with the intention of developing a useful system to produce progeny of SSCs.

Materials and Mehtods: Testes from 3, 5 and 7 months old calves were utilized to examine isolation, purity and *in –vitro* propogation of SSCs. Cells were isolated and purified with two-stemp enzymatic digestion combined with centrifugal separation on discontinuous Percoll density gradient layers.

Findings: Cell propagation and SSCs marker expression were determined at 5, 10 and 15 days post-culture. Immunostaining in conjunction with transcrioption based marker expression of cultured cells showed that stem cell markers (DBA, UCHL1, Oct4 and Sox2) were expressed in SSCs.

Conclusion: The results demonstrated marker expression of bull spermatogonial stem cells and showed that prior to transplantation *in-vitro* culturing of bull SSCs is implementable.

Keywords: Bovine; *in-vitro* propogation; Pre-pubertal; SSCs; Testis

INTRODUCTION

Adult spermatozoa production is depended on single cell self- renewal and differentiation, located in the basment membrane of seminiferous tubules called spermatogonial stem cell (SSC) (He et al., 2015; Kaavya et al., 2016; Olive et al., 2015; Ryu et al., 2004; Schulz & Miura, 2002; R. Zhang et al., 2016; Zhao et al., 2016; Zheng et al., 2014). SSCs transplantation in rodents by Brinster & Zimmermannt (1994) opened a new insight for germ cells technology application (Schlatt, 2002). One, such as transgenic progeny production, has already been achieved through SSCs genetic modification (Shinohara et al., 2004; Seandel et al., 2007). For that reason, SSCs could be a great genetic resource. However, compared to other testicular cells in testes, the number of SSCs is remarkably lower; 2 in 1000 testicular cells are SSCs (Khaira et al., 2005), comprise only 0.3% of germ cells (Aponte et al., 2005). Nevertheless, transplantation requires large quantity of cells; therefore, prior to transplantation, *in-vitro* culture is an important practice. *In- vitro* progeny production techniques have mainly been developed for mouse SSSs (He et al., 2015). Recent studies have shown, *in-vitro* SSCs could propagate and maintain pluripotent (Dobrinski and Travis, 2007; He et al., 2015; McLean et al., 2001; Yamada et al., 2016; Zhao et al., 2016). Despite, the methods has been developed for bovine SSCs, none of them have been recommended it for long-term culture. (Fujihara et al., 2011). Cultured bovine germ cells, gonocyte could proliferate until 1.5 months. However, SSCs were able to make colonies for a week. In addition, Aponte (2008) proposed a culture technique based on growth

factors. The results presented somatic cells, required for germ cells proliferation. Furthermore, functional proliferation of *in-vitro* SSCs transplanted into infertile testes have been reported in rodents (Shinohara et al., 2003) and porcine (Kim et al., 2008). In addition, cultured SSCs from neonatal mouse (Shinohara et al. 2004), adult mouse (Seandel et al., 2007) and humans (Conrad et al., 2008) have given rise to embryonic stem cell-like cells. An important essay of *in - vitro* SSCs detection is expression SSCs specific markers. One such is DBA used to classify and identify spermatogonia from neonatal calves testes (Herrid et al., 2007; Izadyar et al., 2002) at differentiation point of development (Manku & Culty, 2015). Moreover, the binding of UCHL1 (Ubiquitin carboxyl- terminal hydrolase deubiquiting enzyme) (Vansandt, 2014), known as protein gene product 9.5 (Zheng et al., 2014) expresses in cytoplasm of SSC is also a general marker for type A spermatogonia in bull (Reding et al., 2010). Sox2 and Oct4, which are critical factors for maintaining the pluripotency of stem cells and their synergetic action controls another pluripotency factor Nanog (Shi & Jin, 2010). In any case, in spite of a few endeavors to culture male germ cells in animals species counting cattle (Izadyar et al., 2003; Oatley et al., 2004; Aponte et al., 2006) have not been successful and recommended for long term culture.

We aimed to build a practical system to generate progeny of SSCs by investigating expression of stem cell markers in- vitro cultured SSCs. To this end, we examined expression of different markers specific for stem cells, further we cultured SSCs from pre-pubertal testis. SSCs ability of proliferation after culture was evaluated by expression of markers.

MATERIALS AND METHODS

Testes from 3, 5 and 7 months old calves were collected in Agriculture and Forestry Research Center at University of Tsukuba and National Institute of Livestock and Grassland Science, all the animal work was performed under the approval of Institutional Animal Care and use Committee of the University of Tsukuba (approval no. 18-397). Immediately, after castration, the testicles transported to laboratory within 1 – 2 hour in phosphate buffer saline (PBS) containing 50 iu/ml penicillin – streptomycin (Gibco USA, 1864865), on ice. Approximately 20 g of the testicular tissue used for each cell isolation and culture process.

Isolation, purification and culture of SSCs

Single SSC was isolated and purified, using two-step isolation and discontinuous Percoll density layers purification as described by (Izadyar et al. 2002) with minor modification. In brief, after de capsulation, testicular tissues were minced in DMEMF12 containing 10 % FBS (Biowest, S15064s1870), 50 iu/ml penicillin – streptomycin (Gibco USA, 1864865), 40 iu/ml gentamycin (Sigma aldrich, 125174778V), and 1.5 mg/ml collagenase IV, 2 mg/ ml hyaluronidase type II, 1.5 mg/ml trypsin and 30 µlg/ml DNase I (Worthington, X6A16385) enzymes. Then the suspension containing testicular sample digested at 37 C^{o} in shaking water bath, 122 cycle/ min for 60 minutes. After removal of interstitial cells by centrifugation at 80xg for 5 minutes, the testicular fragments suspended in DMEMF12 as mentioned and enzymatic digestion was performed as above for 45 minutes. Germ cells separated from seminiferous tubules fragments with centrifugation at 30Xg for 2 minutes and filtration with 70-µlm nylon cell strainer. To pellet germ cells centrifugation at 1500xg for 5 minutes was performed. Further purification was performed with discontinuous Percoll density layers of 20%, 30%, 40%, 50% and 60%. Isolated cells purity was assessed by immunocytochemistry for SSCs markers. Cells were stained with DBA-biotin (1:200 µll) diluted in PBS for 15 minutes. Positive cells in 100 cells were counted. 1 x 10^{5} cells/ ml

were seeded in DMEMF12 supplemented with 10% FBS (v/v), 10 ng/ml EGF (Discovery Labware, Inc, 354001), 40 iu/ml gentamicin and 50 iu/ml penicillin – streptomycin and the medium was changed every two days.

Immunohistochemistry of cultured spermatogonial stem cells

After 5, 10 and 15 days in culture, cells were immunohistochemically analyzed for DBA, Oct4, UCHL1, and Sox2 described by Fujihara et al. (2011) with some modification. Briefly, after two washes with PBS, cells were fixed in 4 % parafarmaldehyde for 30 minutes at room temperature, then washed with PBS, non– specific binding block was performed using 10% goat serum (v/v made in PBS). Then, the cells were rinsed in PBS and stained with primary antibodies against DBA biotin (Abgent, 1:200 µl), UCHL1 (Abgent, 1:300 µl), Oct4 (Cell Signaling Technology 1:400 µl) and Sox2 (Cell Signaling Technology, 1:200 µl) (v/v in PBS) kept at 4 C° overnight. After washing twice with PBS, cells incubated with corresponding secondary antibodies Neutra Avidin Fluorescence FITC – conjugated (1:500 µl), goat anti mouse IgG (173667, 1:300 µl and texas red anti mouse IgG H+L (vector, made in horse 1:300 µl) for 90 minutes at room temperature. Analyzed under fluorescence microscope (Leica Microsystem, Germany).

Expression of spermagonia specific markers in cultured SSCs

To examine expression spermagonia specific marker UCHL1 and stemness factors Sox2, Oct4 in cultured SSCs, total RNA was extracted from the cells cultured using TRI Reagent Sigma (T9424). 800 µl of the reagent was added to cells, homogenized with disposable homogenizer (Nippi BioMasher Series Linup 891300). After being centrifuged at 2000 xg for 10 minutes at 4 C^O, DNA was dissociated by incubating the supernatant at room temperature for 5 minutes and biochemical phase separation was performed. To isolate RNA, Chloroform (0.1 x TRI reagent volume) was added, mixed with vigorous shaking, and then incubated for 5 minutes at room temperature. The mixture was centrifuged at 12000 x g for 15 minutes and the top part containing RNA was taken and mixed with iso – propanol (50% of TRI volume). After incubation for 5 minutes at room temperature, the cocktail centrifuged at 12000 x g for 10 minutes. Samples were washed twice with freshly prepared 75% ethanol (7500 x g centrifugation for 5 minutes at each wash), and pelleted RNA was placed under a fume hood until it dried completely. Then, the extracted RNA diluted with DNase and RNase free water (Millipore, F1AH74109) and RNAs amount was measured with Nanodrop lite spectrophotometer (Thermo Scientific). To amplify DNA, the 100 ng of extracted RNAs (14 µll) were mixed with 1 µll dNTP Mixture (TaKaRa) and 1 µll Oligo dt20 primer (Sigma). For RT-PCR, samples incubated for 5 minutes at 65 C^O and kept on ice. For reverse transcription, 4 µll 10X RT buffer (TaKaRa), 1 µll H2O (Millipore, F1AH74109) and 1 µll MMLV high performance reverse transcriptase (NIPPON GENE) were added and were incubated at 42 C^O for 50 minutes then 70 C^O for 15 min. RT - PCR amplification was carried out on 1 µll of the above cDNA solution per 19 µll PCR reaction mixture containing LA Taq HS, 10 × LA PCR Buffer II (Mg 2+ plus), dNTP Mixture (TaKaRa Hot Start version RR006A kit, TaKaRa BIOTECHNOLOGY) and H2O. They were mixed well and centrifuged briefly prior to PCR.

The following primers used for amplification of specific genes: Source: (http://bioinfo.ut.ee/ primer3-0.4.0/).

Table 1. Primers for amplification of germ cell marker genes

Genes	Forward	Reverse (5’ -3’)
UCHL1	GTCCCGGTCAAGAAACAAAA	TCTGGAACCAGGTCTTCACC
Oct4	GTTTTGAGGCTTTGCAGCTC	CTCCAGGTTGCCTCTCACTC
Sox2	GTTTGCAAAAGGGGGAAAGT	GAGGCAAACTGGAATCAGGA

The PCR product separated and visualized on 2% agarose gel electrophoresis containing 10 µll GelRed (Nucleic Acid Gel Stain, biotium.com). All PCR products were sequenced to confirm identity.

Statistical Analysis

The results are presented as mean ± SEM and statistical analysis was performed with single factor one-way ANOVA followed by Tukey HSD test for differences between means. Differences were considered significant when the P value was 0.05.

RESULTS

Cells isolation and purity

The antibody used in this study reported to identify spermatogonia at isolation time in many species including cattle. Isolated spermatogonial stem cell purity was assessed by comparing number of positive cells in total 100 isolated cells obtained from each discontinuous Percoll density gradient layer. Throughout different age of calves, spermatogonia enriched in fraction 40% Percoll density gradient was significantly higher than in others **(Fig. 1).** Interestingly, the average number of positive cells recovered in 40% Percoll gradient from 3 month was, 37.3 ± 3.7 from 5 months old 31.5 ± 0.8 similar to 7 months 32± 0.8. These positive cells manifested a typical morphology of SSCs: round shape, dense nucleus and with high nuclear cytoplasmic ratio.

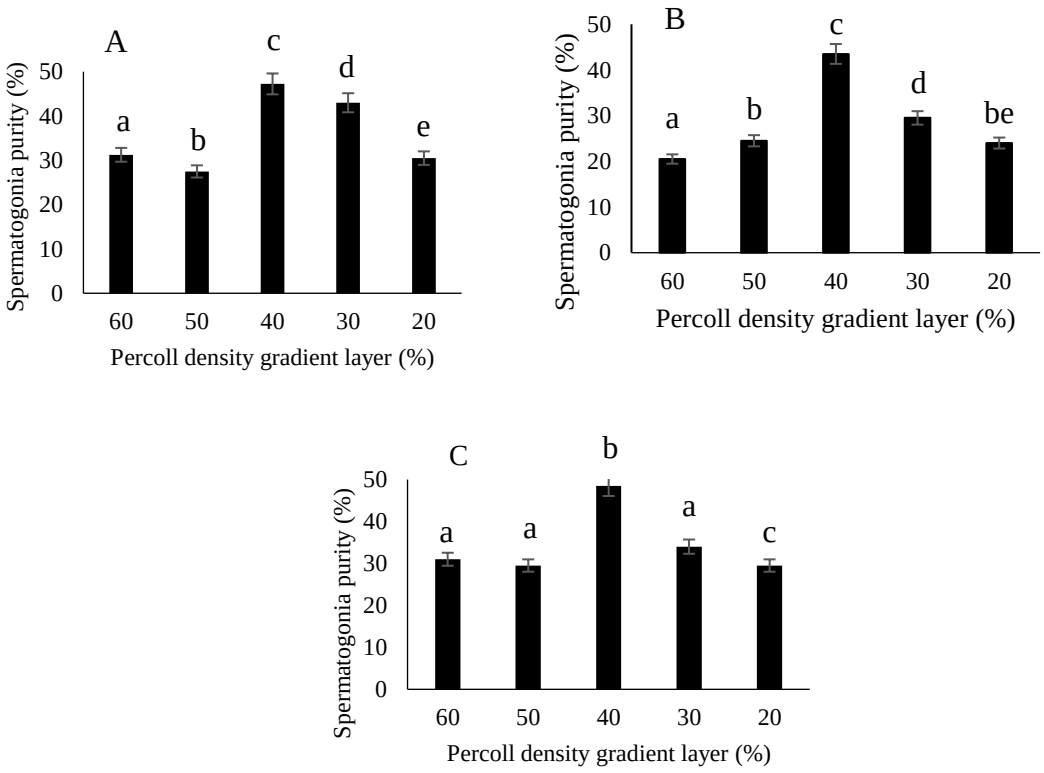

Fig. 1. Purity of isolated bovine spermatogonia at various stage of age. The result represent pooled data from 9 different experiments and the data is presented as mean ± SEM ($^{a-e}P<0.05$). (A-C) DBA positive cells/ 100 cells were counted under fluorescence microscope (Leica Microsystem, Germany). A, B, C, cells purified from 3, 5, and 7 months old calves respectively.

Colony formation and immunohistochemistry of cultured SSCs

All cultures begun with mixed population of spermatogonial stem cells and somatic cells from testicular tissue. On average 45% of cultivated cells were pure spermagonia. Colony like structures were formed in culture plates.

The colony forming cells approached each other and became packed as the colonies continued to grow. Between days 5 and 10 of culture, the colonies grew almost doubled in number; with further growth between days 10 and 15 of culture. As the development of colonies progress, the cells remained attached to the plate surface, constructing monolayer.

To characterize those colonies and cells, they were immunocytochemically analyzed after 5, 10 and 15 days of culture, showing that they were DBA, UCHL1, Sox2 and Oct4 were also positive **(Fig. 2A).** The number of colonies was almost doubled from 5 (110 ± 0.57) to 10 days (192 ± 1.43) and then further increased by 15 days (210.5 ± 1.21) **(Fig. 2B).** At 10 days of culture, the colonies had spherical shape, however, changed to long shape at 15 days of culture.

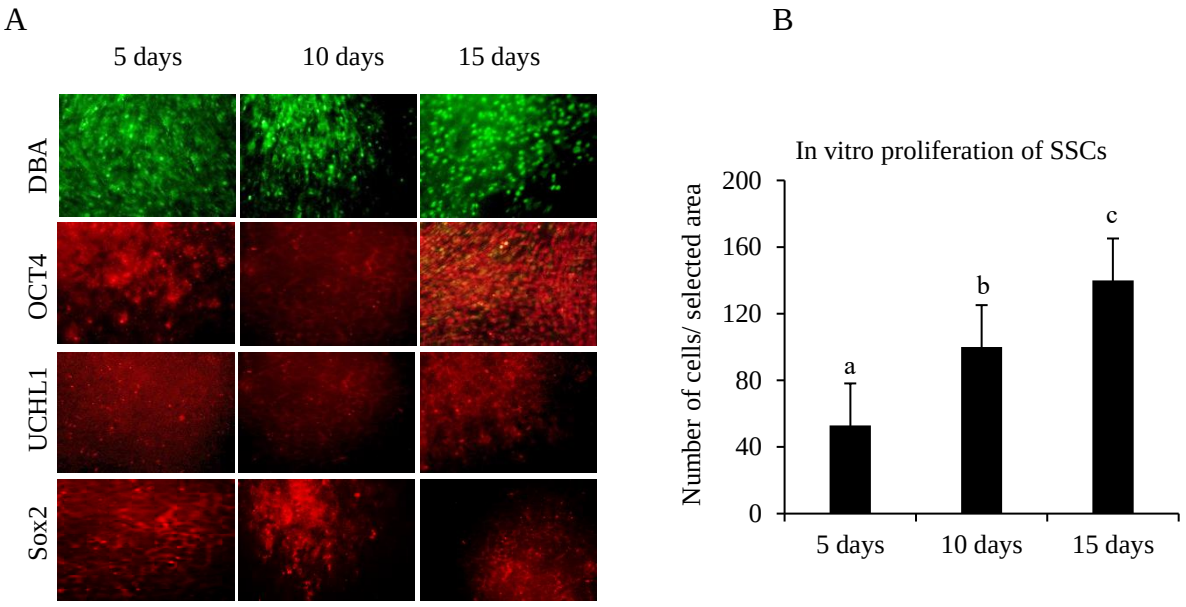

Fig. 2. *In vitro* culture of spermatogonial stem cells from pre- pubertal bovine testis. SSCs cultured, after 5, 10 and 15 day in culture the cells were stained with DBA, OCT4, UCHL1 and Sox2. Expression of SSCs markers maintained by 15 days post *in - vitro* culture. Images are representative of three independent experiments. (B) Number of DBA positive cells counted. 10 and 15 days post- in vitro showed significant increase of number of SSCs compared to 5 days in vitro. (A) Data are presented as mean ± SEM (n=9). (a-c $P<0.05$).

Expression of spermatogonia specific markers in cultured SSCs

In order to verify the stemness prosperities of the proliferating purified spermatogonial stem cells, the mRNA expression level for UCHL1, Oct4, and Sox2 quantified at day 15th of in- vitro culture, using RT – PCR. The analysis demonstrated that, spermatogonial stem cells specific marker UCHL1 and pluripotency factors Oct4 and Sox2 expressed around 300 bps **(Fig.3).**

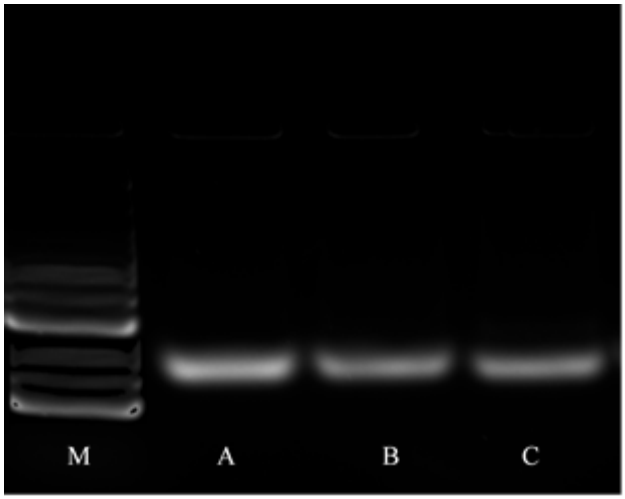

Fig. 3. Expression of transcription based markers in *in-vitro* spermatogonial stem cells from pre-pubertal calves. Total RNA quantified and amplified for UCHL1 (A), Oct4 (B) and Sox2 (C) expression. UCHL1 (A), Oct4 (B) and Sox2 (C) expressed at the predicted 300 base pairs. Images are representative of three independent experiments.

DISCUSSION

Isolation and purity of spermatogonial stem cells

The estimated number of SSCs is very low in testis, has limited efficient isolation of these cells for research activities. Based on cells characteristics, different isolation and purification methods have been used (R. Zhang et al., 2016). Despite the prevalence of isolation and purification techniques, viability of the cells during isolation is another concern that affects *in-vitro* function of SSCs. We used two -step digestion method for cell isolation followed by discontinuous Percoll density gradient layers enrichment. In our study, highly enriched population of spermatogonia to a final purity of 47 %, 43 % and 48 % could be isolated routinely from the pre- pubertal calves at the age of 3, 5 and 7 months old respectively (Fig. 1A, 1B, 1C). In terms of the most pure SSCs, 40 % of discontinuous Percoll density layer would be the most appropriate density to enrich SSCs. Combining two step enzymatic digestion, density separation and differential plating (Izadyar et al., 2002) achieved type A spermatogonia enrichment up to 67% relative to other cells. In addition to this, after first discontinuous Percoll density layers treatment (Fujihara et al., 2011) could enrich 2 – 11% of total cells, however, after second treatment the purity was as much higher as 40% with 75 % viability. Despite lack in differential plating and two times discontinuous Percoll density layers, we could obtain high number of spermatogonia. In that case, our results are comparable. Two step enzymatic digestion was established for the first time over 30 years ago (Bellvé et al., 1977), and is widely used for isolating SSCs of different species (Ahmad et al., 2013; Heidari et al., 2012; Izadyar et al., 2002; Kaavya et al., 2016; Liu et al., 2011; Rodriguez-Sosa et al., 2006; Shafiei et al., 2013; Zhao et al., 2016). Therefore, these techniques could be used to obtain undifferentiated spermatogonia from different species and will help further use of enriched cells.

SSCs culture techniques are not valid for large animal and yet to be successful for long- term. In present study, we successfully propagated pre-pubertal bovine SSCs in culture on stem cell specific medium and tested proliferation ability of SSCs, we found that, after 5 days in culture, SSCs were able to cover large portion of culture wells and make round shape colonies. The colonies grew further from 5 to 10 and 15 days in culture (Fig.2B). The number of colonies increased 100 folds from day 5 to day 10 with further increase until day 15.

However, the growth from day 10 to day 15 was 50 folds. For the first time, Izadyar et al. (2003) developed method for bovine SSCs long –term culture. Their results showed presence of SSCs in culture. However, at some point SSCs were not committed to differentiate. Additionally, SSCs cultured in medium containing different levels of growth factors (Aponte, 2008) and co-cultured with Sertoli cells in the presence of colony stimulating factor-1 (Shafiei et al., 2013), results in proliferation of SSCs. However, our results are not in arrangement. The fact, that the shape of colonies at 15 days of culture changed from spherical to elongated shape. Apparently, the growth factor and other components of the medium induce the growth and formation of organized structures *in- vitro.* Generally, bovine SSCs are in single form, however, it has been suggested that bull SSCs in and chain also have stem cells capability (Worbel, 2000). The round shape colonies do not seems to originate from aggregation of cultured cells but likely self- generate during early proliferation. As Sertoli cells are in spermatogonia suspension in range of 20 – 50 % (Izadyar et al., 2003), the contamination contribute in forming colonies. Immunohistochemistry of cultured SSCs shown the presence of somatic cells in colony (Aponte, 2008). Sertoli cells produce various elements required for maintaining proliferation capacity in SSCs (Aponte et al., 2008). Generally, the colonies happen to be round in shape. However, the use of growth factor EGF induced difference in morphology of the colonies. Due to the effect of EGF, colonies get together and make elongated form (Wahab et al., 2003).

Further, to investigate *in- vitro* stem cells characteristics of bovine SSCs, we examined expression of SSCs marker UCHL1 and self-renewal inducing factors Sox2 and Oct4 at both immunohistochemically and transcription levels. Interestingly, it was found that, the colonies were consistently positive for the spermatogonia specific markers until 15 day *in –vitro.* DBA, UCHL1, Sox2 and Oct4 (Fig. 2A) and expression of SSCs specific marker UCHL1 and self- renewal factors Oct4, Sox2 was maintained at 15th day post culture (Fig.3). DBA a germ cells specific marker have been used for spermatogonia classification and identification (Herrid et al., 2007; Izadyar et al., 2002). UCHL1 is a general marker for bull spermatogonia, bounding to the cytoplasm of SSCs (Fujihara et al., 2011; Reding et al., 2010) and other species (Goel et al., 2010; Vansandt, 2014; Zhao et al., 2016). Additionally, UCHL1 is present in SSCs that undergo asymmetrical cell division (Lou et al., 2009). Oct4 is transcription factor that is necessary for stemness (Pan et al., 2002) and its expression is observed in spermatogonia at different stages of bovine germ cells (Fujihara et al., 2011). However, in pig it is shown specific to differentiated type germ cells (Goel et al., 2008) and is present in mouse gonocyte (Shinohara et al., 2005). OCT4 and Sox2 members of pluripotency regulating protein network (Manku & Culty, 2015), promote stemness of stem cells and are crucial for maintaining proliferation capability (Deluz et al., 2016; H et al., 2014; Hagey et al., 2018; Oatley & Brinster, 2008; Zhang & Cui, 2014). Synergistically, Sox2 and Oct4 control Nanog activities (Yamaguchi et al., 2005) required for inducing self – renewal in stem cells. In mammals, these factors reported to be present in undifferentiated germ cells (Kim et al., 2014; Phillips et al., 2010). Since Sox2 and Oct4 are factors required for stemness and UCHL1 is spermatogonial stem cells specific marker, mRNA expression in cultured SSCs corroborated that SSCs retained stemness.

CONCLUSION

To conclude, spermatogonial stem cells with high survival rate could be enriched from pre- pubertal bovine testes, using two-step enzymatic digestion followed by Percoll ingredient layers, and isolated cells long term *in –vitro* cultured is possible until 15 days in culture. Further studies will investigate initiation of spermatogenesis in homologous recipient from cultured spermatogonial stem cells.

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Conflict of interest

All authors expresses no conflict of interest in any part of research, manuscript and submission to the journal.

REFERENCES

Ahmad, S., Xiao, Y., Han, L., Hua, H., Riaz, H., Liang, A., & Yang, L. G. (2013). Isolation, identification and enrichment of type a spermatogonia from the testis of chinese cross-bred buffaloes (Swamp×River). *Reproduction in Domestic Animals*, 48(3), 373–381.

Aponte, P. M., Soda, T., van de Kant, H. J. G., & de Rooij, D. G. (2006). Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. *Theriogenology*, 65(9), 1828–1847.

Aponte, P. M., Soda, T., Treeds, K., Mizark, S. C., van de Kan, H. J. G., & de Rooji, D. G. (2008) Propagation of bovine spermatogonial stem cells in vitro. *Reproduction*, 136(5), 543-557.

Aponte, P. M., Van Bragt, M. P., De Rooij, D. G., & Van Pelt, A. M. (2005). Spermatogonial stem cells: characteristics and experimental possibilities. *Apmis*, *113*(11-12), 727-742.

Bellve, A. R., Cavicchia, J. C., Millette, C. F., O'Brien, D. A., Bhatnagar, Y. M., & Dym, M. (1977). Spermatogenic cells of the prepuberal mouse: isolation and morphological characterization. *The Journal of cell biology*, *74*(1), 68-85.

Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences*, *91*(24), 11298-11302.

Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., & Skutella, T. (2008). Generation of pluripotent stem cells from adult human testis. *Nature*, *456*(7220), 344-349.

Deluz, C., Friman, E. T., Strebinger, D., Benke, A., Raccaud, M., Callegari, A., & Suter, D. M. (2016). A role for mitotic bookmarking of SOX2 in pluripotency and differentiation. *Genes & development*, *30*(22), 2538-2550.

Dobrinski, I., & Travis, A. J. (2007). Germ cell transplantation for the propagation of companion animals, non-domestic and endangered species. *Reproduction, Fertility and Development*, 19(6), 732–739.

Fujihara, M., Kim, S.-M., Minami, N., Yamada, M., & Imai, H. (2011). Characterization and in vitro culture of male germ cells from developing bovine testis. *The Journal of Reproduction and Development*, 57(3).

Goel, S., Fujihara, M., Minami, N., Yamada, M., & Imai, H. (2008). Expression of NANOG, but not POU5F1, points to the stem cell potential of primitive germ cells in neonatal pig testis. *Reproduction*, *135*(6), 785.

Goel, S., Reddy, N., Mandal, S., Fujihara, M., Kim, S. M., & Imai, H. (2010). Spermatogonia-specific proteins expressed in prepubertal buffalo (Bubalus bubalis) testis and their utilization for isolation and in vitro cultivation of spermatogonia. *Theriogenology*, *74*(7), 1221-1232.

Hagey, D. W., Klum, S., Kurtsdotter, I., Zaouter, C., Topcic, D., Andersson, O., ... & Muhr, J. (2018). SOX2 regulates common and specific stem cell features in the CNS and endoderm derived organs. *PLoS genetics*, *14*(2), e1007224.

He, Y., Chen, X., Zhu, H., & Wang, D. (2015). Developments in techniques for the isolation, enrichment, main culture conditions and identification of spermatogonial stem cells. *Cytotechnology*, 67(6), 921–930.

Heidari, B., Rahmati-Ahmadabadi, M., Akhondi, M. M., Zarnani, A. H., Jeddi-Tehrani, M., Shirazi, A., Naderi, M. M., & Behzadi, B. (2012). Isolation, identification, and culture of goat spermatogonial stem cells using c-kit and PGP9.5 markers. *Journal of Assisted Reproduction and Genetics*, 29(10), 1029–1038.

Herrid, M., Davey, R. J., & Hill, J. R. (2007). Characterization of germ cells from pre-pubertal bull calves in preparation for germ cell transplantation. *Cell and Tissue Research*, 330(2), 321–329.

Izadyar, F., Den Ouden, K., Creemers, L. B., Posthuma, G., Parvinen, M., & De Rooij, D. G. (2003). Proliferation and differentiation of bovine type A spermatogonia during long-term culture. *Biology of Reproduction*, 68(1), 272–281.

Izadyar, F., Spierenberg, G. T., Creemers, L. B., den Ouden, K., & de Rooij, D. G. (2002). Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction*, 124(1), 85–94.

Kaavya, R., Kannan, T., Basha, S., Vairamuthu, S., Ramesh, G., & William, B. (2016). Isolation of Spermatogonial Stem Cell and Its Viability in Mice by Different Enzymatic Digestion Methods. *International Journal of Livestock Research*, 6(11), 54–60.

Kanatsu-Shinohara, M., Miki, H., Inoue, K., Ogonuki, N., Toyokuni, S., Ogura, A., & Shinohara, T. (2005). Long-Term Culture of Mouse Male Germline Stem Cells Under Serum-or Feeder-Free Conditions1. *Biology of Reproduction*, 72(4), 985–991.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., & Shinohara, T. (2003). Long-Term Proliferation in Culture and Germline Transmission of Mouse Male Germline Stem Cells. *Biology of Reproduction*, 69(2), 612–616.

Kanatsu-Shinohara, M., Toyokuni, S., & Shinohara, T. (2004). Transgenic mice produced by retroviral transduction of male germ line stem cells in vivo. *Biology of Reproduction*, 71(4), 1202–1207.

Khaira, H., µl, D., Ohl, D. A., & Smith, G. D. (2005). Spermatogonial stem cell isolation, storage, and transplantation. *Journal of Andrology*, 26(4), 442–450.

Kim, S. M., Fujihara, M., Sahare, M., Minami, N., Yamada, M., & Imai, H. (2014). Effects of extracellular matrices and lectin Dolichos biflorus agglutinin on cell adhesion and self-renewal of bovine gonocytes cultured in vitro. *Reproduction, Fertility and Developmen*t, 26(2), 268–281.

Kim, Y., Turner, D., Nelson, J., Dobrinski, I., McEntee, M., & Travis, A. J. (2008). Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. *Reproduction*, 136(6), 823–831.

Liu, S., Tang, Z., Xiong, T., & Tang, W. (2011). Isolation and characterization of human spermatogonial stem cells. *Reproductive Biology and Endocrinology*, 9(1), 141.

Lou, J., Megee, S., & Dobrinski, I. (2009). Asymmetric distribution of UCH-L1 in spermatogonia is associated with maintenance and differentiation of spermatogonial stem cells. *Journal of Cellular Physiology*, 220(2), 460–468.

Manku, G., & Culty, M. (2015). Mammalian gonocyte and spermatogonia differentiation: Recent advances and remaining challenges. *Reproduction*, 149(3), R139–R157.

McLean, D. J., Johnston, D. S., Russell, L. D., & Griswold, M. D. (2001). Germ cell transplantation and the study of testicular function. *Trends Endocrinol Metab*, 12(1), 16–21.

Oatley, J. M., & Brinster, R. L. (2008). Regulation of spermatogonial stem cell self-renewal in mammals. *Annual review of cell and developmental biology*, *24*, 263-286.

Oatley, J. M., Reeves, J. J., & McLean, D. J. (2004). Biological activity of cryopreserved bovine spermatogonial stem cells during in vitro culture. *Biology of Reproduction*, 71(3), 942–947.

Olive, V., Cuzin, F., Hogarth, C. A., Griswold, M. D. M., Sadri-ardekani, H., Atala, A., Meistrich, M. L. L., Van Beek, M., Khaira, H., McLean, D. J., Ohl, D. A., Smith, G. D., Phillips, B. T., Gassei, K., Orwig, K. E., Dym, M., Kokkinaki, M., He, Z., Zheng, Y., … Tournaye, H. (2015). Spermatogonial stem cell regulation and spermatogenesis. *Molecular and Cellular Endocrinology*, 419(3), 1663–1678.

Pan, G. U. J. in, Chang, Z., Er, H. ansrsh, & Pei, D. U. (2002). Stem cell pluripotency and transcription factor Oct4. *Cell Research*, 12(5–6), 321–329.

Phillips, B. T., Gassei, K., & Orwig, K. E. (2010). Spermatogonial stem cell regulation and spermatogenesis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1546), 1663-1678.

Reding, S. C., Stepnoski, A. L., Cloninger, E. W., & Oatley, J. M. (2010). THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. *Reproduction*, *139*(5), 893-903.

Rodriguez-Sosa, J. R., Dobson, H., & Hahnel, A. (2006). Isolation and transplantation of spermatogonia in sheep. *Theriogenology*, *66*(9), 2091-2103.

Aponte, P. M., Soda, T., Teerds, K. J., Mizrak, S. C., van de Kant, H. J., & de Rooij, D. G. (2008). Propagation of bovine spermatogonial stem cells in vitro. *Reproduction*, *136*(5), 543.

Ryu, B. Y., Orwig, K. E., Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004). Phenotypic and functional characteristics of spermatogonial stem cells in rats. *Developmental biology*, *274*(1), 158-170.

Schlatt, S. (2002). Spermatogonial stem cell preservation and transplantation. *Molecular and cellular endocrinology*, *187*(1-2), 107-111.

Schulz, R. W., & Miura, T. (2002). Spermatogenesis and its endocrine regulation. *Fish physiology and biochemistry*, *26*, 43-56.

Seandel, M., Daylon, J., Shemlkov, S. V., Falciatori, I., Jiyeon, K., Chavala, S., Scherr, D. S., Fan, Z., Torres, R., Gale, N. W., Yancopoulos, G. D., Murphy, A., David M., V., Hobbs, R. M., Pandolfi, P. P., & Rafil, S. (2007). Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature*, 449(7160), 346–350.

Shafiei, S., Tajik, P., Nava, G., Garoussi, T., Panahi, Q., & Feyli, R. (2013). Isolation of bovine spermatogonial cells and coculture with prepubertal sertoli cells in the presence of colony stimulating factor-1 Key words : Abstract : *Iranian Jounal of Veterinary Medicine*, 7(2), 83–90.

Shi, G., & Jin, Y. (2010). Role of Oct4 in maintaining and regaining stem cell pluripotency. *Stem Cell Research and Therapy*, 1(5), 1–9.

Vansandt, L. M. (2014). *Characterization, enrichment, and in vitro culture of spermatogonial stem cells in the domestic cat: A model for rare and endangered felids* (Doctoral dissertation, University of Maryland, College Park).

Wahab-Wahlgren, A., Martinelle, N., Holst, M., Jahnukainen, K., Parvinen, M., & Söder, O. (2003). EGF stimulates rat spermatogonial DNA synthesis in seminiferous tubule segments in vitro. *Molecular and Cellular Endocrinology*, *201*(1-2), 39-46.

Wrobel, K. H. (2000). Prespermatogenesis and spermatogoniogenesis in the bovine testis. *Brain Structure and Function*, *202*(3), 209.

Yamaguchi, S., Kimura, H., Tada, M., Nakatsuji, N., & Tada, T. (2005). Nanog expression in mouse germ cell development. *Gene Expression Patterns*, 5(5), 639–646.

Zhang, R., Sun, J., & Zou, K. (2016). Advances in Isolation Methods for Spermatogonial Stem Cells. *Stem Cell Reviews and Reports*, 12(1), 15–25.

Zhang, S., & Cui, W. (2014). Sox2, a key factor in the regulation of pluripotency and neural differentiation. *World Journal of Stem Cells*, 6(3), 305.

Zhao, H. M., Yang, H., Luo, F. H., Li, M. X., Zhang, S., & Yang, X. G. (2016). Isolation , proliferation , and induction of Bama mini-pig spermatogonial stem cells in vitro. *Genetics and Molecular Research*, 15(3), 1–13.

Zheng, Y., Zhang, Y., Qu, R., He, Y., Tian, X., & Zeng, W. (2014). Spermatogonial stem cells from domestic animals: Progress and prospects. *Reproduction*, 147(3), 65–74.