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Transfection of early and late stage sheep spermatogonial stem cells in culture

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ABSTRACT

The genetic manipulation of spermatogonial stem cells (SSCs) can be used as an alternative to somatic cell nuclear transfer method for the production of transgenic animals. SSCs are now *in vitro* cultured and transplanted in sheep, however, there are no known protocol for DNA transfection of sheep SSCs. The aim of present study was to define the optimal transfection conditions of spermatogonial stem cells (SSCs) in early and late ovine SSC colony formation stages in culture. SSCs were isolated from the slaughterhouse ram testis tissue using a two-step enzymatic digestion process. Results showed that, 2 µl of DNA with 0.5 µg of Lipofectamin or Turbofect were able to transfect SSC colonies at late stage. Since the colonies of SSCs were not in logarithmic growth phase, around 15% of colonies were transfected and no significant difference between Lipofectamin or Turbofect was observed. However, more cells were transfected on early stages of SSC colony formation (7th days), especially when Turbofect was used (around 40 and 45% for Lipofectamin and Turbofect, respectively). Although the early stages SSCs were more suitable for transfection, but the formation of colonies were impaired on transfected cells.

Key words: Lipofectamine, Sheep, Spermatogonial stem cells, Transfection, Turbofect

Spermatogonial stem cells (SSCs) are derived from postnatal quiescent progenitor cells and have the unique ability to self renew or divide into more differentiated progeny (Potter and DeFalco 2017). The capability for self renewal and differentiation in adult mammalian enables SSCs to maintain spermatogenesis (Azizi *et al.* 2017). SSCs are unique stem cells that transmit genetic information to offspring and can be manipulated to produce transgenic animals (Wang *et al*. 2014). Aim of the present study was to define the optimal transfection conditions of SSCs in early and late ovine SSC colony formation stages in culture. Liposomes are made up of cationic lipids, which can interact with the negatively charged nucleic acid molecules and form complexes coating the nucleic acid inside. The positive outer surface of the complex can associate with the negatively charged cell membrane, allowing the internalization of the nucleic acid (Niu and Liang 2008). Liposome-mediated transfection (lipofection) is a simple and powerful technique for DNA transfer into cultured cells (Kalina *et al*. 2003). Turbofect is a cationic polymer and can be used in the presence or absence of serum (Oba and Tanaka 2012).

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MATERIALS AND METHODS

Collection of testes and isolation of SSCs: Ram lambs testes collected from slaughterhouse immediately after slaughter were washed 3–4 times with normal saline solution (32–37ºC), containing 0.1% streptomycin sulphate. SSCs were isolated by a two-time enzymatic digestion process as described by Izadyar *et al*. (2003) with some modifications. For the first enzymatic digestion, minced seminiferous tissue was suspended in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase type 2, 5 µg/ml DNase and 1 mg/ml trypsin and was incubated at 37ºC in an incubator shaker operated at 200 cycles/min for 45 min. After this, the dispersed tissue was collected and subjected to centrifugation at 1,000 rpm for 2 min. The supernatant were discarded and the tissue pellet was washed once with DMEM. For the second enzymatic digestion, the tissue was suspended in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase type 2 and 5 µg/ml DNase and was incubated in an incubator shaker operated at 200 cycles/ min for 30 min. The tissue was then centrifuged 1,000 rpm for 2 min and the supernatant was collected in a 15 ml tube. The dispersed cells, present in the supernatant were expected to contain SSCs, sertoli cells, myoid cells and other contaminating cells of the seminiferous tubular tissue.

Enrichment of SSCs: For enriching the SSCs, the supernatant was filtered sucessively through a 80 µm and then 60 µm nylon net filter. The filtered cells were then transferred to BSA-lectin coated 35 mm petri dishes. The lectin-BSA coated dishes were prepared by dissolving lectin

(5 µg/ml) from Datura starmonium agglutinin in DPBS and pouring 500 µl of lectin-DPBS in each dish. The dishes were kept for 2 h at room temperature after which these were washed with BSA. The dishes were then kept at room temperature for another 2 h for coating BSA (0.6% BSA in DPBS). The cells seeded on the lectin coated dishes were incubated for 5–6 h at 37°C in a CO_2 incubator (5% CO_2 in air) to enable most of contaminating cells to get attached to the lectin-BSA. After this, the remaining media, which was expected to contain SSCs was collected and transferred to 15 ml tube. It was then centrifuged for 5 min at 1,000 rpm following which the supernatant was discarded and the pellet was re-suspended in DMEM. These cells were then subjected to Percoll density gradient cell separation for further purification based on van Pelt *et al*. (1996) protocol.

Preparation of feeder layers: The cells left over in the lectin coated dishes were rejuvenated with fresh DMEM and were incubated in CO_2 incubator (5% CO_2 in air) at 37°C for 2–3 days to enable these cells, which were expected to be primarily sertoli cells, to grow till a confluent monolayer was formed. For propagation, the cells were subcultured in 25 cm culture flask after being disaggregated with 0.25% trypsin-EDTA. For the preparation of a feeder layer, sertoli cells were inactivated by treatment with 10 mg/ml mitomycin-C for 3 h after which these were washed 5 times with DPBS and finally with DMEM supplemented with 10% FBS.

Culture of SSCs: After density gradient separation, the collected cells were seeded on sertoli cells feeder layer in 35 mm dishes and were incubated in a CO_2 incubator (5% CO_2 in air) at 37°C for varying periods of time depending upon the experiment. The culture medium was replaced every third day with fresh medium supplemented with 10% FBS. SSC colonies were observed after 10 days. Alkaline phosphatase staining was used for characterisation of ES cells. For alkaline phosphatase staining, SSC colonies were washed twice with DPBS and then stained using a kit as per the manufacturer's protocol.

Transfection of SSCs: Medium was changed with transfection medium (DMEM without serum and antibiotics) 1 h before experiments when Lipofectamin was used as transfection reagent. Each of DNA, Lipofectamine and Turbofect was diluted in 50 µl of transfection medium separately, based on experimental design, and incubated for 5 min at room temperature. After this, the diluted DNA was added to diluted Lipofectamine or Turbofect (total volume=100 µl) and mixed gently and incubated for 20 min at room temperature. Complexes (100 µl) were added to each well containing the cells and mixed gently by rocking the plate back and forth. The cells were then incubated at 37°C in a CO_2 incubator for 18–48 h prior to testing for transgene expression. Medium was changed periodically based on experimental design.

Experimental design

First experiment (Optimization of the concentration of Lipofectamin, Turbofect and foreign DNA): For this study, effect of different concentrations of Lipofectamin and Turbofect (0.2, 0.5, 1 and 2 µl for 100 µl media), and different concentrations of DNA (0.2, 0.5, 1, 2 and 4 µg for 100 µl media) in transfection of early and late SSC colony formation stages (7 and 14 days after culture, respectively) was studied. The incubation time for cells with Lipofectamin-DNA complex and Turbofect-DNA complex was 8 and 24 h, respectively.

Second experiment (To study different incubation times of cells with Lipofectamin-DNA complex): In this experiment, based on results of first experiment, 0.5 µl DNA and 1 µg Lipofectamin in different incubation times (6, 8, 18 and 24 h) were studied.

Statistical analysis: Data were analysed with a statistical software program (SPSS 16). Comparisons between multiple numeric datasets were performed using one-way ANOVA followed by Duncan multiple-range test. Statistical significance was accepted at $P<0.05$.

RESULTS AND DISCUSSION

First experiment aimed to define the optimal transfection conditions of SSCs. For this reason, effect of two transfection reagents (at different concentrations), and varying concentration of DNA on early and late SSC colony formation stages were studied. Results showed that, 2 µl of DNA with 0.5 µg of Lipofectamin or Turbofect were able to transfect SSC colonies at late stage. Since the colonies of SSCs were not in logarithmic growth phase, around 15% of colonies were transfected and no significant difference between Lipofectamin and Turbofect was observed ($P>0.05$) (Fig. 1).

While the GFP gene transfer to SSCs after the 14th days, when the whole colonies were formed, resulted to transfect a few colonies, however, transfection of early stages of SSC colonies resulted in higher efficiency (Fig. 2), especially when Turbofect was used (around 40 and 45% for Lipofectamin and Turbofect, respectively), although no significant difference was observed between them ($P>0.05$). However, the number of cells and formation of whole colonies were not changed, ten days after transfection of early stages SSC colonies. It seems that by expression of GFP in transfected cells, the ability of cell division was impaired.

The results of second experiment indicated that the optimum incubation time for cells and Lipofectamin-DNA complex was 8 h and higher incubation time resulted in

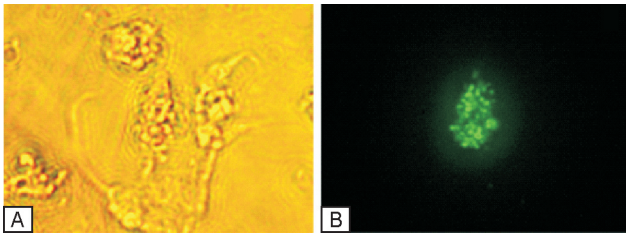

Fig.1. (A-B). Transfection of spermatogonial stem cell colonies. (**A**) Spermatogonial stem cell colonies; (**B**) Same colonies fluorescing under UV light.

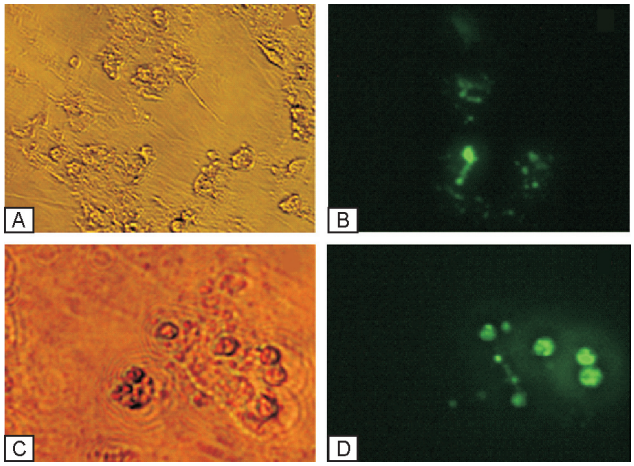

Fig.2 (A-D). Transfection of early stages SSC colonies: (**A,B**) Spermatogonial stem cells transfected with Lipofectamin and the same cells fluorescing under UV light, respectively. (**C,D**) Spermatogonial stem cells transfected with turbofect and the same cells fluorescing under UV light, respectively.

cell death.

SSCs are now *in vitro* cultured and transplanted in different species, however, there are limited protocols for DNA transfection of SSCs in any of them, other than mice Hamra *et al*. 2005). In order to introduced EGFP gene into sheep SSCs in current study, we evaluate the transfection conditions by using two different transfection reagents (Lipofection and Turbofect) in early and late SSC colony formation stages.

Although transfection of early stages of SSC colonies resulted in higher efficiency, especially when Turbofect was used, when compared with late SSC colony formation stages, however, we were unable to increase expansion of SSC after transfection and transfected SSCs showed impaired colony formation. Hamra *et al*. (2005) showed that serum addition, even with minor contamination by testicular somatic cells, results in a loss of stem cell numbers after growth on a variety feeder layers. Previous studies suggested that factors detrimental to spermatogonial stem cell maintenance were present in serum, whereas in other studies, it seemed that, although spermatogonial stem cells could expand in the absence of serum when grown on MEFs, serum was essential for proliferation on a laminin matrix (Kanatsu-Shinohara *et al*. 2005). However in our study, colony formation was not affected in untrasfected colonies, when FBS was used in culture media.

It may be concluded from the results that efficiency of transfection of SSCs were almost same for both Lipofectamin or Turbofect, however, later one is serum independent and slightly higher number of transfected cells resulted by it. Although the early stages SSCs were more suitable for transfection, but the formation of colonies was impaired on transfected cells. Thus, further studies investigating the effect of different growth factors, vitamins and herbal extract for enrichment of transfected SSCs culture conditions are needed.

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