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Zona-free and with-zona parthenogenetic embryo production in goat (*Capra hircus*) — effect of activation methods, culture systems and culture media

Arun Kumar De *, Dhruba Malakar, Manoj Kumar Jena, Rahul Dutta, Shweta Garg, Yogesh S. Akshey

Animal Biotechnology Centre, National Dairy Research Institute, Karnal, Haryana, India

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ABSTRACT

Studies on parthenogenetic activation of oocytes are important to improve the efficiency of nuclear transfer as artificial activation of oocytes is an essential component of nuclear transfer protocol. The present study was carried out to investigate the effects of different activation methods, culture systems and culture media on *in vitro* development of zona-free and with-zona parthenogenetic embryos in goat. In case of zona-free parthenogenesis, there was a significant ($p<0.05$) increase in cleavage rate and blastocyst yield when oocytes were activated by electrical pulse ($76.29\pm0.52\%$ and $19.07\pm0.39\%$ respectively) than when Ca-ionophore was used for activation ($63.45\pm0.73\%$ and $14.09\pm0.65\%$ respectively). The quality of blastocysts was evaluated by determination of cell number and by expression profile of pluripotent related gene Oct-4. No significant ($p<0.05$) difference was found in quality of blastocysts produced by different activation methods. In culturing of zona-free parthenogenetic embryos, flat surface (FS) was proved to be the best system. A significant ($p<0.05$) decrease in cleavage rate and blastocyst yield was found in Microdrop culture of zona-free embryos ($43.67\pm2.08\%$ and $0.72\pm0.72\%$ respectively) in comparison to WOW of zona-free embryos ($73.88\pm1.70\%$ and $15.51\pm1.34\%$ respectively) and FS of zona-free ($75.14\pm0.81\%$ and $23.93\pm2.71\%$ respectively) as well as with-zona ($72.16\pm1.55\%$ and $18.16\pm0.68\%$) embryos. Zona-free flat culture system yielded significantly ($p<0.05$) higher blastocyst rate than zona-free WOW system as well as with-zona flat culture system. The zona-free and with-zona parthenogenetic embryos were cultured in three different media — Research Vitro Cleave media from Cook® Australia (RVCL), Embryo Development Medium (EDM) and Modified Synthetic Oviductal Fluid (mSOF). In case of zona-free parthenogenesis, significant ($p<0.05$) increase was found in blastocyst development rate in RVCL medium ($18.61\pm1.52\%$) than EDM ($11.29\pm0.77\%$) or mSOF ($11.53\pm1.86\%$). In case of with-zona parthenogenesis, RVCL medium and EDM were found superior to mSOF. The results of the study will be helpful to improve the efficiency of nuclear transfer in goat.

1. Introduction

Parthenogenesis is the biological phenomenon by which embryonic development is initiated without male contribution. The parthenogenetic activation of oocytes is an important tool to investigate the comparative roles of paternal and maternal genomes in controlling early embryo development. As artificial activation of oocytes is an essential component

* Corresponding author at: Current address: Animal Science Division, Central Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands-744101, India.
E-mail address: biotech.cari@gmail.com (A.K. De).

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of nuclear transfer protocols, studies on parthenogenetic oocyte activation are important to improve the efficiency of nuclear transfer (Kim et al., 1996). An optimized activation protocol may enhance nuclear reprogramming of the reconstructed embryo which in turn increases the efficiency of nuclear transfer.

The efficiency of blastocyst production by somatic cell nuclear transfer (SCNT) is less and is hampered by various biological and technical problems (Vajta et al., 2003); only approximately 1–2% of the embryos reconstructed by nuclear transfer are able to develop to term (Lai and Prather, 2003). Its success rate is dependent upon many factors, including donor cell cycle, donor cell type, oocyte quality, enucleation, fusion, oocyte activation procedures and *in vitro* culture conditions.

Activation is a crucial parameter affecting blastocyst production in nuclear transfer. Various chemicals like Ca ionophore, ethanol and strontium have been used for activation of oocytes (Alberio et al., 2001; Das et al., 2003; Varga et al., 2008). In traditional method for production of cloned goat embryos, electric pulses were applied for activation of reconstructed oocytes (Melican et al., 2005; Shen et al., 2006). Oocyte activation by electrical pulse is initiated by an elevation of intracellular Ca^{++}. Immediately after electrical stimulation, there is an influx of extracellular Ca^{++} which in turn triggers an increase of intracellular Ca^{++} (Cheong et al., 2002). Ionophores such as Calcium ionophore and ionomycin induces a great single intracellular calcium rise in MII oocytes which originates exclusively from the internal deposits (Hoth and Penner, 1992) and a likely consequence is the activation of several calcium-dependent proteolytic pathways, leading to the destruction of cyclin B, reduction of MPF activity, and resumption of meiosis (Jellerette et al., 2006; Rinaudo et al., 1997; Tomashov-Matar et al., 2005). Ultrasound was also shown to induce nuclear activation and parthenogenetic development of *in vitro* matured pig oocytes (Sato et al., 2005).

Culture system is another important factor for improving the efficiency of blastocyst production by traditional SCNT as well as hand-made cloning (HMC) method. Several culture systems like Well-of the wells (WOW) (Vajta et al., 2001), agarose gels (Peura and Vajta, 2003), glass oviduct (Thouas et al., 2003) and microdrops (Oback et al., 2003) have been developed for culture of zona-free cloned embryo with varying in efficiency of blastocyst production. A suitable culture system needs to be established for culture of zona-free cloned goat embryos.

Culture medium is the most determinant factor for improvement of efficiency of *in vitro* embryo development. Commonly used media for *in vitro* embryo production (IVP), like M-199 and mSOF, has been shown to be less efficient for *in vitro* development of cloned buffalo embryos produced through SCNT (Shah et al., 2008). Commercially available sequential medium (G1/G2, Vitrolife, Sweden) has been reported to improve the efficiency to some extent (Simon et al., 2006). In the present study, three different types of media — RVCL, EDM and mSOF were evaluated for the efficiency of zona-free and with-zona blastocyst production.

The aim of the present study was to investigate the effects of different activation methods, culture systems and culture media on yield and quality of zona-free and with-zona parthenogenetic goat blastocysts. In the first experiment, the effects of different activation methods (Ca-ionophore and electrical activation) were evaluated on blastocyst formation and blastocyst quality. Blastocyst quality was evaluated not only by blastocyst cell number but also by expression of pluripotency gene Oct-4 in inner cell mass (ICM) of blastocysts. In the second and third experiment, the effect of various culture systems and culture media were evaluated for production of zona-free and with-zona parthenogenetic blastocysts. The results of the experiments will help in improving the efficiency of SCNT and HMC in terms of yield and quality of blastocysts.

2. Materials and methods

All the present experiments comply with all relevant institutional and national animal welfare guidelines, policies and ethics committee approval.

All chemicals and media were purchased from Sigma Chemical Co (St. Louis, MO, USA), and disposable plastic wares were from Nunc (Roskilde, Denmark) unless specified otherwise.

2.1. In vitro maturation of oocytes

Goat ovaries were collected from local abattoir and transported to the laboratory in a thermo flask containing warm (35 °C) normal saline containing antibiotics (400 IU/ml penicillin and 500 μg/ml streptomycin) within 3 h. Ovaries were trimmed, washed and then oocytes were aspirated by puncturing the visible follicles with a 18-gage needle in the oocyte collection medium (OCM) containing TCM 199, 100 μg/ml L-glutamine, 10% FBS (Hyclone, Logan, UT, Cat no. CH30160.02), 50 μg/ml gentamicin and 3 mg/ml BSA (Fraction-V). Oocytes were picked up gently under stereo zoom microscope and kept in a 35 mm Petri dish containing OCM and washed 5 times in this medium. The cumulus-oocyte-complexes (COCs) having ≥3 layers of compact cumulus cells were selected for *in vitro* maturation. COCs were washed 2 times with the maturation medium containing TCM-199, 10 μg/ml LH, 5 μg/ml FSH, 1 μg/ml estradiol-17β, 50 μg/ml sodium pyruvate, 5.5 mg/ml glucose, 3.5 μg/ml L-glutamine, 50 μg/ml gentamicin, 3 mg/ml BSA, and 10% FBS. Four drops of 100 μl maturation medium were made in 35 mm Petri dishes and covered with mineral oil. These dishes were placed in the incubator with 5% CO_2 in air at 38.5 °C, 1 h prior to use for equilibration. Then 15–16 oocytes were placed in each drop of maturation medium. The Petri dishes were incubated at 38.5 °C under 5% CO_2 in air with maximum humidity for 24 h (Malakar et al., 2008).

2.2. Parthenogenetic activation

Matured oocytes with expanded cumulus cells were transferred into micro centrifuge tube containing 0.5 mg/ml hyaluronidase in T2 (TCM-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 μg/ml gentamicin and 2% FBS) and incubated for 1 min at 38.5 °C under 5% CO_2 in air. Then vortexing was done for 2–3 min. The contents of the tube were transferred to a 35 mm Petri dish containing T2 and completely denuded oocytes were selected and washed twice in fresh T2 for removal of cumulus cells. For zona-free parthenogenesis the oocytes were made zona free by incubating them to T10 (TCM-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 μg/ml