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Handmade cloned and parthenogenetic goat embryos – A comparison of different culture media and donor cells

M.K. Jena, D. Malakar*, A.K. De, S. Garg, Y.S. Akshey, R. Dutta, S. Sahu, A.K. Mohanty, J.K. Kaushik

Animal Biotechnology Center, National Dairy Research Institute, Karnal 132001, India

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ABSTRACT

The aim of the present investigation was the production of handmade cloned and parthenogenetic goat embryos. One somatic cell was attached to an enucleated demi-oocyte and fused with another enucleated demi-oocyte with the aid of an electric pulse. Triplets were chemically activated with a Ca ionophore and 6-DMAP, and then cultured in three media. Blastocyst formation with fetal fibro cells was induced in RVCL ($15.5 \pm 4.2\%$), mSOF ($11.0 \pm 2.2\%$) and EDM ($10.7 \pm 2.4\%$) media, respectively ($P > 0.05$). Similarly, adult fibroblast cells formation was also recorded in RVCL ($14.1 \pm 3.3\%$), mSOF ($9.6 \pm 2.7\%$) and EDM ($9.7 \pm 2.2\%$) media, respectively ($P > 0.05$). PCR analysis of the highly polymorphic MHC class II DRB gene of cloned embryos and donor cells, recorded similar bands. Zona and zona-free parthenogenetic embryos were also produced with the aid of a Ca ionophore and 6-DMAP, following culture in the three media. Zona parthenogenetic hatched blastocysts were recorded in RVCL ($6.8 \pm 0.9\%$), mSOF ($1.2 \pm 0.7\%$) and EDM ($5.5 \pm 0.7\%$) ($P < 0.05$) media, respectively. In the zona-free parthenogenetic blastocyst, formation was recorded in the RVCL ($8.8 \pm 0.9\%$), mSOF ($5.6 \pm 0.5\%$) and EDM ($5.1 \pm 0.8\%$) ($P < 0.05$) media, respectively. In conclusion, cloned and parthenogenetic goat embryonic development was higher in the RVCL medium and cloned embryos production was similar with both types of donor cells in this medium.

1. Introduction

Somatic cell nuclear transfer (SCNT) is a technique for the faster multiplication of superior germplasm. "Dolly", the first successful cloned sheep was obtained from a differentiated adult mammary epithelial cell and created a revolution in science (Wilmut et al., 1997). Willadsen (1986) also performed nuclear transplantation on sheep embryos, using single blastomeres (from 8- to 16-cell embryos) as donor cells. Further developments led to the establishment of SCNT for the production of cloned mammalian embryos from various cell types, including fetal fibroblast cells (Keefer et al., 2001), granulosa cells (Wells et al., 1999), cumulus cells (Wakayama et al., 1998), and adult skin fibroblast cells (Wakayama and Yanagimachi, 1999). Subsequently different cloned animals have been produced by SCNT like, *e.g.* in cattle (Cibelli et al., 1998), goats (Baguisi et al., 1999), mice (Wakayama et al., 1998), pigs (Polejaeva et al., 2000), rabbits (Chesne et al., 2002), mules (Woods et al., 2003), horses (Galli et al., 2003), rats (Zhou et al., 2003) and dogs (Lee et al., 2005).

The micromanipulator-based or traditional cloning technique, is a multi-step, time consuming and complicated procedure that requires expensive and sophisticated equipment, like, *e.g.* the micromanipulator, micropipettes and micro forge. Moreover, proper use of these tools requires highly skilled and qualified expertise. To overcome this problem micromanipulator free (handmade)

* Corresponding author. Tel.: +91 9416741839; fax: +91 184 2250042.
E-mail address: dhrubamalakar@gmail.com (D. Malakar).

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cloning procedures can be implemented for nuclear transfer.

Handmade cloning (HMC) is a simplified nuclear transfer process, avoiding the need for sophisticated instrumentation and technical expertise. Bovine nuclear transfer was first described with zona-free oocytes, using embryonic cells (Peura et al., 1998). Later the improved handmade cloning technique was developed for somatic cell nuclear transfer (Vajta et al., 2001). Handmade cloned offspring have been produced in cattle (Tecirlioglu et al., 2003; Vajta et al., 2003), horses (Lagutina et al., 2005), pigs (Du et al., 2005) and buffaloes (George et al., 2011).

Goats as such, the focus of this study, are valuable livestock as they provide meat, milk, fiber, manure, *etc.* The first transgenic goat was produced, using fetal fibroblasts as the donor cell (Baguisi et al., 1999). Cloned goats were produced using cytoplasts derived from abattoir ovaries and donor cells derived from a transgenic fetus (Reggio et al., 2001). Cloned kids were also produced from ear skin fibroblasts of adult alpine does (Shen et al., 2006).

The cloned embryo culture medium has always been very important, so, *e.g.* in the zona-free method the frequent change of medium during culture, is not preferred. The medium G1.2 supplemented with 8 mg ml^{-1} BSA has been used for the culture of traditional cloned goat embryos (Keefer et al., 2002; Reggio et al., 2001). Similarly activated nuclear transferred embryos were cultured in TCM-199, supplemented with 5% FBS and gentamicin (5 μg ml^{-1}) (Shen et al., 2006). HMC (handmade) cloned bovine embryos have been cultured in a well of the well (WOW) system, using mSOF (modified synthetic oviductal fluid), supplemented with 5% cattle serum (Vajta et al., 2000, 2003). Zona-free buffalo embryonic development rates were then recorded to be significantly higher ($P < 0.05$) regarding cleavage (82%) and blastocyst formation rate (40.3%) in RVCL (Research Vitro Cleave from Cook Australia) medium (Shah et al., 2008). Selokar et al. (2011) then performed interspecies handmade cloned embryo production, by nuclear transfer of cattle, goat and rat fibroblasts to buffalo oocytes, using RVCL as the culture medium. Dutta et al. (2011) used RVCL (containing 1% BSA and 4% FCS) media to produce zona-free cloned goat embryos. Zona-free cloned goat embryos were cultured in TCM-199 based embryo development medium, supplemented with 10 mg ml^{-1} BSA, in the WOW culture system (Akshey et al., 2010b). Bovine parthenogenetic embryos were developed using the mSOF culture medium (Wang et al., 2008). Goat parthenogenetic embryos were also cultured in G1.2/G2.2 sequential culture medium supplemented with 8 mg ml^{-1}, crystallized BSA (Ongeri et al., 2001).

At present there are few available reports regarding the media requirements for the development of zona-free cloned goat embryos. Handmade cloned goat embryos were cultured using embryo development medium (EDM) and fetal fibroblasts as the donor cells (Akshey et al., 2008).

The objective of the present investigation was to analyze the developmental efficiency of handmade cloned goat embryos, with zona and without zona parthenogenetic embryos in three types of media, (RVCL, mSOF and EDM), regarding cloned embryo production efficiency, using two types of donor cells, fetal fibroblast and adult fibroblast cells.

2. Materials and methods

Animal Ethics: 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 ware were from Nunc (Roskilde, Denmark), unless specified otherwise.

2.1. In vitro maturation (IVM) of oocytes

Goat ovaries were collected from an abattoir and washed with warm (35 °C) normal saline, containing 400 IU ml^{-1} penicillin and 500 μg ml^{-1} streptomycin. Ovaries were then transported to the laboratory in a thermo flask (containing warm normal saline, within 3 h), trimmed, washed and the oocytes collected by puncturing the visible follicles with an 18-gauge needle. Thereafter ovaries were placed in the oocyte collection medium (OCM), containing TCM 199 (HEPES modification), 100 μg ml^{-1} L-glutamine, 10% FBS (Hyclone, Logan, UT, Cat no. CH30160.02), 50 μg ml^{-1} gentamicin and 3 mg ml^{-1} BSA (Fraction-V). Oocytes were kept in a 35 mm Petri dish containing OCM and washed 5 times in this medium. The cumulus-oocyte-complexes (COC's) having ≥3 layers of compact cumulus cells were selected for IVM and oocytes matured *in vitro* (Malakar et al., 2008). Oocytes were washed twice with the maturation medium containing TCM-199 (HEPES modified), 10 μg ml^{-1} LH, 5 μg ml^{-1} FSH, 1 μg ml^{-1} estradiol-17β, 50 μg ml^{-1} sodium pyruvate, 5.5 mg ml^{-1} glucose, 3.5 μg ml^{-1} L-glutamine, 50 μg ml^{-1} gentamicin, 3 mg ml^{-1} BSA, and 10% FBS. Four drops (100 μl) maturation medium were placed in the 35 mm Petri dishes and covered with mineral oil. These dishes were then placed in the incubator with 5% CO_2 in air, at 38.5 °C 1 h prior to use for equilibration. A total of 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, at maximum humidity, for 24 h (Akshey et al., 2008).

2.2. Preparation of recipient cytoplasts

The recipient cytoplasts were prepared as described by Shah et al. (2008), with minor modifications. Matured oocytes with expanded cumulus cells were transferred into the micro-centrifuge tube containing 0.5 mg ml^{-1} hyaluronidase in T2 (T denotes TCM-199 supplemented with 2.0 mM L-glutamine, 0.2 mM sodium pyruvate, 50 μg ml^{-1} gentamicin and the number denotes the percentage of FBS) and incubated for 1 min at 38.5 °C, under 5% CO_2 in air. Then vortexing was performed for 2–3 min. The contents of the tube were then transferred to a 35 mm Petri dish, containing T2. The completely denuded oocytes were selected and washed twice in fresh T2 for removal of the cumulus cells. The denuded oocytes were transferred to another Petri dish, containing 2 mg ml^{-1} pronase in T10 and incubated for 2 min at 38.5 °C under 5% CO_2 in air, to digest the zona pellucida. Completely zona-free oocytes were transferred into another Petri dish containing T20 – to stop the activity of the pronase enzyme. Zona-free oocytes were washed twice in T20 and incubated in the same medium at 38.5 °C, under 5% CO_2 in air, for 20 min, until the visible protrusion cone, containing nuclear material was observed under the zoomstereo microscope.

Bisection of the zona-free oocytes was performed by the protrusion cone guided method, which eliminates the use of the Hoechst 33342 stain. Protrusion cone bearing oocytes was transferred into the Petri dish containing 3 ml T20 and 2.5 μg ml^{-1} cytochalasin B and manually bisected using an ultra sharp micro blade (Micro Blades, MTB-05; Micromanipulator Microscope Company, Inc., Carson City). This was done in such a manner that the smaller half carried the protrusion cone containing the nuclear material, and the larger half, the enucleated oocyte. The enucleated oocytes were kept in one well of a 4-well dish and transferred to T20 and incubated for 20 min at 38.5 °C, under 5% CO_2 in air.

2.3. Preparation of somatic cell cultures

2.3.1. Primary cell cultures of fibroblast cells from an adult female goat

Skin biopsies were taken aseptically from the ear pinna of a healthy female goat. The ear pinna was shaved and skin tissue was pinched off the ear pinna and the wound aseptically dressed. The tissue was maintained in