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Short communication

Comparative assessment of development competence of zona-intact and zona-free cloned goat embryos produced by innovative micromanipulation tools

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ABSTRACT

The present study demonstrated a new approach for production of zona free cloned embryos in goat wherein, instead of hand guided bisection applied under Hand-made cloning technique, we have adopted micromanipulator based enucleation using innovative microtools viz., Bisection blade and Aspiration pipette. Using bisection blade, oocyte was bisected into two halves and one half having polar body-MII chromosome was instantly discarded by aspiration pipette, resulted into 100% enucleation efficiency. This process did not require Hoechst staining for confirmation of enucleation. The electrofusion process was carried out using indigenously prepared electrodes for individual electrofusion of demicytoplasts-somatic cell triplet, which revealed significantly higher ($P \leq 0.01$) fusion rate ($92.78 \pm 0.5\%$) as compared to conventional technique ($64.61 \pm 0.93\%$) for zona intact embryos. The developmental rates of zona-free embryos were observed as significantly higher ($P \leq 0.01$) 2–4 cells (79.13 ± 0.82 vs 29.42 ± 0.55), 8–16 cells (66.68 ± 01.02 vs 23.83 ± 0.9), morula (31.93 ± 1.12 vs 17.93 ± 1.07), blastocysts (24.0 ± 1.56 vs 7.79 ± 0.98) and mean blastomere counts (210.3 ± 2.3 vs 154.0 ± 1.60) as compared to zona-intact embryos.

1. Introduction

The conventional technique of somatic cell nuclear transfer (SCNT) used for creation of cloned sheep "Dolly" involved micromanipulation for enucleation of oocytes and somatic cell nuclear transfer into enucleated oocytes (Wilmut et al., 1997). However, the confirmation of enucleation was an essential step for selecting the enucleated oocytes by fluorescent staining of extruded ooplasm. Although the conventional SCNT has been used successfully in many animal species, the requirements of technical skill for micromanipulation as well as the cumbersome and time consuming steps are limiting factors for achieving the high rate of success. The technology was simplified by developing Hand-made cloning technique (HMC) that did not require micromanipulator (Vajta et al., 2001). In brief, the zona free matured oocytes were bisected by hand guided blades under stereo microscope. All halved oocytes (demicytoplasts) were confirmed for enucleation by Hoechst staining, before cell attachment and electrofusion. Although the HMC technique accelerated the overall processes of SCNT, but in most of experiments staining was performed as a confirmatory step of enucleation.

In our laboratory, the technique was further simplified by adopting the basic concept of HMC by using the innovated micromanipulation tools for enucleation and electrofusion process. The aim of our study was to assess the developmental competence of zona-free embryos derived from our modified technique as compared to conventional zona-intact cloned embryos.

2. Materials and methods

All plasticwares were of tissue culture grade purchased from Becton Dickinson Labware (NJ., USA) and the chemicals, reagents and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Preparation of fibroblast cell line

Primary fibroblast cell culture was established from ear tissue explants of a single goat (No. 202) as described by Gopalakrishna et al. (2014) (Fig. 2(a)). At fifth to ninth passage, the cells were made serum starved by treating with DMEM+0.5% FBS for 3 days followed by trypsinization before using as a somatic cell nuclear

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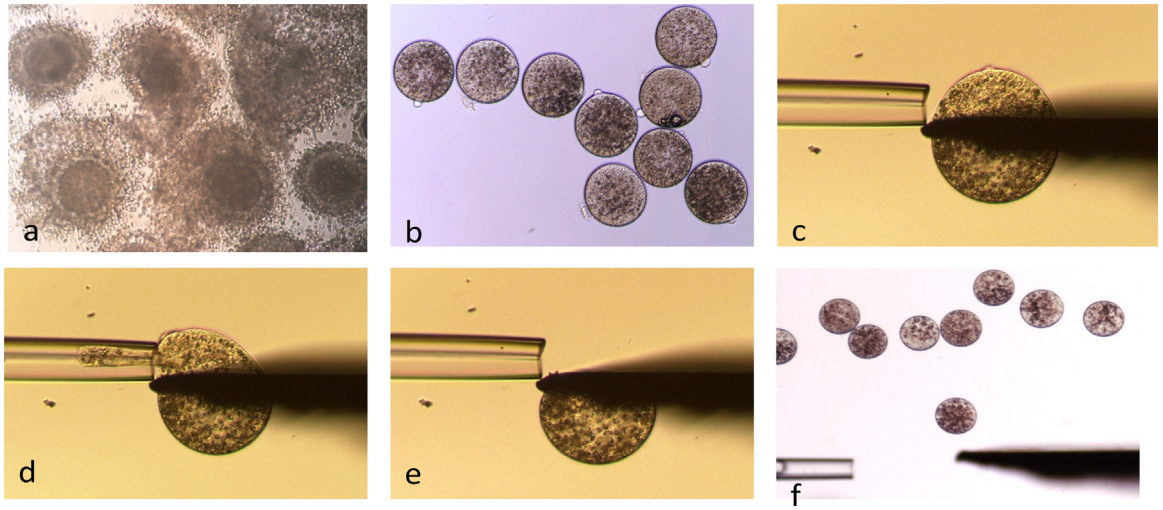

Fig. 1. Enucleation of oocytes using innovative microtools: (a) In vitro matured oocytes (b) zona free oocytes showing protrusion cone (c) Position of bisection blade and aspiration pipette (d) Bisection and simultaneous aspiration of half cytoplast along with polar body (e) and (f) enucleated demicytoplasts. (a–e: 200X; f: 100X).

donor (Fig. 2b and c).

2.2. SCNT by innovative microtools

For enucleation, a set of innovative micro tools namely Bisection blade and Aspiration pipette, were indigenously designed. Bisection blade was prepared by cutting the metallic blade by fine cutter into small pieces of triangular shapes (3 mm × 4 mm × 2 mm) having sharp cutting tip edge. Each piece was glued into a glass capillary of 1 mm diameter and 10 cm length (Narishige, Model GD-1, Japan) in such a way that the cutting edge remains parallel to the surface of the petri dish. Aspiration pipette was prepared from sodium borosilicate glass tubes of 7.5 cm long having 1 mm outer and 0.78 mm inner diameter (Sutter instrument CO., CA). The pipette was pulled and bevelled using horizontal pipette puller (Narishige, model PN-30, Japan) and micro forge (Narishige, model MF-900, Japan) to make the inner diameter of 60 μm with 25–30° angle.

The oocytes derived by slaughterhouse ovaries were matured in vitro as described by Kumar et al. (2014) (Fig. 1(a)). The matured oocytes were denuded by using 0.1% hyaluronidase and subsequently treated with proteinase K (2 mg/ml) to dissolve the zona pellucida (Fig. 1(a)). After incubating the zona free oocytes (cytoplast) in TCM+20% FBS, the oocytes having protrusion cones (Fig. 1(b)) were transferred into a 100 μl droplets of enucleation media (2.5 μg/ml cytochalasin B+TCM with 20% FBS). Prior to bisection, the tip of bisection blade was dipped into 10% polyvinyl pyrrolidone for 4–5 s so as to avoid ooplasm adherence. The cytoplast under micromanipulator was oriented in such a way that the polar body was visible at 12 'O' clock position (Fig. 1(c)). The oocytes were bisected into two equal halves and demicytoplasts with PB-MII were aspirated instantly by aspiration pipette (Fig. 1d and e). The remaining demicytoplasts without MII were incubated in TCM with 20% FBS for 20 min at 38.5 °C which rounded up within 5 min (Fig. 1(f)). Half of the total number of demicytoplasts were immersed in TCM+2% FBS supplemented with 0.5 mg/ml phytohaemagglutinin-L for 4–5 s and transferred into TCM with 2% FBS. In the same media, each demicytoplast was manually attached with single fibroblast to form couplets (demicytoplast-donor cell pair). The fusion protocol was simplified by developing individual oocytes-somatic cell fusion using gold plated electrodes. The original thickness of L shaped genetrode tips (BTX 2001, San Deigo, CA, USA) was reduced from 300 μm to 80–100 μm by continuous rubbing and grinding by fine metal grinding paper and subsequently gold plated to make neutral. The electrode was mounted in to a thick glass tube having compatible thickness to the pipette holder of micromanipulator. Finally the other end of electrodes was connected with the highly conductive wires of the electro fusion machine. The equal numbers of couplets and the demicytoplasts were overlaid in Zimmerman's cell fusion medium in 60 mm petridish, which was used as a fusion chamber. A couplet and a demicytoplast were aligned in a manner so that the cell was sandwitched between two demicytoplasts (Fig. 2(d)). A single step fusion was induced by a double electrical pulse of 2.2 kV/cm for 10 μs, delivered by a BTX Electro Cell Manipulator (BTX 2001, CA, USA) (Fig. 2(e)). The fused triplets (Couplet+demicytoplast) were incubated in TCM+20% FBS at 38.5 °C for 4 h in CO_2 incubator.

2.3. SCNT by conventional method

The conventional method of SCNT was performed as described by Du et al. (2006) with slight modifications. In brief, the zona pellucida of oocyte was pierced by enucleation needle covering the polar body and creating a microincision (Fig. 3(a)). This was followed by squashing and compressing out the first polar body along with 15–20% of cytoplasm (Fig. 3(b)). The extruded material was Hoechst 33342 stained to confirm the enucleation. A single fibroblast was transferred into perivitelline space using a transfer pipette having 15–20 μm diameter, followed by brief treatment with phytohaemagglutinin-L (Fig. 3(c)). A single step electrofusion was induced individually to each oocyte-cell couplet through modified electrodes, keeping the same fusion parameters as applied for zona free technique (Fig. 3(d)). The reconstructed embryos were incubated in TCM+20% FBS at 38.5° for 4 h in CO_2 incubator.

2.4. Chemical activation, embryo culture and staining

The reconstructed embryos derived from both the methods were chemically activated as per the protocol described by Kumar et al. (2014). In brief, the oocytes were treated with 5 μM calcium ionophore A23187 (Sigma C7522) for 5 min followed by treatment with 10 μg/ml cycloheximide +2 mM 6-dimethyl amino purine for 4 h. The activated embryos of both groups were sequentially cultured in Research vitro cleave medium (Cook®, Australia) and Blastocyst medium (Cook®, Australia) up to day 8 under humidified atmosphere at 5% CO_2 and 38.5 °C temperature