Study of the Efficiency of the Chemically Assisted Enucleation Method for Handmade Cloning in Goat (Capra hircus)

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Contents
The present investigation was carried out to find an efficient chemically assisted procedure for enucleation of goat oocytes related to handmade cloning (HMC) technique. After 22-h in vitro maturation, oocytes were incubated with 0.5 μg/ml demecolcine for 2 h. Cumulus cells were removed by pipetting and vortexing in 0.5 mg/ml hyaluronidase, and zona pellucidae were digested with pronase. Oocytes with extrusion cones were subjected to oriented bisection. One-third of the cytoplasm with the extrusion cone was removed with a micro blade. The remaining cytoplasts were used as recipients in HMC. Goat foetal fibroblasts were used as nuclear donors. The overall efficiency measured as the number of cytoplasts obtained per total number of oocytes used was significantly (p < 0.05) higher in chemically assisted handmade enucleation (CAHE) than oriented handmade enucleation without demecolcine (OHE) (80.02 ± 1.292% vs. 72.9 ± 1.00%, respectively, mean ± SEM). The reconstructed and activated embryos were cultured in embryo development medium (EDM) for 7 days. Fusion, cleavage and blastocyst development rate were 71.63 ± 1.95%, 92.94 ± 0.91% and 23.78 ± 3.33% (mean ± SEM), respectively which did not differ significantly from those achieved with random handmade enucleation and OHE. In conclusion, chemically assisted enucleation is a highly efficient and reliable enucleation method for goat. HMC which eliminates the need of expensive equipment (inverted fluorescence microscope) and potentially harmful chromatin staining and ultraviolet (UV) irradiation for cytoplasm selection.

Introduction
Nuclear transfer is a very effective method for propagation of valuable, desired, extinct and endangered animals as well as production of 100% transgenic animals and stem cells for research and application (Baguisi et al. 1999; Lanza et al. 2000 and Keefer et al. 2001). Enucleated oocytes at the second metaphase stage (MII) are often used as recipient cytoplasts for nuclear transfer. One of the key steps in nuclear transfer is the removal of all genetic material associated with chromatins from recipient oocytes. Because the chromatines in mammalian oocytes are invisible under common light microscopy, the position of the chromatines is indirectly determined by locating the first polar body (FPB) or directly by observing under UV light after staining with a DNA-binding dye (Hoechst 33342). In traditional micromanipulator-based cloning, the nuclear material of oocytes is usually aspirated by the loss of cytoplasm along with the FPB (Tsunoda and Kato 2000). In handmade cloning (HMC), oocytes are generally randomly bisected followed by selection of the chromatin-free cytoplasm by ultraviolet (UV) microscopy after Hoechst staining (Vajta et al. 2001 and Du et al. 2005). The disadvantage of this technique is that there is unnecessary high loss of cytoplasm; harm caused by staining and illumination to UV and the two-step procedure consumes considerable time (Dominko et al. 2000).

Oriented bisection requires some detectable orientation points that reliably show the presence of nuclear DNA because in domestic animals, cytoplasmic lipid droplets hamper the visualization of the unstained chromatin. The polar body is not a perfect indicator of the site of the metaphase plate, and enucleation should be confirmed by subsequent staining, although the orientation provided by the polar body usually allows for the preservation of the majority of the cytoplasm (Prather et al. 1989; Dominko et al. 1999 and Mitatipov et al. 1999). At zona-free work, separation of the polar body from oocytes that occur after pronase digestion can be prevented by pre-incubation of metaphase-II zona intact oocytes in biological glue, phytohaemagglutinin (PHA) before pronase digestion (Peura et al. 1998). According to observation of Peura et al. 1998, the polar body-oriented manual enucleation of zona-free bovine oocytes results in 90% accuracy; however, the efficiency and reliability of PHA was rather inconsistent. In addition, an unfavourable effect of the PHA incubation is that it slows down and hampers complete digestion of the zona by pronase.

Chemically assisted enucleation seems to be a potentially superior approach with a minimal decrease in oocyte cytoplasmic volume and no need for Hoechst staining and selection by UV microscopy. It is based on the phenomenon that treatment of MII phase oocytes with some cytokinesis-relaxing agents induces a visible extrusion cone on the surface of oocyte-containing chromatin. Removal of this cone and surrounding cytoplasm results in reliable enucleation (Yin et al. 2002a,b; Peura 2003; Li et al. 2004, 2009; Lan et al. 2008; Borges et al. 2009 and Saraiva et al. 2009). The aim of the present investigation was to find out the efficiency of chemically assisted enucleation in micromanipulator-free HMC technique in goat.

Materials and Methods
All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and disposable plastic wares from Falcon-ware Becton-Dickinson (Bedford, MA, USA) unless otherwise stated.

In vitro maturation of oocytes
In vitro maturation (IVM) of goat oocytes was carried out as described earlier by Malakar and Majumdar.
Briefly, goat ovaries were collected from local abattoir and transported to laboratory in a thermoflask containing 0.9% normal warm (32–35°C) sterile saline fortified with antibiotics (50 µg/ml gentamicin sulphate) within 3 h. Then, the ovaries were washed 4–5 times with normal saline supplemented with antibiotics (50 µg/ml gentamicin sulphate). Cumulus oocyte complexes (COCs) were aspirated by puncturing method in aspiration medium consisting of TCM-199 (HEPES modified) and bovine serum albumin (BSA) (0.3% w/v). COCs with three or more than three cumulus layers and homogeneous ooplasm were taken for IVM. COCs were washed two times with washing medium containing TCM 199 (HEPES modified), 10% foetal calf serum (FCS), 50 µg/ml sodium pyruvate, 3.5 µg/ml l-glutamine and 50 µg/ml gentamicin sulphate and three times with maturation medium containing TCM-199 (HEPES modified), 10% foetal calf serum (FCS), 50 µg/ml sodium pyruvate, 3.5 µg/ml l-glutamine and 50 µg/ml gentamicin sulphate and three times with maturation medium containing TCM 199 (HEPES modified), 10% foetal calf serum (FCS), 50 µg/ml sodium pyruvate, 3.5 µg/ml l-glutamine and 50 µg/ml gentamicin sulphate and three times with maturation medium containing TCM-199 (HEPES modified), 10% foetal calf serum (FCS), 50 µg/ml sodium pyruvate, 3.5 µg/ml l-glutamine and 50 µg/ml gentamicin sulphate and three times with maturation medium containing TCM-199 (HEPES modified), 10% foetal calf serum (FCS), 50 µg/ml sodium pyruvate, 3.5 µg/ml l-glutamine and 50 µg/ml gentamicin sulphate and three times with maturation medium containing TCM-199 (HEPES modified), 10% foetal calf serum (FCS), 50 µg/ml sodium pyruvate, 3.5 µg/ml l-glutamine and 50 µg/ml gentamicin sulphate.

Chemically assisted handmade enucleation (CAHE)

Once maturation period was completed, COCs with expanded cumulus cells were removed from the maturation droplet, and cumulus cells were removed by pipetting and vortexing in 0.5 mg/ml hyaluronidase. Denuded oocytes (15 per well) were incubated in maturation medium containing 0.5 µg/ml demecolcine for 2 h at 38.5°C in 5% CO2 in humidified air. Then, oocytes were washed first in T2 where T denotes HEPES-modified M-199 supplemented with 2.0 mM l-glutamine, 0.2 mM sodium pyruvate, 50 µg/ml gentamicin and the following number denotes 2% foetal bovine serum (FBS), then subsequently transferred to T10 (T containing 10% FBS) containing 2 mg/ml pronase for 5–8 min. The oocytes with completely digested zona pellucida were transferred to T20 (T containing 20% FBS) and incubated at 38.5°C until a prominent protrusion cone (Fig. 1A) was clearly observed. The protrusion cone containing oocytes was transferred into a 35-mm dish containing 2.5 µg/ml cytochalasin-B and mechanically bisected using an ultra sharp splitting blade (SurgeEdge®) under stereo zoom microscope (Olympus, Japan) in such a way that the protrusion cone remains in the smaller half (Fig. 1B). The larger demi-oocytes without protrusion cone were transferred into T20 and incubated for 15 min to regain spherical shape.

Oriented handmade enucleation without demecolcine treatment (OHE)

All steps were similar to the previously described procedure, but demecolcine pre-treatment was not applied.

Fig. 1. (A) Protrusion cone produced on the surface of zona-free in vitro matured goat oocytes. (B) Bisection of zona-free matured goat oocytes with sharp blade for enucleation. (C) Nuclear material present in protrusion cone of matured goat oocytes visualized after Hoechst staining under UV light. (D) Four cells stage handmade cloning embryo in well of well (WOW) system. (E) Morula produced in WOW system. (F) Blastocysts produced with chemically assisted handmade enucleation.
Random handmade bisection for enucleation (RHE)

Demecolcine pre-incubation was omitted from pre-treatment of this group. COCs with expanded cumulus cells were removed from the maturation droplet and pipetted vigorously in TCM-199 medium supplemented with 25 mM HEPES and 5% FCS to remove the cumulus cells. Completely denuded oocytes with evenly granular ooplasm were incubated with pronase (2 mg/ml) in T10 for 8 min at 38.5°C. The oocytes with completely digested zona pellucida were transferred to T20 containing 2.5 μg/ml cytochalasin-B, and random handmade equal bisection was applied using an ultra-sharp splitting blade (SurgeEdge, Munder stereo zoom microscope (Olympus). All demi-oocytes were selected and stained with 10 μg/ml Hoechst 33342 in T2 drops for 10 min, then placed into 1-μl drops of T2 medium covered with mineral oil. Using an inverted microscope with UV light, the positions of chromatin-free demi-oocytes were identified (Fig. 1C) and stored in T2 drops.

Pairing and fusion

The enucleated demi-oocytes were incubated in PHA (0.5 mg/ml in T2) for 3–4 s and transferred into T2 containing low-density donor cells prepared and stored during the 10–15 min incubation interval for rounding of demi-oocytes. Each demi-oocyte was then allowed to attach to a single-, rounded-, medium-sized cell by gently rolling the demi-oocyte over it. The couplets (demi-oocyte–donor cell pairs) were transferred to fusion medium containing 0.3 mM mannitol, 0.1 mM MgSO₄, 0.05 mM CaCl₂, and 3 mg/ml BSA for equilibration. Electrofusion of the couplets and triplets (demi-oocyte–somatic cell–demi-oocyte) was then carried out by single-step method. The couplets and the remaining demi-oocytes were then transferred to northern and southern parts, respectively, of the fusion chamber (BTX micro slide 0.5-mm gap, model 450; BTX, San Diego, CA, USA) containing the fusion medium. The demi-oocyte and a couplet were picked up using a fine-tipped capillary pipette (Unopette, Becton-Dickinson, NJ, USA) having an inner diameter of 100–120 μm. Initially, the couplet was expelled and aligned with an alternate current (AC) pulse (7 volts) using BTX Electrocell Manipulator 200 (BTX), so that the somatic cell faces the negative electrode. Immediately after alignment, another demi-oocyte was introduced into the fusion chamber close to the somatic cell. As soon as the somatic cell was sandwiched between the demi-oocytes, a double direct current (DC) pulse of 2.31 kV s was applied. The triplets were then incubated in T20 for treatment at 38.5°C for rounding up and subsequent reprogramming.

Activation

The reconstructed oocytes were activated after 4 h of fusion, using 2 μM Ca ionophore in T20 for 5 min at 38.5°C. Then, they were washed thrice in T20 and incubated individually in 5-μl droplets of T20 containing 2 mM 6-dimethylaminopurine (6-DMAP) in 3% CO₂ in air at 38.5°C for 3 h.

Culture of in vitro cloned embryo

The activated reconstructed embryos were washed with embryo development medium (EDM) containing TCM-199 (HEPES modified), 10% FCS, essential and non-essential amino acids and 10 mg/ml BSA and cultured individually into well of wells (WOW) system (Vajta et al. 2001) – handmade microwells of 300 μm wide and 300 μm deep prepared with the help of a smooth, ‘V’-shaped darning needles (Booth et al. 2001) in a well of a four-well dish (Nunc; Roskilde, Denmark), containing 400 μl of embryo development medium. The WOWs were prepared a day before in vitro culture (IVC) of embryos, rinsed by pipetting and replaced with fresh medium just before IVC and covered with 400 μl of mineral oil.

Quality assessment of HMC embryos and determination of blastocyst cell number

Rates of embryo development in terms of cleavage and blastocyst formation were observed on day 7 of IVC and percent development of each stage determined. The health of the blastocysts was determined by counting the cell numbers of blastocysts by differential staining as described by Thouas et al. (2001). The blastocysts were incubated in sol I (DPBS with 1% Triton X-100 and 100 μg/ml propidium iodine) for 15 s, then immediately transferred to 500 μl of solution II (fixative solution of 100% ethanol with 25 μg/ml Hoechst 33342) and incubated at 4°C overnight. The cell number was counted using an inverted microscope (Nikon) fitted with an UV lamp and excitation filters.

Experiment 1: Time-dependent occurrence of extrusion cones

After maturation, a total of 200 morphologically intact oocytes were incubated in demecolcine and then subjected to pronase digestion. The presence of detectable extrusion cones was registered at 5, 15, 25, 35, 45 and 55 min after end of pronase digestion.

Experiment 2: Comparison of the efficiency of CAHE vs. OHE

The efficiency of CAHE was tested using 250 oocytes (four replicates). After IVM, oocytes were incubated with demecolcine. Oriented bisection was performed in oocytes where an extrusion cone was detected after partial pronase digestion. Percentages of bisected versus total oocytes and surviving versus bisected oocytes were recorded. Subsequently, both putative cytoplasts and karyoplasts were collected separately and presence or absence of chromatin was detected by Hoechst 33342 staining.

The efficiency OHE was investigated using 225 oocytes (four replicates). After IVM, oriented bisection was performed in matured oocytes where an extrusion cone and/or a PB were detected after partial pronase digestion. Results were evaluated the same way as described in case of CAHE.
Experiment 3: Comparison of the in vitro development of embryos produced with CAHE, OHE and RHE

A total of 485 in vitro matured oocytes (in four replicates) were randomly distributed into three groups and were subjected to three different enucleation procedures: CAHE, OHE, RHE. Fusion rates between cytoplasts and donor fibroblasts were recorded. Cleavage and blastocyst development rate were recorded on day 2 and day 7, respectively.

Statistical analysis
The data were analysed using SYSTAT 7.0 (SPSS Inc., Chicago, IL, USA) after arcsin transformation. Differences between means were analysed by one-way ANOVA followed by Fisher’s LSD test. Significance was determined at p < 0.05.

Results
Experiment 1
The results of Experiment 1 are shown in Table 1. The mean number of oocytes after 25 min of pronase digestion was significantly higher than the other groups. It showed that the extrusion process is time dependent (Fig. 1A).

Experiment 2
Results of the Experiment 2 are shown in Table 2. No significant difference between CAHE and OHE was found in oocyte bisection efficiency (Fig. 1B). The ratio of successful enucleation per bisected oocyte (cytoplasts/bisected oocyte) was significantly higher in CAHE than OHE.

The overall efficiency measured as the number of cytoplasts obtained per total number of oocytes used was also significantly higher in CAHE than in OHE group.

Experiment 3
Results of Experiment 3 were shown in Table 3. No significant difference was found among the treatments in respect of fusion rate, cleavage rate (Fig. 1D), blastocysts rate (Fig. 1F) and cells per blastocysts.

Discussion
Among the various factors that influence the efficiency of somatic cell nuclear transfer (SCNT), the removal of chromosomal material from oocytes is an important one. In most domestic species, a large amount of cytoplasmic lipid droplets hamper visualization of chromatin. So, some orientation points are required for successful enucleation. In traditional cloning, PB-oriented enucleation is the most common approach assuming that the second meiotic metaphase (MII) spindle and chromosomes lie in close proximity to first polar body (FPB). But the FPB does not accurately predict the location of MII meiotic spindle and chromosome in mammalian species (Hewitson et al. 1999; Silva et al. 1999 and Miao et al. 2004). It has been observed that up to 30% of zona included oocytes are improperly enucleated using this technique (Prather et al. 1989; Dominko et al. 1999; Mitalipov et al. 1999 and Nour and Takahashi 1999).

Though chemically induced enucleation has been attempted repeatedly in experimental and domestic animals since the early 1990s (Fulka and Moor 1993), the efficiency remained very low. It has been seen that treatment of metaphase-II phase oocytes with certain cytoskeleton-relaxing agents induce a visible extrusion cone on the surface. This is the basis of chemically assisted enucleation. Chemically assisted enucleation has been successfully applied to porcine and bovine oocytes to prepare recipient cytoplasts for nuclear transfer procedures (Saraiva et al. 2009). Demecolcine...
treatment produces a membrane protrusion in metaphase II-stage matured oocytes. The maternal chromosome mass is condensed within the protrusion cone, which makes it easy to remove the maternal chromosomes for nuclear transfer (NT). Demecolcine treatment did not increase the potential of NT oocytes to develop into blastocysts, but demecolcine-assisted removal of chromosomes is effective for bovine cloning (Tani et al. 2006). Matured goat oocyte enucleation has demonstrated that treatment with 0.8 ng/ml demecolcine induces cytoplasmic protrusions in over 90% of the oocytes. Moreover, oocyte selection and enucleation can be achieved simultaneously with demecolcine treatment (Lan et al. 2008). The protrusion formation rates as high as 84% in mouse and 92% in goat oocytes were reported (Borges et al. 2009). Treatment of mouse oocytes with demecolcine had no effect on their in vitro development after parthenogenetic activation, or on their ability to repolymerize a new spindle after the removal of the drug or the reconstruction of the treated cytoplasm with a somatic nucleus. Therefore, demecolcine-assisted enucleation appears as an efficient alternative to mechanical enucleation, which can simplify nuclear transfer procedures (Borges et al. 2009). Chemically assisted enucleation has been used successfully in both micro- and manipulator-based SCNT (Yin et al. 2002a; Peura 2003; Li et al. 2004) as well as zona-free HMC (Peura 2003 and Vajta et al. 2005). The cell numbers of blastocysts in colcemid-treated oocytes were numerically higher than untreated oocytes, and higher pregnancy rates were reported from the colcemid-treated oocytes. Moreover, significantly higher rates of full-term pregnancies leading to successful birth of healthy calves were reported in colcemid-treated oocytes (Li et al. 2009).

In the present investigation, demecolcine-assisted enucleation did not compromise in vitro developmental rates of HMC goat embryos. Goat embryos reconstructed by CAHE had the same developmental competence and as many nuclei in blastocyst stage as those produced with OHE and RHE. We did not undertake any experiment to evaluate whether enucleation by CAHE compromises the in vivo development of goat embryos. Earlier studies (Yin et al. 2002b) showed that demecolcine-assisted enucleation did not compromise in vivo development. A significant increase in number of oocytes with extrusion cones was observed at 15 and 25 min after pronase digestion (Fig. 1A). The results of the study were in consistent with the observations reported in cattle (Vajta et al. 2005). The chemically assisted enucleation was less time consuming and was less expensive because inverted fluorescent microscope was no longer required.

In conclusion, an efficient chemically assisted enucleation procedure was established for goat HMC.

Acknowledgement

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Conflict of interest

Yogesh S. Akshey: cloning experiment; Dhruba Malakar: design and guidance of project; Arun Kumar De: culture research work; Manoj Kumar Jena: data analysis; Shailendra Sahu: drafting research paper; Rahul Dutta: culture research work.

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