

Chemically induced enucleation of activated bovine oocytes: chromatin and microtubule organization and production of viable cytoplasts

Naiara Zoccal Saraiva^{1,2}, Clara Slade Oliveira³, Cláudia Lima Verde Leal⁴, Marina Ragagnin de Lima², Maite Del Collado^{2,4}, Roberta Vantini², Fabio Morato Monteiro⁵, Simone Cristina Méo Niciura⁶ and Joaquim Mansano Garcia²

Embrapa Amazônia Oriental, Belém; Universidade Estadual Paulista, Jaboticabal; Embrapa Dairy Cattle, Valença; Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga; Instituto de Zootecnia, Sertãozinho; and Embrapa Southeast Livestock, São Carlos, Brazil

Date submitted: 13.3.2013. Date revised: 1.7.2014. Date accepted: 18.8.2014

Summary

As the standard enucleation method in mammalian nuclear transfer is invasive and damaging to cytoplasm spatial organization, alternative procedures have been developed over recent years. Among these techniques, chemically induced enucleation (IE) is especially interesting because it does not employ ultraviolet light and reduces the amount of cytoplasm eliminated during the procedure. The objective of this study was to optimize the culture conditions with demecolcine of pre-activated bovine oocytes for chemically IE, and to evaluate nuclear and microtubule organization in cytoplasts obtained by this technique and their viability. In the first experiment, a negative effect on oocyte activation was verified when demecolcine was added at the beginning of the process, reducing activation rates by approximately 30%. This effect was not observed when demecolcine was added to the medium after 1.5 h of activation. In the second experiment, although a reduction in the number of microtubules was observed in most oocytes, these structures did not disappear completely during assessment. Approximately 50% of treated oocytes presented microtubule reduction at the end of the evaluation period, while 23% of oocytes were observed to exhibit the complete disappearance of these structures and 28% exhibited visible microtubules. These findings indicated the lack of immediate microtubule repolymerization after culture in demecolcine-free medium, a fact that may negatively influence embryonic development. However, cleavage rates of 63.6–70.0% and blastocyst yield of 15.5–24.2% were obtained in the final experiment, without significant differences between techniques, indicating that chemically induced enucleation produces normal embryos.

Keywords: Bovine, Chemically induced enucleation, Chromatin; Microtubule, Nuclear transfer

¹All correspondence to: Naiara Zoccal Saraiva. Embrapa Amazônia Oriental, Trav. Dr. Enéas Pinheiro, s/no, Caixa Postal 48, CEP 66095–100, Belém, PA, Brazil. Tel: +55 91 3204 1113. e-mail: naiara.saraiva@embrapa.br

²Departamento de Medicina Veterinária Preventiva e Reprodução Animal, Universidade Estadual Paulista, Jaboticabal, Brazil.

³Embrapa Dairy Cattle, Valença, Brazil.

⁴Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, Brazil.

⁵Centro APTA Bovinos de Corte, Instituto de Zootecnia, Sertãozinho, SP, Brazil.

⁶Embrapa Southeast Livestock, São Carlos, Brazil.

Introduction

Somatic cell nuclear transfer has been used extensively as a valuable tool for the production of animal clones from adult somatic cells and for the generation of transgenic animals for agricultural and biomedical research. However, the frequency of early postimplantation developmental arrest and abortion is high, especially in bovine (Hill *et al.*, 2000). Biological and technical aspects related to somatic cell nuclear transfer may account for the limited success of the technique; oocyte enucleation is one of the crucial

technical steps that need to be improved. Successful reprogramming of the donor nuclei depends on the sufficient supply of factors in the recipient oocyte. In this respect, any cytoplasmic alterations such as changes in nuclear maturation status, microtubule organization and microfilaments, or biochemical and physiological changes can potentially affect nuclear reprogramming and result in cloning failure (Li *et al.*, 2009).

The standard method of oocyte enucleation is time consuming, technically demanding, invasive and damaging to cytoplasmic spatial organization, including the loss of molecular factors that play a key role in subsequent oocyte activation, reprogramming of the transferred nucleus, mitotic spindle assembly, and early embryo development (Simerly *et al.*, 2003; Miyara *et al.*, 2006; Van Thuan *et al.*, 2006). Furthermore, although nuclear DNA is removed during oocyte enucleation, there is the possibility of damage to mitochondrial DNA (Fischer-Russel *et al.*, 2005). Therefore, simplification of this step is constantly a center of interest for scientists involved in the production of clones (Fulka *et al.*, 2004).

Chemical enucleation using microtubule-depolymerizing drugs is an attractive procedure that has been used to simplify conventional enucleation methods. In chemically assisted enucleation (Yin *et al.*, 2002a,b), metaphase II oocytes are exposed to an antimitotic drug and spindle depolymerization leads to the formation of a cortical protrusion on the surface of the oocyte that contains all oocyte chromosomes. This protrusion can be easily removed by micromanipulation techniques (Yin *et al.*, 2002a) or by oocyte bisection (handmade cloning; Vajta *et al.*, 2003). Anti-mitotic drugs have also been applied to pre-activated oocytes, resulting in the extrusion of a second polar body (PB) that contains all oocyte chromosomes. This technique is called chemically induced enucleation (IE).

In chemically IE, the treatment of pre-activated oocytes with high concentrations of demecolcine (0.4 to 0.5 $\mu\text{g}/\text{ml}$), a microtubule-depolymerizing drug, has been proven to be effective for oocyte enucleation in mice (Baguisi & Overström, 2000; Gasparrini *et al.*, 2003; Ibáñez *et al.*, 2003), goats (Ibáñez *et al.*, 2002), and cattle (Fischer-Russel *et al.*, 2005). Although mouse offspring were produced by nuclear transfer using this protocol (Baguisi & Overström, 2000; Gasparrini *et al.*, 2003), the percentage of embryos that developed to blastocyst stage was much lower among chemically enucleated nuclear transfer embryos than in those enucleated by conventional mechanical methods (Gasparrini *et al.*, 2003).

To our knowledge, there has been to date only one report using chemically IE in cattle, and in which the authors employed intracytoplasmic injection for

embryo reconstitution (Fischer-Russel *et al.*, 2005). An understanding of the mechanism of chemically IE and modifications of the current protocol would lead to an increase in the frequency of induced enucleation and thus minimize the invasiveness of currently employed enucleation procedures. As further data are needed to fine tune the dose and duration of exposure to demecolcine in combination with modifications of the oocyte activation protocol (Fischer-Russel *et al.*, 2005), the objective of the present study was to optimize the culture conditions of bovine oocytes exposed to demecolcine after oocyte activation in an attempt to improve the efficiency of chemically IE, and to evaluate tubulin and chromatin organization and the viability of cytoplasts produced by this technique.

Material and methods

Chemicals

Chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Experimental design

Experiment I: Evaluation of different culture conditions with demecolcine for chemically induced enucleation

First, different experiments were conducted to standardize the chemically IE technique in bovine. Oocytes were treated with demecolcine at the beginning (Experiment Ia) or after the onset (Experiment Ib) of the activation process.

Experiment Ia: Exposure to demecolcine at the beginning of parthenogenetic activation. Oocytes matured *in vitro* for 26 h were denuded and activated artificially with ionomycin (5 μM for 5 min) and cycloheximide (10 $\mu\text{g}/\text{ml}$ for up to 5 h). Three groups were established: (1) a control (not activated and not treated with demecolcine); (2) an activated (exposed only to the oocyte-activating agents); and (3) a treated group (DEME; exposed to the oocyte-activating agents and 0.05 $\mu\text{g}/\text{ml}$ demecolcine). Treatment with demecolcine was initiated at the beginning of oocyte activation and samples were collected in quintuplicate from the three groups after 0, 4 or 5 h of treatment. The oocytes were stained with 10 $\mu\text{g}/\text{ml}$ Hoechst 33342 for 15 min and the percentage of metaphase II oocytes and activation and enucleation rates was determined under an epifluorescence microscope (330–385 nm).

Experiment Ib: Exposure to demecolcine after the onset of activation. After 26 h of *in vitro* maturation, oocytes were denuded and activated as described in Experiment Ia for up to 4 h. Control, activated and demecolcine-treated (DEME) groups were also defined. However, in the DEME group the activation

lasted for up to 4 h and treatment with 0.05 $\mu\text{g}/\text{ml}$ demecolcine was started 0, 0.5, 1.0, 1.5, or 2.0 h after the onset of oocyte activation. Therefore, the following subgroups were established: (1) DEME 0–2 h (exposure to demecolcine between 0 and 2 h of activation); (2) DEME 0–4 h; (3) DEME 0.5–2.5 h; (4) DEME 0.5–4 h; (5) DEME 1–3 h; (6) DEME 1–4 h; (7) DEME 1.5–3.5 h; (8) DEME 1.5–4 h, and (9) DEME 2–4 h. Quintuplicate samples were obtained. The oocytes were stained with 10 $\mu\text{g}/\text{ml}$ Hoechst 33342 for 15 min and activation and enucleation rates were determined under an epifluorescence microscope (330–385 nm).

Experiment II: Tubulin and chromatin organization in parthenogenetically activated oocytes submitted to chemically induced enucleation

In the third experiment, we evaluated the effects of demecolcine on nuclear and microtubule organization of activated oocytes submitted to chemically IE. For this purpose, three groups were defined as described before (control, activated, and treated with demecolcine–DEME). In the DEME group, activated oocytes were exposed to the drug in the last 2 h of activation (DEME 2–4 h group). The samples were fixed and the configuration of chromatin and meiotic spindle (microtubules) was evaluated in triplicate at 0, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, or 10 h after the onset of activation.

Experiment III: Early development of embryos reconstituted by nuclear transfer from chemically enucleated cytoplasts

In the final experiment, after chemical or conventional enucleation, part of the cytoplasts were fixed in 4% paraformaldehyde for 1 h at room temperature and stored in phosphate-buffered saline (PBS) + 1% PVP for evaluation of the efficiency of enucleation by staining with Hoechst 33342 for 15 min and observation under an epifluorescence microscope (330–385 nm). The remaining cytoplasts were reconstituted by nuclear transfer (NT) and presumptive zygotes were cultured *in vitro*. The rates of embryo development were determined after 48 h (cleavage) and on day 7 of culture (blastocysts).

Oocyte recovery and *in vitro* maturation

Bovine oocytes were obtained by follicular aspiration from ovaries obtained at a local slaughterhouse. The ovaries were transported to the laboratory in 0.9% saline solution at 30–35°C. Follicles with diameters between 3 and 8 mm were aspirated using an 18-gauge needle attached to a 20-ml syringe and cumulus oocyte complexes (COCs) with at least three layers of cumulus cells and homogenous cytoplasm were selected. The COCs were washed in HEPES-buffered tissue culture medium-199 (TCM-199; Gibco BRL, Grand Island, NY,

USA) supplemented with 10% fetal calf serum (FCS), 0.20 mM sodium pyruvate, and 83.4 $\mu\text{g}/\text{ml}$ amikacin (Instituto Biochimico, Rio de Janeiro, Brazil). Groups of 15 COCs were matured in droplets (100 μL) of TCM-199 supplemented with 10% FCS, 1.0 $\mu\text{g}/\text{ml}$ FSH, 50 $\mu\text{g}/\text{ml}$ hCG (ProfasiTM, Serono, São Paulo, Brazil), 1.0 $\mu\text{g}/\text{ml}$ estradiol, 0.20 mM sodium pyruvate, and 83.4 $\mu\text{g}/\text{ml}$ amikacin under mineral oil (Dow Corning Co., Midland, MI, USA) for up to 26 h at 38.5°C in an atmosphere of 5% CO₂ in air and maximum humidity.

Isolation of nucleus donor cells

Blastomeres were used as donor nucleus cells in the NT procedures. Cells were obtained from embryos produced *in vitro* with female and male sexed semen. The *in vitro* fertilization procedure was performed 24 h after *in vitro* maturation. For this purpose, groups of 15 oocytes were washed twice and transferred to 30 μL drops of TALP-IVF medium supplemented with 0.6% bovine serum albumin (BSA), 10 $\mu\text{g}/\text{ml}$ heparin, 18 μM penicillamine, 10 μM hypotaurine and 1.8 μM epinephrine, and covered with sterile mineral oil. Frozen straws of semen from the same bull sexed by flow cytometry (Lagoa da Serra, Sertãozinho, Brazil) were used. Each straw containing approximately 2 million spermatozoa was centrifuged separately on a discontinuous 45/90 Percoll gradient for 7 min at 3600 g. The pellet was resuspended in 500 μL TALP-IVF medium and again centrifuged for 5 min at 520 g. After centrifugation, 100 μL of the medium containing the pellet was collected from the bottom of the tube and homogenized in a conic tube. The final suspension was divided among 10 oocyte-containing drops, at a final concentration of approximately 10⁴ spermatozoa per oocyte. The plates were incubated for 18–20 h at 38.5°C in an atmosphere of 5% CO₂ under saturated humidity. After this period, embryos were cultured in synthetic oviductal fluid (SOF) supplemented with 2.5% FCS and 5 mg/ml BSA at 38.5°C in a humidified atmosphere of 5% CO₂ in air. Groups of 10–20 1-cell embryos were cultured in 100 μL microdrops of culture medium for 5 days. The medium was renewed on the third day of embryo culture.

For the isolation of blastomeres used for reconstitution by NT from cytoplasts enucleated by the conventional technique, day-5 embryos were cultured in SOF medium containing 0.4 $\mu\text{g}/\text{ml}$ demecolcine for 12 h to synchronize cells in the metaphase stage. Approximately 3 h before the beginning of micromanipulation, the embryos were washed several times and cultured in demecolcine-free SOF medium so that at the time of use the cells were in G₁, a stage compatible with oocytes exhibiting high activity of maturation-promoting factor (MPF). For the chemical enucleation technique, in which activated oocytes

(low MPF activity) are used, the embryos were not synchronized as about 80% of the cells are naturally in the S phase of the cell cycle (Campbell *et al.*, 1996).

At the time of use, the zona pellucida was removed from the embryos by incubation in 0.5% pronase for 30 s. Next, the embryos were transferred to microdroplets of Ca²⁺- and Mg²⁺-free PBS containing 0.3% PVA and disaggregated into single blastomeres with a pipette. Blastomeres derived from early and compact morulae were used. The blastomeres were incubated for 30 min in 7.5 µg/ml cytochalasin B and then transferred to droplets for micromanipulation and used as donor nucleus cells.

Nuclear transfer procedure

Oocyte enucleation techniques

For conventional oocyte enucleation, oocytes matured *in vitro* for 18 to 20 h were used. After denuding with 2 mg/ml hyaluronidase, the structures were incubated for 30 min at 38.5°C in SOF medium with HEPES buffer (HSOF) supplemented with 10% FCS, 7.5 µg/ml cytochalasin B, and 7.5 µg/ml Hoechst 33342. Enucleation was performed under an inverted light microscope (Olympus IX-70), removing the first PB and part of the adjacent cytoplasm. Enucleation was confirmed by examining the oocyte under ultraviolet light to determine the absence of the metaphasic plate.

For chemically IE, oocytes matured *in vitro* for 26 h were denuded and activated artificially (5 µM ionomycin for 5 min and 10 µg/ml cycloheximide for 5 h). After 1.5 h of activation, the oocytes were treated with demecolcine (0.05 µg/ml) for complete extrusion of the nuclear material of the oocyte together with the second PB. At the end of treatment, the second PB and a small part of the adjacent cytoplasm were removed by micromanipulation and the cytoplasts were subsequently used for NT.

Embryo reconstitution

Embryonic cells were individually transferred to the perivitelline space of each recipient cytoplast and cytoplast–cell complexes were electrofused in 0.3 M mannitol solution containing 0.05 mM CaCl₂, 0.1 mM MgSO₄ and 0.1% PVA. Electrofusion was induced by a direct pulse of 1.5 kV/cm for 70 µs (Bordignon *et al.*, 1999) and successfully reconstituted structures were incubated in SOF + 10% FCS at 38.5°C in a humidified atmosphere of 5% CO₂ in air.

Parthenogenetic activation

Only reconstituted oocytes obtained by the conventional technique were activated at the end of the procedure, whereas in chemically IE oocytes were activated at the beginning of the protocol. The oocytes were chemically activated with 5 µM ionomycin in

HSOF supplemented with 10% FCS for 5 min and with 10 µg/ml cycloheximide + 10 µg/ml cytochalasin B in SOF supplemented with 10% FCS for 5 h at 38.5°C in a humidified atmosphere of 5% CO₂ in air. Next, embryos were cultured until day 7.

Immunofluorescence analysis of tubulin and chromatin

The protocol described by Liu *et al.* (1998) was used. Briefly, oocytes were incubated for 30 min in microtubule-stabilizing buffer (0.1 M PIPES, pH 6.9, 5 mM MgCl₂·6H₂O (Merck, Darmstadt, Germany), 2.5 mM EGTA) containing 2% formaldehyde, 0.1% Triton X-100, 1 µM Taxol, 0.01% aprotinin, 1 mM dithiothreitol, and 50% deuterium oxide (Aldrich Chem. Co., Milwaukee, WI, USA). Next, oocytes were kept overnight at 4°C in washing medium (WM; PBS with 0.02% NaN₃, 0.01% Triton X-100, 0.2% non-fat dry milk, 2% FCS, 2% BSA, and 0.1 M glycine) and were then incubated in WM containing anti-α-tubulin mouse monoclonal antibody (1:50) conjugated with fluorescein isothiocyanate (FITC) for 2 h at 38°C. After washing in WM, oocytes were stained with 10 µg/ml Hoechst 33342 in WM for 10 min, mounted on slides with 90% glycerol in PBS, and examined under an epifluorescence microscope at 330–385 and 420–490 nm. For the evaluation of nuclear kinetics, the oocytes were classified as immature, metaphase II (MII) or activated (AII/TII with extrusion of the 2nd PB or pronucleus stage) (Fig. 1). In addition, the oocytes were classified according to microtubule organization (present, reduced, or absent) as described in Fig. 2.

Statistical analysis

The rates of activation, enucleation and embryo development were analyzed by chi-squared (χ^2) test or Fisher's exact test, when appropriate. The percentages obtained in Experiment II were submitted to analysis of variance (ANOVA) and means were compared by the Tukey test. All analyses were performed using Statistical Analysis System (SAS) 9.1 software, adopting a level of significance of 5%.

Results

Addition of demecolcine at the beginning of parthenogenetic activation

At the beginning of assessment (0 h), most oocytes were at the MII stage (133/163, 81.6%). A significant increase in the percentage of oocytes with extrusion of the second PB was observed in the groups activated for 4 or 5 h. These groups did not differ from one another

Table 1 Percentage of metaphase II (MII), activated, and enucleated (EN) oocytes in the control group (CON), group activated with 5 μ M ionomycin for 5 min and 10 μ g/ml cycloheximide (ACTIV), and group activated and treated with 0.05 μ g/ml demecolcine (DEME) for 0, 4 and 5 h

Time (h)	Treatments	MII (%)	Activated (%)	EN (%)	Total
0	–	133 (81.6) ^a	02 (1.3) ^c	01 (0.6) ^c	163
4	CON	121 (80.7) ^a	04 (2.7) ^c	0 ^c	150
	ACTIV	31 (19.6) ^b	97 (61.4) ^a	07 (4.4) ^b	158
5	DEME	26 (17.7) ^b	53 (36.0) ^b	58 (39.4) ^a	147
	CON	126 (79.2) ^a	05 (3.1) ^c	0 ^c	159
	ACTIV	34 (21.6) ^b	100 (63.7) ^a	13 (8.3) ^b	157
	DEME	29 (18.9) ^b	47 (30.7) ^b	64 (41.8) ^a	153

^{a,b,c}Values with different superscript letters within the same column differ ($P < 0.05$, chi-squared test).



Figure 1 Evaluation of nuclear kinetics. Photomicrographs of activated bovine oocytes treated with demecolcine. Categories of oocytes observed: (a) metaphase I (MI) oocyte; (b) metaphase II (MII) oocyte; (c) anaphase/telophase II (AII/TII) oocyte. Oocytes were stained with Hoechst 33342 and observed under an epifluorescence microscope. The arrows indicate nuclear material (blue). Bar = 50 μ m.

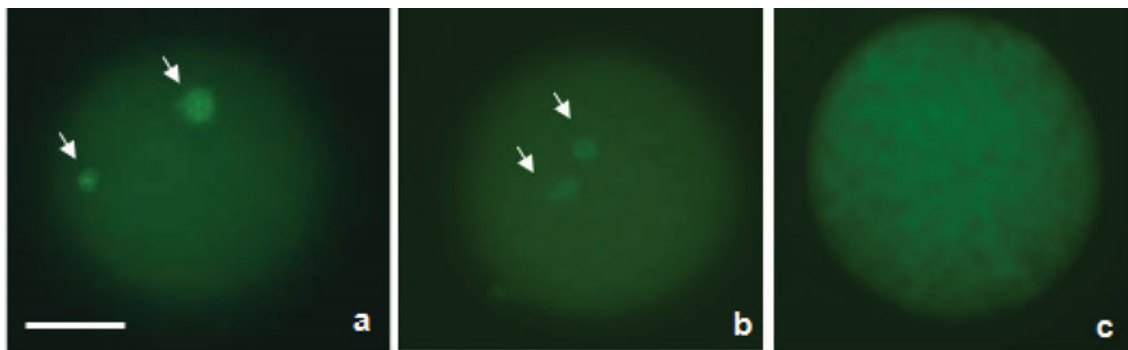


Figure 2 Evaluation of microtubule configuration. Photomicrographs of activated bovine oocytes treated with demecolcine. Categories of oocytes observed: (a) oocyte with microtubules (MTs); (b) oocyte with reduced MTs; (c) oocyte without MTs. Oocytes were stained with FITC-conjugated anti- α tubulin antibody (1:50) and observed under an epifluorescence microscope. The arrows indicate MTs (green). Bar = 50 μ m.

and the percentage of oocytes was higher than in the groups treated with demecolcine (Table 1).

Enucleation rates were higher in the DEME group ($P < 0.05$) than in the control and activated groups after 4 and 5 h of activation (Table 1), which also differed from one another. However, demecolcine probably interfered with oocyte activation, resulting in low enucleation rates (39.4–41.8%). No significant

difference was observed between groups treated with demecolcine for 4 and 5 h (Table 1).

Addition of demecolcine after the onset of parthenogenetic activation

Demecolcine exerted no damaging effects on oocyte activation when added to the medium 1.5 h after

Table 2 Percentage of activated oocytes after incubation in medium containing 5 μ M ionomycin for 5 min and 10 μ g/ml cycloheximide for 4 h and different times of treatment with demecolcine: DEME 0–2 h (exposure to demecolcine between 0 and 2 h of activation); DEME 0–4 h; DEME 0.5–2.5 h; DEME 0.5–4 h; DEME 1–3 h; DEME 1–4 h; DEME 1.5–3.5 h; DEME 1.5–4 h; DEME 2–4 h

Treatments	Activated (%)	Total
CON	01 (0.9)*	114
ACTIV	97 (74.6) ^{a,b}	130
DEME 0–2 h	42 (38.9)*	108
DEME 0–4 h	36 (32.7)*	110
DEME 0.5–2.5 h	59 (54.1)*	109
DEME 0.5–4 h	56 (48.7)*	115
DEME 1–3 h	69 (59.0)*	117
DEME 1–4 h	62 (55.8)*	111
DEME 1.5–3.5 h	64 (62.7) ^a	102
DEME 1.5–4 h	76 (69.7) ^{a,b}	109
DEME 2–4 h	87 (77.0) ^b	113

*The asterisk indicates difference in relation to activated group.

^{a,b}Values followed by the same letter between rows did not differ significantly ($P > 0.05$, chi-squared test).

ACTIV: activated group; CON: control group.

the beginning of the process, with no significant differences ($P > 0.05$) between the DEME 1.5–3.5 h, DEME 1.5–4 h or DEME 2–4 h groups and the group submitted to activation only (mean of 62.7–77.0%) (Table 2).

Enucleation rates differed significantly between all groups treated with demecolcine and the control (0/114) and activated (0/130) groups (Fig. 3). The DEME 1.5–4 h (42.2%) and DEME 2–4 h (54.0%) groups presented higher enucleation rates ($P < 0.05$) than all other groups (26.8–36.3%), but there was no difference between these two groups. As the percentage of activated and enucleated oocytes was higher in the DEME 2–4 h group, this treatment was chosen for the following experiments.

Evaluation of nuclear and microtubule kinetics in pre-activated oocytes treated with demecolcine

The frequency of immature oocytes was similar in the three groups (control, activated and DEME) throughout the period of evaluation (Fig. 4). Effects of activation were detected after 2 h, when the mean percentage of activated oocytes was higher in the activated and DEME groups in comparison to the control group (Fig. 4).

The percentage of oocytes with microtubules was significantly reduced ($P < 0.05$) in the DEME group when compared with the activated group 2.5 h after the onset of activation (0.5 h of exposure to demecolcine). After 10 h, lower mean percentages of oocytes

with microtubules were observed in the activated and DEME groups compared to control and this percentage also differed between the first two groups (Fig. 5). In parallel, there was a significant increase ($P < 0.05$) of oocytes with reduced microtubules in the DEME group 2.5 h after the onset of activation. This higher mean percentage was observed up to 4 h after the onset of activation when compared to the control and activated groups (Fig. 5).

Microtubule reduction was observed in approximately 50% of oocytes of the DEME group 10 h after the onset of activation. However, no significant difference was observed between the treated and activated groups, only when compared with the control group. In addition, 23% of oocytes displayed absent microtubules in the DEME group, a mean value higher than that observed in the control and activated groups ($P < 0.05$) (Fig. 5).

Early development of embryos reconstituted by nuclear transfer from cytoplasts produced by chemically induced enucleation

In relation to cleavage and blastocyst rates, no significant differences were observed between groups ($P > 0.05$) despite a tendency towards lower embryo production in the chemically enucleated group. As no significant difference was observed between sexes, the results of female and male embryos were pooled for each technique (Table 3).

Discussion

First, oocytes were exposed to demecolcine at the start of activation. However, as the enucleation rates were relatively low (39.45–42.38%), a second experiment was conducted in which demecolcine was added after the onset of oocyte activation and higher enucleation rates were obtained. No damaging effect of demecolcine on oocyte activation was observed when treatment was started 1.5 h after the exposure of oocytes to the activating agents, with a mean enucleation rate of 54%. Similar findings have been reported by Ibáñez *et al.* (2003) and Fischer-Russel *et al.* (2005) for mouse and bovine oocytes, respectively, and can be explained by the fact that demecolcine impairs spindle rotation in activated oocytes, an event that normally precedes formation of the PB. Thus, the beginning of demecolcine treatment in relation to oocyte activation is the key point for successful enucleation. The present results agree with previous studies showing that an intact spindle is required for the degradation of cyclin B and the consequent inactivation of MPF and exit from the M phase (Kubiak *et al.*, 1993).

Table 3 Number and percentage of cleaved embryos and blastocysts produced by nuclear transfer from donor embryo cells after conventional or chemically induced enucleation in bovine

Treatments	No. fused	No. cleaved (%)	No. blastocyst (%)
Conventional	66	42 (63.6)	16 (24.2)
Chemical-induced	90	63 (70.0)	14 (15.5)

No significant differences were observed between groups ($P > 0.05$, chi-squared test).

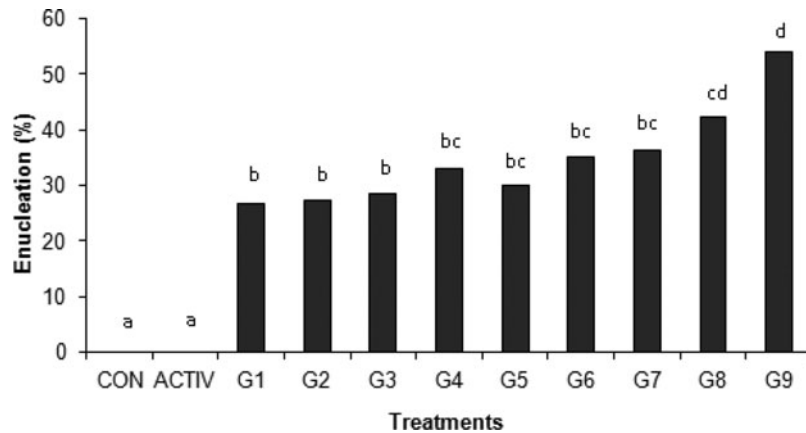


Figure 3 Graphic representation of the percentage of enucleated oocytes (EN) after activation with 5 μM ionomycin for 5 min and 10 $\mu\text{g/ml}$ cycloheximide for up to 4 h and different treatments with demecolcine: group 1 (DEME 0–2 h); group 2 (DEME 0–4 h); group 3 (DEME 0.5–2.5 h); group 4 (DEME 0.5–4 h); group 5 (DEME 1–3 h); group 6 (DEME 1–4 h); group 7 (DEME 1.5–3.5 h); group 8 (DEME 1.5–4 h); group 9 (DEME 2–4 h). ^{a–d}Columns followed by different superscript letters differ from one another ($P < 0.05$, chi-squared test). ACTIV: activated group; CON: control group.

The enucleation rates obtained in the present study were lower than those reported by Fischer-Russel *et al.* (2005) for activated bovine oocytes treated with demecolcine (up to 91.7%). However, the high rates reported by these authors were obtained 5 h after the onset of oocyte activation. A new assessment performed 17 h after the onset of activation showed a decline in enucleation, with rates ranging from 3.4–46.1%. These findings indicate that the chromosomes were reintegrated into the oocyte after incomplete extrusion of the second PB, demonstrating the inefficiency of the enucleation process in the absence of micromanipulation of these structures.

In this study, several oocytes contained subcortical nuclear material which was connected to the chromosomes present in the PB by a spindle remnant, a feature also observed in activated mouse oocytes (Ibáñez *et al.*, 2003). These oocytes were considered to be non-enucleated. Incomplete release of the PB may have occurred in these treated oocytes as a consequence of the lack of intact microtubules for the formation of contractile rings, which are responsible for cytokinesis (Larkin & Danilchik, 1999). However, since this material is juxtaposed to the PB, the latter can be easily removed by micromanipulation, facilitating the enucleation procedure and promoting an increase

of oocyte enucleation rates. In addition, we found that exposure to demecolcine can be interrupted after 1 h of treatment, contributing to the optimization of the current protocol, which recommends treatment for 2 h (Fischer-Russel *et al.*, 2005), reducing the time of exposure to the drug.

After optimization of the chemical enucleation protocol, we analyzed chromatin and microtubule kinetics in these activated oocytes treated with demecolcine. Previously our group observed that in MII bovine oocytes, the microtubule-depolymerizing action of demecolcine occurred after only 0.5 h of treatment, with the complete absence of these structures in most of the oocytes analyzed (Saraiva *et al.*, 2009). Different results were obtained in the present study for pre-activated oocytes. Although the density of microtubules was reduced in these oocytes, complete disappearance of these structures was not seen in most of them. Similar results have been reported for mouse oocytes in which microtubules also had not disappeared completely even after 2 h of treatment with demecolcine (Ibáñez *et al.*, 2003).

The rapid effect of demecolcine on metaphase II oocytes (Saraiva *et al.*, 2009), in which microtubules disappeared completely within 0.5 h of treatment, is likely to reflect differences in the sensitivity of the

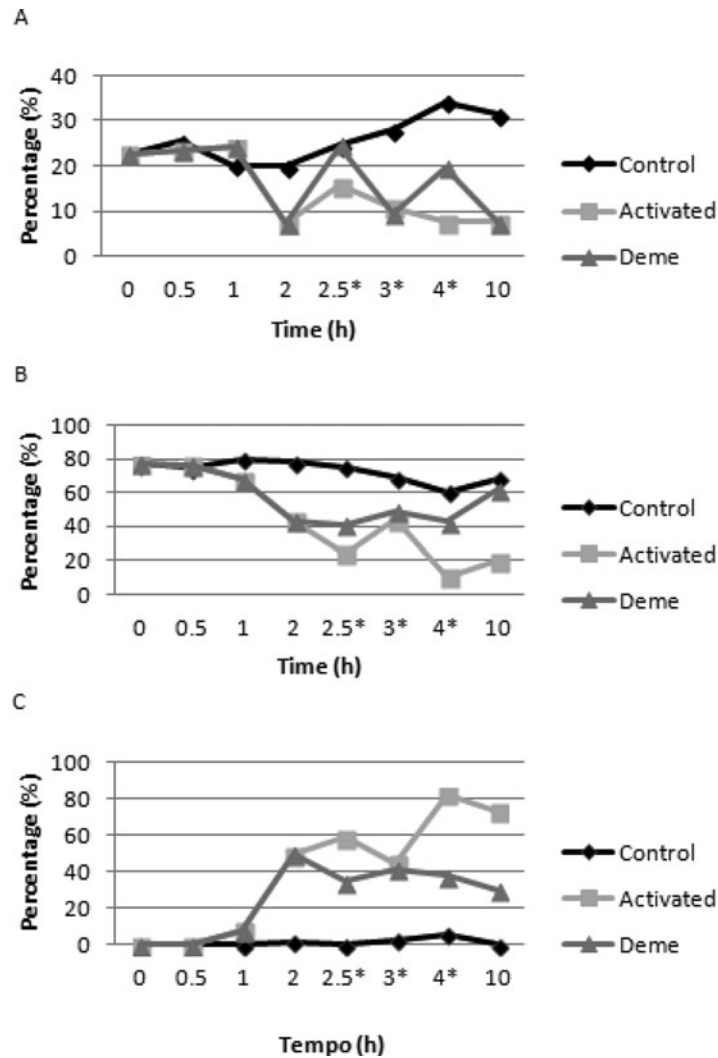


Figure 4 Graphic representation of nuclear kinetics in activated bovine oocytes treated with demecolcine and evaluated at intervals of 0.5 and 1.0 h between 0 and 10 h after the onset of activation. Control (not activated or treated with demecolcine); Activated (exposed only to the oocyte-activating agents); DEME (activated and treated with demecolcine for 2 h). (A) Immature oocytes; (B) mature oocytes; (C) activated oocytes. The asterisk indicates exposure to demecolcine.

meiotic spindle of these structures, with the spindle of metaphase II oocytes being a more sensitive target for demecolcine than the spindle of activated oocytes. The presence of these spindle remnants may indicate differences in the stability of some microtubules in the spindle, which probably correspond to interpolar microtubules (Combelles & Albertini, 2001).

In the present study, reduction of microtubules was observed in approximately 50% of treated oocytes at the end of assessment and complete disappearance of these structures in 23%, with visible microtubules being seen in only 27% of oocytes. These findings indicate the absence of immediate repolymerization of microtubules after culture of pre-activated oocytes in demecolcine-free medium, in contrast to metaphase II oocytes, as evaluated previously by our group (Saraiva *et al.*, 2009). This fact may have some

negative consequences for subsequent embryonic development as microtubules are important during the first cleavages. However, a reduction of microtubule density was also observed in oocytes of activated group 10 h after the onset of the process, suggesting that this reduction is a 'physiological' process.

It is important that future research also assesses the behaviour of microfilaments during treatment with demecolcine, especially in cases of pre-activated oocytes. Demecolcine-induced enucleation requires the artificial activation of the oocyte prior to its exposure to the microtubule-destabilizing drug. Parthenogenetic activation of bovine oocytes can be achieved by using compounds that increase intracellular calcium, such as ionomycin (calcium ionophore), followed by treatment for several hours with the protein synthesis inhibitor CHX, which induces the resumption of meiosis by

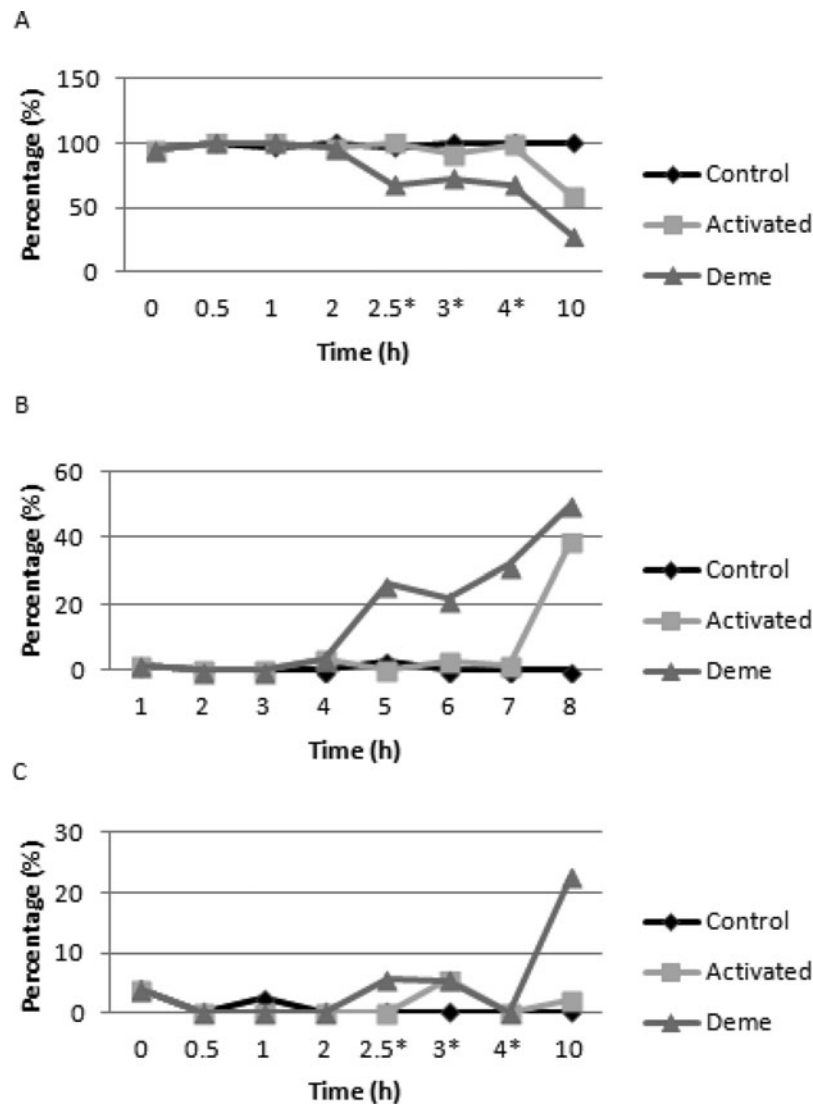


Figure 5 Graphic representation of microtubule kinetics in activated bovine oocytes treated with demecolcine and evaluated at intervals of 0.5 and 1.0 h between 0 and 10 h after the onset of activation. Control (not activated or treated with demecolcine); Activated (exposed only to the oocyte-activating agents); DEME (activated and treated with demecolcine for 2 h). (A) oocytes with microtubules; (B) oocytes with reduced microtubules; (C) oocytes without microtubules. The asterisk indicates exposure to demecolcine.

inactivation of MPF and mitogen-activated protein kinase (MAPK) (Fischer-Russel *et al.*, 2005). Recently, Vasilev *et al.* (2012) observed that artificial elevation of intracellular Ca^{2+} levels with ionomycin immediately led to drastic alteration of the actin cytoskeleton in the immature oocytes, which exhibited depolymerization of the actin meshwork in the subplasmalemmal regions along with highly enhanced polymerization and bundling of the actin filaments in the inner cytoplasm.

As demonstrated in the sea urchin, zygote cell cleavage depends heavily on the regulation of the actin cytoskeleton (Dales & De Santis, 1981). Hence, actin is intimately implicated in fertilization and in the early stages of development. These alterations led to

difficulties in cell cleavage in the monospermic zygotes and eventually to a higher rate of abnormal development. Then, further studies are needed to better characterize the mechanism of demecolcine-induced enucleation of preactivated oocytes, considering the involvement of microfilaments on the subsequent embryonic development. In this context, Meng *et al.* (2011) observed that in bovine MII oocytes, the microtubules were concentrated to form the normal spindle structure with microfilaments distributed uniformly in the cytoplasm, while in the demecolcine-treated oocytes, the spindle microtubules were nearly depleted by demecolcine, but the microfilaments were hardly affected. The demecolcine treatment seemed to have altered the balance between the

interaction of microtubules and microfilaments in the treated oocytes, which appears to be the result of the cytoplasmic protrusion. Without the aid of the microtubules, the chromosomes were compressed by the microfilaments to form a protrusion on the oocyte membrane.

Evaluation of embryonic development showed cleavage rates ranging from 63.6–70.0% in the groups reconstituted by the different techniques. Blastocyst rates ranged from 15.5–24.2%. Although embryo production tended to be lower for oocytes obtained by chemically IE, no significant differences were observed when compared with the conventional procedure.

There are various factors that justify the use of demecolcine for NT. According to Simerly *et al.* (2003, 2004), removal of the meiotic spindle during conventional enucleation seems to be responsible for the low development of embryos reconstituted from somatic cells in primates, since the centrosome and some motor proteins, such as NuMA and KIFC1, which are fundamental for the formation of the spindle poles during mitosis, are depleted from the ooplasm, resulting in spindle dysfunction and aneuploid embryos. In addition, traditional mechanical enucleation has been shown to reduce the amount of spindle-associated γ -tubulin in mouse oocytes (Van Thuan *et al.*, 2006). Within this context, alternative methods of oocyte enucleation may permit the preservation of spindle-associated factors in enucleated oocytes and consequently improve embryo quality.

Cloned mammalian species have been produced from either demecolcine-assisted (Yin *et al.*, 2002a; Kawakami *et al.*, 2003; Vajta *et al.*, 2003; Tani *et al.*, 2006) or demecolcine-induced enucleated oocytes (Baguisi & Overström, 2000; Gasparrini *et al.*, 2003) reconstituted with somatic or embryonic stem cells. The only report of demecolcine-induced enucleation in bovine (Fischer-Russel *et al.*, 2005) showed that intracytoplasmic injection of the donor nucleus prior to oocyte activation and treatment with demecolcine or after 1.5 and 2.0 h post-activation yielded 19 and 15.7% of blastocysts, respectively. For both treatments, cleavage and blastocyst rates were similar to those observed for the control group (26.1%). These data agree with the present results, indicating that demecolcine-induced enucleation does not compromise embryo development in cattle.

In summary, we optimized the chemically IE protocol and showed that the procedure may be shortened. However, demecolcine impaired meiotic progression when it was added to the medium during the onset of the activation process. Furthermore, demecolcine reduced microtubule density in pre-activated oocytes, but these structures did not disappear completely in most oocytes, and apparently there was no immediate repolymerization of micro-

tubules after culture in demecolcine-free medium. Nevertheless, the chemically IE technique permitted to obtain viable cytoplasts for NT, providing results similar to those observed for the conventional method. Studies evaluating the ability to establish pregnancy and to produce live offspring are needed to confirm the use of demecolcine-induced enucleation in bovine.

Acknowledgements

This study was supported by the São Paulo Research Foundation (FAPESP), Brazil.

References

- Baguisi, A. & Overström, E.W. (2000). Induced enucleation in nuclear transfer procedures to produce cloned animals. *Theriogenology* **53**, 209.
- Bordignon, V., Clarke, H.J. & Smith, L.C. (1999). Developmentally regulated loss and reappearance of immunoreactive somatic histone H1 on chromatin of bovine morula-stage nuclei following transplantation into oocytes. *Biol. Reprod.* **61**, 22–30.
- Campbell, K.H.S., Loi, P., Otaegui, P.J. & Wilmut, I. (1996). Cell cycle co-ordination in embryo cloning by nuclear transfer. *Rev. Reprod.* **1**, 40–6.
- Combelle, C.M. & Albertini, D.F. (2001). Microtubule patterning during meiotic maturation in mouse oocytes is determined by cell cycle-specific sorting and redistribution of γ -tubulin. *Dev. Biol.* **239**, 281–94.
- Dales, B. & De Santis, A. (1981). The effect of cytochalasin B and D on the fertilization of sea urchins. *Dev. Biol.* **83**, 232–7.
- Fischer-Russel, D.F., Ibáñez, E., Albertini, D.F. & Overström, E.W. (2005). Activated bovine cytoplasts prepared by demecolcine-induced enucleation support development of nuclear transfer embryos *in vitro*. *Mol. Reprod. Dev.* **72**, 161–70.
- Fulka, J., Loi, P., Fulka, H., Ptak, G. & Nagai, T. (2004). Nucleus transfer in mammals: noninvasive approaches for the preparation of cytoplasts. *Trends Biotechnol.* **22**, 279–83.
- Gasparrini, B., Gao, S., Ainslie, A., Fletcher, J., Mc Garry, M., Rithie, W.A., Springbett, A.J., Overström, E.W., Wilmut, I. & de Sousa, P.A. (2003). Cloned mice derived from embryonic stem cell karyoplasts and activated cytoplasts prepared by induced enucleation. *Biol. Reprod.* **68**, 1259–66.
- Hill, J.R., Burghardt, R.C., Jones, K., Long, C.R., Looney, C.R., Shin, T., Spencer, T.E., Thompson, J.A., Winger, Q.A. & Westhusin, M.E. (2000). Evidence for placental abnormality as the major cause of mortality in first trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* **63**, 1787–94.
- Ibáñez, E., Sanfins, A., Combelle, C., Albertini, D.F. & Overström, E.W. (2002). Induced enucleation of mouse and goat oocytes: kinetic and phenotypic characterizations. *Theriogenology* **57**, 421.
- Ibáñez, E., Albertini, D.F. & Overström, E.W. (2003). Demecolcine-induced oocyte enucleation for somatic cell cloning: coordination between cell-cycle egress, kinetics of

- cortical cytoskeletal interactions, and second polar body extrusion. *Biol. Reprod.* **68**, 1249–58.
- Kawakami, M., Tani, T., Yabuuchi, A., Kobayashi, T., Murakami, H., Fujimura, T., Kato, Y. & Tsunoda, Y. (2003). Effect of demecolcine and nocodazole on the efficiency of chemically assisted removal of chromosomes and the developmental potential of nuclear transferred porcine oocytes. *Cloning Stem Cells* **5**, 379–87.
- Kubiak, J.Z., Weber, M., De Pennart, H., Winston, J. & Maro, B. (1993). The metaphase II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin B in the presence of CSF. *EMBO J.* **12**, 3773–8.
- Larkin, K. & Danilchik, M.V. (1999). Microtubules are required for completion of cytokinesis in sea urchin eggs. *Dev. Biol.* **214**, 215–26.
- Li, G-P., White, K.L., Aston, K.I., Bunch, T.D., Hicks, B., Liu, Y. & Sessions, B.R. (2009). Colcemid-treatment of heifer oocytes enhances nuclear transfer embryonic development, establishment of pregnancy and development to term. *Mol. Reprod. Dev.* **76**, 620–8.
- Liu, L., Ju, J-C. & Yang, X. (1998). Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. *Biol. Reprod.* **59**, 537–45.
- Meng, Q., Wu, X., Bunch, D., White, K., Sessions, B. R., Davies, C. J., Rickords, L. & Li, G-P. (2011). Enucleation of demecolcine-treated bovine oocytes in cytochalasin-free medium: mechanism investigation and practical improvement. *Cell. Reprogram.* **13**, 411–8.
- Miyara, F., Han, Z., Gao, S., Vassena, R. & Latham, K.E. (2006). Non-equivalence of embryonic and somatic cell nuclei affecting spindle composition in clones. *Dev. Biol.* **289**, 206–17.
- Saraiva, N.Z., Perecin, F., Méo, S.C., Ferreira, C.R., Tetzner, T.A.D. & Garcia, J.M. (2009). Demecolcine effects on microtubule kinetics and on chemically assisted enucleation of bovine oocytes. *Cloning Stem Cells* **11**, 141–51.
- Simerly, C., Dominko, T., Navara, C., Payne, C., Capuano, S., Gosman, G., Chong, K.Y., Takahashi, D., Chace, C., Compton, D., Hewitson, L. & Schatten, G. (2003). Molecular correlates of primate nuclear transfer failures. *Science* **300**, 297.
- Simerly, C., Navara, C., Hyun, S.H., Lee, B.C., Kang, S.K., Capuano, S., Gosman, G., Dominko, T., Chong, K.Y., Compton, D., Hwang, W.S. & Schatten, G. (2004). Embryogenesis and blastocyst development after somatic cell nuclear transfer in nonhuman primates: overcoming defects caused by meiotic spindle extraction. *Dev. Biol.* **276**, 237–52.
- Tani, T., Shimada, H., Kato, Y. & Tsunoda, Y. (2006). Demecolcine-assisted enucleation for bovine cloning. *Cloning Stem Cells* **8**, 61–6.
- Vajta, G., Lewis, I.M., Trounson, A.O., Purup, S., Maddox-Hyttel, P., Schmidt, M., Pedersen, H.G., Greve, T. & Callesen, H. (2003). Handmade somatic cell cloning in cattle: analysis of factors contributing to high efficiency in vitro. *Biol. Reprod.* **68**, 571–8.
- Van Thuan, N., Wakayama, S., Kishigami, S. & Wakayama, T. (2006). Donor centrosome regulation of initial spindle formation in mouse somatic cell nuclear transfer: roles of gamma-tubulin and nuclear mitotic apparatus protein 1. *Biol. Reprod.* **74**, 777–87.
- Vasilev, F., Chun, J. T., Gagnaniello, G., Garante, E. & Santella, L. (2012). Effects of ionomycin on egg activation and early development in starfish. *PLoS One* **7**, e39231.
- Yin, X.J., Kato, Y. & Tsunoda, Y. (2002a). Effect of enucleation procedures and maturation conditions on the development of nuclear-transferred rabbit oocytes receiving male fibroblast cells. *Reproduction* **124**, 41–7.
- Yin, X.J., Tani, T., Yonemura, I., Kawakami, M., Miyamoto, K., Hasegawa, R., Kato, Y. & Tsunoda, Y. (2002b). Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. *Biol. Reprod.* **67**, 442–6.