CLONING AND STEM CELLS
Volume 11, Number 1, 2009

DOI: 10.1089/clo.2008.0044

Demecolcine Effects on Microtubule Kinetics and on Chemically Assisted Enucleation of Bovine Oocytes

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Abstract

This study aimed to evaluate the effect of demecolcine, a microtubule-depolymerizing agent, on microtubule kinetics; to determine the best concentration of demecolcine as a chemically assisted enucleation agent in metaphase I (MI) and metaphase II (MII) bovine oocytes, and to evaluate the embryonic development after nuclear transfer (NT) using chemically assisted enucleation of recipient oocytes. Oocytes *in vitro* matured for 12 h (MI) and 21 h (MII) were exposed to several concentrations of demecolcine and evaluated for enucleation or membrane protrusion formation. Demecolcine concentration of 0.05 μg/mL produced the highest rates of enucleation in group MI (15.2%) and protrusion formation in group MII (55.1%), and was employed in the following experiments. Demecolcine effect was seen as early as 0.5 h after treatment, with a significant increase in the frequency of oocytes with complete microtubule depletion in MI (58.9%) and MII (21.8%) compared to initial averages at 0 h (27.4% and 1.9%, respectively). Microtubule repolymerization was observed when MII-treated oocytes were cultured in demecolcine-free medium for 6 h (42.4% oocytes with two evident sets of microtubules). Chemically assisted enucleated oocytes were used as recipient cytoplasts in NT procedures to assess embryonic development. For NT, 219 of 515 oocytes (42.5%) formed protrusions and were enucleated, and reconstructed, resulting in 58 nuclear-transferred one-cell embryos. Cleavage (84.5%) and blastocyst development (27.6%) rates were assessed. In conclusion, demecolcine can be used at lower concentrations than routinely employed, and the chemically assisted enucleation technique was proven to be highly efficient allowing embryonic development in bovine.

Introduction

TWO CELL COMPONENTS are essential for producing a clone: the donor nucleus (karyoplast), and the enucleated recipient oocyte (cytoplast), whose cytoplasm content must be sufficiently competent to allow nucleus reprogramming and development to term. Therefore, oocyte enucleation is a key factor in the success of animal cloning (Ibáñez et al., 2003). The enucleation process in traditional nuclear transfer (NT) involves nucleus staining with a DNA-intercalating dye, aspiration of the first polar body (1PB), and an adjacent metaphase plate. To confirm enucleation, UV irradiation is used, but it causes a significant reduction in embryo development to the blastocyst stage, mainly due to alterations induced in the membrane and intracellular components of bovine oocytes (Smith, 1993). Even fluorescent bisbenzimide (Hoechst 33342), normally used in this procedure, has a short wavelength (350-nm excitation) and transfers a large amount of energy to the biological material under excitation (Dominko et al., 2000).

The damage to the recipient oocyte is aggravated by the concomitant removal of a large volume of cytoplasm surrounding the metaphase plate, as this cytoplasm contains mRNA, proteins, and molecular precursors essential to early development until embryo genome activation (Barnes and Eyestone, 1990). The enucleation process is a crucial event in cloning due to the elimination of nuclear genetic contribution from the recipient cytoplasm. Nonetheless, enucleation can result in 30% residual nuclear DNA in cytoplasts due to failures in technique (Li et al., 2004).

It is recognized that selection of high-quality recipient oocytes for nuclear transfer will increase the cloning efficiency and the number of offspring obtained (Miyoshi et al., 2003). An alternative strategy to physical enucleation is to

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treat oocytes with agents that modify the processes of karyokinesis and cytokinesis, and result in chemically enucleated oocytes at high rates. One method consists of total chemical enucleation, with maternal chromosome removal during first (Fulka and Moor, 1993) or second polar body (PB) extrusion (Gasparrini et al., 2003; Ibáñez et al., 2003; Russel et al., 2005; Yin et al., 2002a). The first successful non-invasive oocyte enucleation approach for the use in nuclear transfer experiments has been developed by Fulka and Moor (1993), who showed that the treatment of mouse MI oocytes with etoposide, a topoisomerase II inhibitor, and cycloheximide, a protein synthesis inhibitor, yields chemically enucleated cytoplasts at high rates (more than 90%). After exposure to etoposide, all the genetic content is maintained together and expulsed from the cytoplasm in the first PB by exposure to cycloheximide. However, the etoposide–cycloheximide enucleated oocytes did not contain maturation promoting factor (MPF) at the end of the enucleation procedure. Nonetheless, MPF levels could be restored during subsequent incubation in drug-free medium (Fulka and Moor, 1993; Karnikova et al., 1998).

A major advantage of the chemical enucleation procedure is that it provides a method of conditioning nuclei for such a prolonged period (8–10 h) in a cytoplasmic environment in which MPF is present before activation (Fulka and Moor, 1993). Other advantages of chemical enucleation are its simplicity and the capacity to enucleate large numbers of oocytes at the same time, being an economical source of competent recipient cytoplasm. The analysis of the time required for each technique revealed a clear advantage of chemical enucleation over the classical microaspiration technique. This method was 20 times faster than the aspiration technique normally used to enucleate a single oocyte (Savard et al., 2004). Studies in domestic animals showed that demecolcine can be used as a potential reagent to induce enucleation of ovine (Hou et al., 2006), and porcine (Savard et al. 2004) oocytes in meiosis I, reaching approximately 50% and 40% of enucleation, respectively.

Another method is named chemically assisted oocyte enucleation (Kawakami et al., 2003; Li et al., 2004, 2006; Tani et al., 2006; Vajta et al., 2005; Yin et al., 2002b), which is based on treating oocytes with agents that interfere in microtubule kinetics, inducing the formation of a visible protrusion containing condensed chromatin on the oocyte surface. This method seems to be a potentially superior approach with minimal decrease in the oocyte cytoplasmic volume and no need for Hoechst staining and selection by UV microscopy (Li et al., 2006).

Therefore, this study evaluated the use of demecolcine, a microtubule depolymerizing agent, for bovine oocyte enucleation. The objectives were: (1) to ascertain the lowest efficient concentration of demecolcine for enucleation and protrusion formation in oocytes exposed to it in MI and MII, (2) to establish nuclear and microtubule kinetics in oocytes submitted to demecolcine, and (3) to evaluate embryo development potential after NT using the chemically assisted technique.

Materials and Methods

Chemicals

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

Oocyte recovery and in vitro *maturation (IVM)*

Bovine oocytes were obtained by follicular aspiration from ovaries obtained at a local slaughterhouse. The ovaries were transported in saline solution 0.9% at 30–35°C to the laboratory. Follicles with diameters between 3 and 8 mm were aspired 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) supplemented with 10% fetal calf serum (FCS) heat-inactivated at 55°C for 30 min, as well as 0.20 mM of sodium pyruvate and 83.4 μg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil). Groups of 40–50 COCs were placed in 400 μL of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 1.0 μg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Ont, Canada), 50 μg/mL hCG (Profasi™, Serono, São Paulo, Brazil), 1.0 μg/mL estradiol, 0.20 mM of sodium pyruvate, and 83.4 μg/mL amikacin under mineral oil (Dow Corning CO, Midland, MI) for *in vitro* maturation (IVM).

Experiment I: Influence of Different Demecolcine Concentrations on Bovine Oocytes

Oocytes obtained after 12 h (group MI) and 21 h (group MII) maturation were incubated in IVM medium supplemented with demecolcine for 12 and 2 h, respectively, at concentrations of: 0 (control); 0.025 μg/mL; 0.05 μg/mL; 0.2 μg/mL; and 0.4 μg/mL. After treatment, oocytes from both groups were stripped from cumulus cells with hyaluronidase (2 mg/mL) at 38.5°C for 5 min, followed by pipetting for 3 to 5 min. The denuded oocytes were then stained with 10 μg/mL Hoechst 33342 for 10 min, observed under epifluorescence microscope (330–385 nm), and evaluated for enucleation or protrusion formation in the membrane. The oocytes were moved with the aid of a glass pipette to confirm the protrusion. Ten replicates were performed in each group with 30 to 50 oocytes in each one. The best results were used in the following experiments.

Experiment II: Evaluation of Nuclear and Microtubule Kinetics in Oocytes Exposed to Demecolcine

Group MI oocytes (12 h IVM) were evaluated for nuclear stage and meiotic spindle (microtubules) pattern after 0, 0.5, 1.0, 1.5, 2.0, 4.0, and 12 h of exposure to 0.05 μg/mL demecolcine. Group MII oocytes (21 h IVM), which received 0.05 μg/mL demecolcine in IVM medium for up to 2 h, were also evaluated at 0, 0.5, 1.0, 1.5, 2.0, 4.0, and 8.0 h of treatment. After 2 h of demecolcine exposure the MII oocytes were incubated in demecolcine-free maturation medium for more 6 h until complete 8 h. Control oocytes were cultured without demecolcine and collected under the same conditions as treated groups; however, due to the large number of treatments, they were evaluated in a separate experiment.

Three replicates of 40 to 60 oocytes for each treatment were evaluated for nuclear and microtubule kinetics. The protocol used, described by Liu et al. (1998), started with oocyte incubation for 30 min in microtubule stabilizing buffer (0.1 M Pipes, pH 6.9; 5 mM $MgCl_2.6H_2O$ (Merck, Darmstadt, Germany); 2.5 mM EGTA), containing 2% formaldehyde, 0.1%

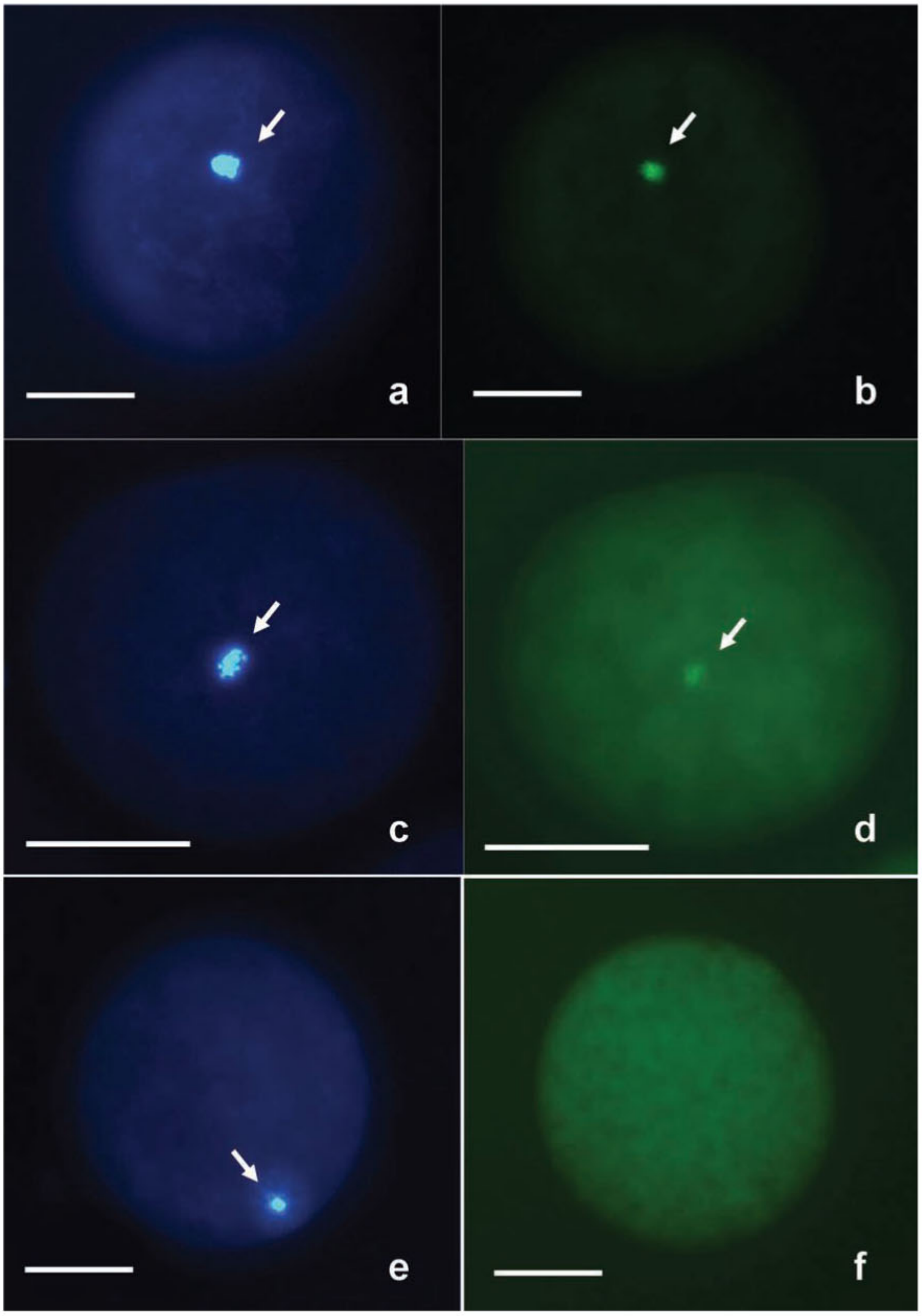

FIG. 1. Evaluation of nuclear (blue) and microtubule (green) kinetics in bovine oocytes treated in metaphase I (MI) with demecolcine. Oocyte categories: (**a, b**) Type 1—oocyte in MI with microtubules evident; (**c, d**) Type 2—oocyte in MI with reduced microtubules; (**e, f**) Type 3—oocyte in MI without microtubules. (**a, c, e**) Oocytes stained with Hoechst 33342; (**b, d, f**) oocytes stained with anti-α tubulin antibody conjugated to FITC (1:50), observed under an epifluorescence microscope. Arrowheads indicate nuclear material (blue), and microtubules (green). Scale bar is 50 μm.

Triton X-100, 1 μM taxol, 0.01% aprotinine, 1 mM dithiothreitol, and 50% deuterium oxide (Aldrich Chem. Co., Milwaukee, WI). Afterward, oocytes were kept overnight at 4°C in washing medium (WM; PBS with 0.02% NaN_3; 0.01% Triton X-100; 0.2% nonfat dry milk; 2% FCS; 2% bovine serum albumin (BSA); and 0.1 M glycine) and then incubated in WM containing anti-α-tubulin mouse monoclonal antibody (1:50) conjugated with fluoroscein isothiocyanate (FITC) at 38°C for 2 h. 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 evaluated under epifluorescence at 330–385 and 420–490 nm. Meiotic spindle and chromosome behaviors were evaluated and oocytes were classified as follows: Type 1—oocyte in metaphase I with evident microtubules in the same location; Type 2—oocyte in MI with reduced microtubules; Type 3—oocyte in MI without microtubules; Type 4—anaphase/telophase I transition (AI/TI); Type 5—oocyte in MII with evident microtubules in both oocyte cytoplasm and polar body; Type 6—oocyte in MII with evident microtubules in only one place; Type 7—oocyte in MII with microtubule reduction; Type 8—oocyte in MII without microtubules; and Type 9—oocyte with DNA fragmentation (Figs. 1 and 2).

Experiment III: Efficiency of Demecolcine-Assisted Enucleation and Embryo Development after Nuclear Transfer (NT)

Nuclei donor cells

Bovine fibroblasts, obtained from a Nellore (*Bos indicus*) cow, were used as nucleus donors. Fragments from a skin biopsy were initially cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 50% FCS and 83.4 μg/mL amikacin at 38.5°C under 5% CO_2 in air. After establishment of primary cell line, cells were maintained in DMEM supplemented with 10% FCS and amikacin and replicated when they reached confluence. Medium was renewed every 48 h and cells underwent three to five passages (0.05% trypsin with 1% poultry serum) before freezing and storage in liquid nitrogen. Before NT, cells were cultured in DMEM supplemented with 0.5% FCS for 3 to 5 days to induce cell quiescence and cell cycle synchronization in stage G_0–G_1 (Campbell et al., 1996), compatible with metaphase II enucleated cytoplasm.

Oocyte enucleation, nuclear transfer, and electrofusion

After 19 h maturation, COCs were denuded as previously described, and then incubated in IVM medium supplemented with 0.05 μg/mL demecolcine for 2 h, completing 21 h maturation. Oocytes presenting the first polar body (1PB) and protrusion in the membrane were incubated in synthetic oviductal fluid (SOF) supplemented with HEPES (HSOF) with 10% FCS and 7.5 μg/mL cytochalasin B for removal of the 1PB and protrusion, with minimal cytoplast removal. The procedure was performed under an inverted optical microscope (Olympus IX-70), removing the 1PB and protrusion with a 25 μm (external diameter) glass pipette. Samples of cytoplasts were stained with 10 μg/mL Hoechst 33342 for 10 min and analyzed for enucleation efficiency. The remaining enucleated oocytes were incubated in HSOF supplemented with 10% FCS and 7.5 μg/mL cytochalasin B for 30 min before reconstitution. Traditional enucleation was performed on the control group without exposure of the oocytes to demecolcine. The same conditions were employed, except that UV light was used to visualize the nucleus and to confirm enucleation.

Individual fibroblasts were transferred to the perivitelline space, and the electrofusion was done in mannitol solution (0.28 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.05 mM HEPES acid, and 3 mg/mL BSA) using two 20 μsec pulses of 2.0 kV/cm produced by a BTX Electrocell Manipulator 2001 (BTX, San Diego, CA). Fusion rates were evaluated 30 to 60 min after electrofusion.

Oocyte activation

Successfully reconstructed oocytes were artificially activated by incubation in TCM-199 with HEPES buffer and 10% FCS supplemented with 5 μM ionomycin for 5 min, followed by incubation in SOF supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h at 38.5°C under a humidified atmosphere of 5% CO_2 in air.

Culture and evaluation of embryo development

Embryo culture was performed in SOF supplemented with 2.5% FCS and 5 mg/mL BSA at 38.5°C under a humidified atmosphere of 5% CO_2. Groups of 10 to 20 one-cell embryos were cultured in 100 μL microdrops of culture medium for 7 days until blastocyst stage. The medium was renewed on the third and fifth days of embryo culture.

Statistical analysis

Results were submitted to ANOVA, and means were compared by Duncan test in Experiment I and by Tukey test in Experiment II. In Experiment III, the proportion of cleaved embryos reaching blastocyst stage underwent Fisher's Exact test analysis. A level of 5% significance was used for all experiments.

Experimental design

Experiment I. This experiment was designed to evaluate enucleation and protrusion formation rates at different demecolcine concentrations. Oocytes were *in vitro* matured for 12 h (group MI) and 21 h (group MII), and then exposed to demecolcine for 12 and 2 h, respectively, at concentrations of: 0 (control); 0.025 μg/mL; 0.05 μg/mL; 0.2 μg/mL; and 0.4 μg/mL.

Experiment II. Results from Experiment I showed that 0.05 μg/mL was the best demecolcine concentration for enucleation, in both groups MI and MII. The objective was to examine the nuclear and microtubule kinetics during the demecolcine treatment with this concentration. Group MI oocytes (12 h IVM) were fixed and evaluated for nuclear stage and meiotic spindle (microtubules) pattern at 0, 0.5, 1.0, 1.5, 2.0, 4.0, and 12 h of exposure to demecolcine. Group MII oocytes (21 h IVM), treated for up to 2 h, were also evaluated at 0, 0.5, 1.0, 1.5, 2.0, 4.0, and 8.0 h after drug exposure.

Experiment III. This experiment was designed to verify the efficiency of demecolcine-assisted enucleation and embryo development after nuclear transfer (NT) in group MII. Some

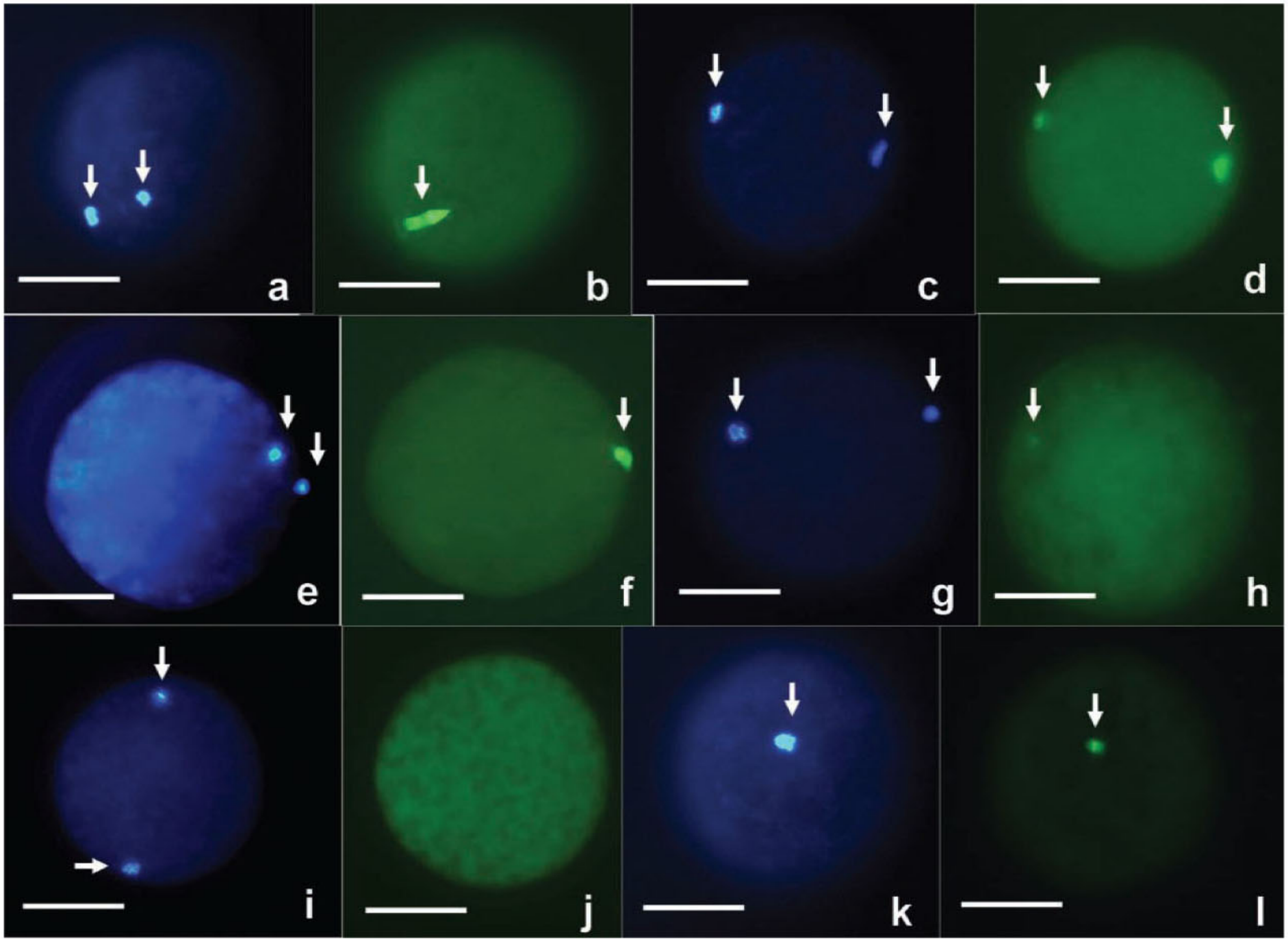

FIG. 2. Evaluation of nuclear (blue) and microtubule (green) kinetics in bovine oocytes exposed to demecolcine at metaphase II (MII). Oocyte categories: (**a, b**) Type 4—anaphase/telophase I transition (AI/TI); (**c, d**) Type 5—oocyte in metaphase II (MII) with microtubules evident both in cytoplasm and in the PB; (**e, f**) Type 6—oocyte in MII with microtubules evident in only one place; (**g, h**) Type 7—oocyte in MII with reduced microtubules; (**i, j**) Type 8—oocyte in MII without microtubules; (**k, l**) Type 1—oocyte in MI with microtubules evident. (**a, c, e, g, i, k**) Oocytes stained with Hoechst 33342; (**b, d, f, h, j, l**) oocytes stained with antibody anti-α-tubulin conjugated to FITC (1:50), observed under an epifluorescence microscope. Arrowheads indicate nuclear material (blue) and microtubules (green). Scale bar is 50 μm.

TABLE 1. ENUCLEATION (EN) AND PROTRUSION FORMATION (PR) RATES IN BOVINE OOCYTES EXPOSED TO DIFFERENT DEMECOLCINE CONCENTRATIONS (0, 0.025, 0.05, 0.2, AND 0.4 μG/ML) BY 12 AND 2 HOURS AFTER 12 (GROUP MI) AND 21 HOURS (GROUP MII) OF MATURATION, RESPECTIVELY

Concentration (μg/mL)	*Group MI (mean % ± SD)*		*Group MI (mean % ± SD)*
	EN	*PR*	*PR*
0 (Control)	5/354 (1.2 ± 2.0)[b]	0/354 (0 ± 0)[b]	0/354 (0 ± 0)[c]
0.025	60/398 (16.2 ± 9.5)[a]	32/398 (8.5 ± 8.1)[a]	159/369 (42.6 ± 13.4)[b]
0.05	57/392 (15.2 ± 10.6)[a]	22/392 (7.1 ± 9.2)[a]	211/388 (55.1 ± 13.0)[a]
0.2	21/398 (5.3 ± 4.5)[b]	28/398 (8.0 ± 7.6)[a]	162/367 (45.1 ± 13.4)[ab]
0.4	16/385 (4.7 ± 6.0)[b]	21/385 (7.6 ± 9.4)[a]	141/363 (39.3 ± 12.1)[b]

[a,b,c]Values with different superscripts within columns are significantly different ($p < 0.05$).

cytoplasts were stained with Hoechst 33342, and analyzed for enucleation efficiency. The remaining enucleated oocytes were used for reconstitution by NT, and cleavage and blastocyst rates were determined on the third and fifth days of embryo culture.

Results

Determination of demecolcine concentration for enucleation or protrusion formation in bovine oocytes

Group MI presented enucleated oocytes (EN) and oocytes with protrusion (PR) in cortical region containing all condensed nuclear material. Demecolcine concentrations of 0.025 and 0.05 μg/mL provided similar ($p > 0.05$) enucleation rates, which were superior ($p < 0.05$) to the other concentrations (Table 1). All demecolcine concentrations were similar ($p > 0.05$) when evaluating PR formation in cortical region, differing only from control (Table 1).

Group MII presented a protrusion formed in oocyte membrane, usually close to the 1PB. The demecolcine concentration of 0.05 μg/mL was superior ($p < 0.05$) to 0.025 and 0.4 μg/mL, and similar ($p > 0.05$) to 0.2 μg/mL (Table 1), and was thus chosen for the following experiments.

Evaluation of nuclear and microtubule dynamics in oocytes exposed to demecolcine

Oocytes exposed to demecolcine in MI (Group MI). The predominant categories in Group MI were Types 1, 2, and 3 oocytes (Table 2; Fig. 1). Other categories seen in Group MII were also seen in this group but they were not mentioned as their numbers were nearly null.

As expected, Type 1 oocytes predominated at the beginning of treatment, with the nucleus presenting MI stage and microtubules still evident in close proximity (Table 2). After only 0.5 h treatment, a significant reduction ($p < 0.05$) was seen in Type 1 oocytes, which persisted up to 12 h exposure to demecolcine (Table 2). In control group the levels of Type 1 oocytes ranged from 55.0 to 85.5% up to 4 h evaluation, with a reduction with only 12 h (12.3%), that is, after 24-h maturation when almost all of the oocytes reached the MII stage.

Although a reduction in microtubule density (Type 2) proportional to the drug exposure time was not verified (Table 2), there was a difference ($p < 0.05$) between control (6.5%) and treated (20.9%) group means when this type was analyzed. The number of Type 9 (DNA fragmentation) oocytes also remained constant ($p > 0.05$) throughout the 12-h evaluation period (Table 2).

There was a significant increase ($p < 0.05$) in oocytes with complete microtubule absence (Type 3) after 1-h treatment (Table 2). The control group had no significant differences between evaluation moments, with 11.2% and 1.1%, respectively, at the beginning (0 h) and after 12 h of evaluation.

Oocyte exposure to demecolcine in MII (group MII). Oocytes with the following nuclear and meiotic spindle characteristics were seen in group MII: Types 1, 4, 5, 6, 7, 8, and 9 (Table 3; Fig. 2). The other categories described in group MI and not mentioned here were also present but were negligible and therefore were not considered.

TABLE 2. NUCLEAR (DNA) AND MICROTUBULE (MT) PATTERNS (TYPES 1, 2, 3, AND 9) AT DIFFERENT TIMES AFTER DEMECOLCINE TREATMENT FOR 12 HOURS OF BOVINE OOCYTE IN MI (12 HOURS MATURATION)

	% Observed pattern at different times (h) of demecolcine exposure						
DNA and MT	*0 h (n = 118)*	*0.5 h (n = 117)*	*1.0 h (n = 130)*	*1.5 h (n = 137)*	*2.0 h (n = 133)*	*4.0 h (n = 139)*	*12.0 h (n = 140)*
Type 1	55.3[a]	11.2[b]	8.0[b]	9.2[b]	10.4[b]	22.2[b]	19.3[b]
Type 2	12.0	25.6	18.4	20.8	20.7	24.3	24.2
Type 3	27.4[b]	58.9[a,b]	64.3[a]	60.1[a]	61.8[a]	46.0[a,b]	45.5[a,b]
Type 9	1.6	2.2	3.3	1.8	1.8	0.7	4.1

*Type 1: oocytes in MI with microtubules evident; Type 2: oocytes in MI with reduced microtubules; Type 3: oocytes in MI without microtubules; and Type 9: DNA fragmentation.

[a,b]Values with different superscripts within rows are significantly different ($p < 0.05$).

TABLE 3. NUCLEAR (DNA) AND MICROTUBULE (MT) PATTERNS (TYPES 1, 4, 5, 6, 7, 8, AND 9) AT DIFFERENT TIMES AFTER DEMECOLCINE TREATMENT FOR 12 HOURS OF BOVINE OOCYTE IN MII (21 HOURS MATURATION)

	% Observed pattern at different times (h) of demecolcine exposure						
DNA and MT	*0 h* (n = *159*)	*0.5 h* (n = *147*)	*1.0 h* (n = *138*)	*1.5 h* (n = *130*)	*2.0 h* (n = *154*)	*4.0 h* (n = *128*)	*8.0 h* (n = *144*)
Type 1	17.3	8.7	7.9	7.6	5.7	7.1	8.3
Type 4	22.4[a]	8.2[b,c]	10.7[a,b,c]	14.7[a,b]	3.5	1.4[c]	0.7[c]
Type 5	40.7[a]	13.5[b]	11.8[b]	7.0[b]	2.6[b]	11.2[b]	42.4[a]
Type 6	5.1[c]	18.5[b,c]	22.5[b]	28.2[a,b]	26.4[a,b]	42.6[a]	21.7[b,c]
Type 7	8.2	16.9	16.2	13.3	11.0	8.7	9.5
Type 8	1.9[c]	21.8[a,b]	18.2[b,c]	16.5[b,c]	38.3[a]	22.3[a,b]	3.5[c]
Type 9	1.3[b]	1.8[b]	1.9[b]	0.6[b]	2.6[b]	1.7[b]	11.1[a]

*Type 1: oocytes in MII with microtubules evident; Type 4: anaphase/telophase I transition—AI/TI; Type 5: oocyte in MII with microtubules evident both in the oocyte cytoplasm and in the PB; Type 6: oocyte in MII with microtubules evident in one place; Type 7: oocyte in MII with reduced microtubules; Type 8: oocyte in MII without microtubules; and Type 9: DNA fragmentation.

[a,b]Values with different superscripts within rows are significantly different ($p < 0.05$).

There was no variation ($p > 0.05$) in the frequency of oocytes in MI (Type 1) and MII with reduced microtubules (Type 7) between the beginning of treatment and end of evaluation (Table 3).

There was a significant ($p < 0.05$) reduction in Types 4 and 5 with only 0.5 h treatment compared to the beginning (0 h) of demecolcine exposure (Table 3). The number of Type 5 oocytes started to increase again ($p < 0.05$) 8 h after the beginning of treatment, while the number of Type 4 oocytes reduced ($p < 0.05$) until the end of evaluation (Table 3). Control group levels of Type 4 oocytes varied from 3.4% to 18.7%, and Type 5 oocytes from 36.9% to 64.1% during the entire evaluation, without any significant differences among evaluation moments.

Also, the number of oocytes classified as Type 6 significantly increased ($p < 0.05$) after 1 h and 4 h of exposure to the drug (Table 3). However, 8 h after the beginning of treatment, these values were reduced ($p < 0.05$), not differing from the first value (Table 3). Levels in the control group varied from 8.0% to 19.0% in all evaluation moments without significant differences.

The quantity of oocytes without microtubules (Type 8) increased ($p < 0.05$) after only 0.5 h demecolcine exposure (Table 3). This level remained constant until 4 h, and was reduced ($p < 0.05$) after 8 h, when it was similar to the beginning of treatment (Table 3). In the control group, only 0% to 2.7% oocytes without microtubules were observed throughout evaluation.

The number of oocytes with DNA fragmentation (Type 9) rised ($p < 0.05$) only 8 h after the start of treatment (Table 3), at which time the controls presented a mean of 2.3%.

Efficiency of the demecolcine assisted enucleation technique and embryo development after nuclear transfer

Since extremely low rates of enucleation and protrusion formation rates were obtained in group MI oocytes, just oocytes enucleated in MII were used as recipient cytoplasts for NT.

The high efficacy of the enucleation technique was verified with 77 enucleated out of 85 oocytes (90.6%) showing protrusion. The technique was also used in following NT procedures without the need of nucleus staining, guided only by PB and formed protrusion.

For nuclear transfer, 515 oocytes were evaluated after demecolcine treatment, of which 219 (42.5%) had protrusions and were enucleated. After transfer of one fibroblast to each of 203 enucleated oocytes, cell fusion was verified in 80 structures (46.8%). However, some of them lysed after fusion, and just 58 nonlysed and excellent grade one-cell embryos were submitted to culture.

There was no significant difference ($p > 0.05$) between the control and demecolcine exposed group for cleavage rate and blastocyst development (Table 4).

Discussion

Determination of demecolcine concentration for enucleation or protrusion formation in bovine oocytes

An earlier study (Fulka and Moor, 1993) showed that treating murine oocytes in MI with etoposide (ETO), a type II topoisomerase inhibitor, and cycloheximide (CHX), a protein synthesis inhibitor, allowed high enucleation rates. However, the cytoplasts prepared according to this technique had a limited ability to support embryo development after reconstruction (Elsheikh et al., 1997), because a very high proportion of the reconstructed embryos (85.5%) expressed metaphase plate abnormalities. Similar to previous studies in bovine, we had no success with etoposide or other

TABLE 4. CLEAVAGE AND BLASTOCYST DEVELOPMENT RATES AFTER NUCLEAR TRANSFER INTO ENUCLEATED OOCYTES BY TRADITIONAL (CONTROL GROUP—C) AND CHEMICALLY ASSISTED (DEMECOLCINE GROUP—DEME) TECHNIQUES

Treatment	n	*Cleavage* n *(%)*[a]	*Blastocysts* n *(%)*[b]
C	78	55 (70.5)	16 (20.5)
Deme	58	49 (84.5)	16 (27.6)

Three replicates.

[a]Cleavage rate: No. of embryos cleaved/No. embryos cultured.

[b]Blastocyst rate: No. of blastocysts cleaved/No. embryos cultured.

chemical agents (camptothecine and doxorrubicine), only achieving enucleation rates of 11.9% (Perecin et al., 2004). Demecolcine has been successfully used in ovine MI oocytes, presenting 58.1% enucleation efficiency (Hou et al., 2006). These authors observed that spindle microtubule organization and 1PB extrusion were inhibited when oocytes in the germinal vesicle (GV) stage were cultured with different demecolcine concentrations for 20 to 22 h in a dose-dependent manner. But when treatment was applied after germinal vesicle breakdown (GVBD) or in metaphase (MI), enucleation rates were high (~58%). However, the enucleation of other domestic animal oocytes has been very inconsistent when the same scheme was applied. Savard et al. (2004) obtained much lower (39.4 ± 7.5%) enucleation success in porcine oocytes when compared to mouse (~90%). Fulka et al. (2004) reported that the probably main reason for this is the inability to synchronize the maturation of ungulate oocytes as perfectly as is possible in mouse. It was suggested that the extent of chromatid segregation is a key determinant of enucleation success.

In this study we used bovine oocytes in MI to evaluate chemical enucleation with demecolcine, but low efficiency rates were observed (16.2%). However, despite low enucleation rates, protrusions containing all the condensed chromatin were formed in the cortical region of some group MI oocytes. This was probably due to incomplete polar body extrusion caused by a lack of microtubules in demecolcine-treated oocytes, which are important in the 1PB extrusion process. Li et al. (2005) showed that chromatin destined to enter into 1PB began to be expelled as a membrane projection when oocytes reach the early AI stage, resembling a 1PB-like structure. Several reports have suggested that the interaction between microtubules and actin filaments of the cell cortex mediates spindle rotation and coordinates karyokinesis and cytokinesis (Gard et al., 1995; Maro et al., 1984). This spindle disruption caused by demecolcine probably inhibits its rotation thus explaining the inhibition of PB extrusion. Our results show that the initial phase of PB formation was observed in some demecolcine-treated oocytes, but later "furrow constriction" and abscission was impaired in bovine oocytes treated with demecolcine. Several proteins necessary for cytokinesis have been localized to the central spindle, and it has been suggested that microtubules (MTs) may serve as tracks along which these proteins and others components of the cell move into the cleavage furrow (Ibáñez et al., 2003). Shorter treatments with demecolcine, which would allow MT regeneration by the time of telophase achievement, may promote the completion of first PB extrusion.

Previous studies with ovine MI oocytes (Hou et al., 2006) revealed that the impairment of PB extrusion was the main limitation to induce enucleation. It has been well established that an intact microtubule spindle is required for the maturation-promoting factor (MPF) inactivation, and thus cell cycle exit from the arrest at the MII phase, but little information in this regard is known in the oocytes during meiosis I. CHX is frequently used to artificially reduce the MPF activity, whose peak level occurs at the M phase, and which is responsible for oocyte arresting at this stage. However, the same authors observed that the decline of MPF activity is not sufficient for PB formation, inconsistent with studies on ETO-induced enucleation.

Demecolcine binds tightly to tubulin dimmers, and prevents MT polymerization, resulting in the loss of dynamic spindle MTs in mitotic and meiotic cells (Ibáñez et al., 2003). Kawakami et al. (2003) proposed that after meiotic spindle depolymerization induced by demecolcine, chromosomes become condensed and closer to the plasma membrane and anchored to the actin-rich domain providing a rigid structure to the oocyte cortex and forming a protrusion in this region. Our results for protrusion formation (55.1%) in bovine MII oocytes exposed to demecolcine were lower than from other authors (Kawakami et al., 2003; Li et al., 2004; Yin et al., 2002b), who reported around 70% of treated oocytes with protrusions. This could be due to the use of oocytes without selection for extruded 1PB. A heterogeneous population of oocytes was used for trying to optimize the procedure; which could explain the different results obtained in this work.

We would like to emphasize the importance of the result obtained in Experiment I where 0.05 μg/mL demecolcine was highly effective in the induction and formation of protrusions. Li et al. (2004) used a 0.2 μg/mL demecolcine concentration in bovine oocytes, but most authors use concentrations equal to or higher than 0.4 μg/mL (Kawakami et al., 2003; Vajta et al., 2005; Yin et al., 2002b). Recently, Hou et al. (2006) also obtained higher enucleation rate in ovine oocytes using lower demecolcine concentrations (0.04 μg/mL). This minimizes the injuries caused to oocytes by exposure to the drug, due to little alteration in cytoskeletal organization, which probably results into higher embryo and fetal development.

Evaluation of nuclear and microtubule dynamics in oocytes exposed to demecolcine

Li et al. (2005) reported that the 1PB extrusion of *in vitro* matured bovine oocytes was first observed as early as 12 h IVM, and 1PB emission rates gradually increased until 24 h IVM. They also described 2.1, 6.9, 10.4, 22.0, 33.3, 52.2, 70.0, 74.3, 85.3, 85.5, and 87.0% oocytes with extruded 1PB after IVM for 12, 14, 15, 16, 17, 18, 19, 20, 22, 24, and 26 h, respectively, and, during 12–13 h IVM, most of the oocytes reached the premetaphase I stage. When oocytes reached the premetaphase I stage, chromosomes became separated and MTs were very clearly observed. In our study, Type 1 oocytes predominated at the beginning of treatment (55.3%) in group MI, with the nucleus presenting MI stage and microtubules strongly evident in close proximity, according to the previous research.

The effect of demecolcine on oocytes was seen soon after 0.5 h of treatment with complete microtubule depletion. The reduction and lower density of microtubules in demecolcine-treated mouse oocytes reported in previous studies (Gasparrini et al., 2003; Ibáñez et al., 2003) was also verified in this one; however, the percentages did not change throughout treatment, probably due to fast demecolcine action in bovine oocytes, leading to complete depletion of microtubules. In the mouse, Ibáñez et al. (2003) observed that although MT density had reduced during demecolcine treatment, spindles did not completely disappear in this species, even after 2-h exposure to the drug. Therefore, bovine oocytes seem to be more sensitive to demecolcine action, which is supported by the fast disorganization and depolymerization of the microtubules. Fast disorganization of mi-

crotubules by demecolcine has also been seen in ovine oocytes (Hou et al., 2006).

In contrast to other species such as the mouse and porcine, where no MTs are seen in 1PB (Lee et al., 2000; Sun et al., 2001; Wang et al., 2000), Li et al. (2005) showed that when bovine oocytes reach the TI stage, MTs of the spindles form a thin, long triangle-shaped cone and the base of the cone is attached to the 1PB-destined chromosomes. MTs composed of spindles gradually move toward 1PB and 2PB, and then almost all of the MTs are expelled outside of the oocyte accompanied with 1PB and 2PB emission. Then, 1PB and 2PB become fully surrounded by MTs. Oocytes with these characteristics (Type 6—with evident MTs in only one place) were also found in our experiment, reaching 42.6% 4 h after the beginning of treatment. It is suggested that there exists some relationship or some unidentified factors connected between spindle MTs and chromosomes. The spindle formation and chromosome segregation seem to be chromosome dependent (Li et al., 2005).

Results showed microtubule repolymerization when oocytes treated with demecolcine for 2 h were cultured another 6 h in demecolcine-free medium. Hou et al. (2006) also verified reversible demecolcine action with subsequent 1PB extrusion after culturing oocytes in drug-free medium. This result is important considering that microtubules, together with microfilaments (MFs), are involved in the cytokinesis and are also fundamental in many other cellular functions. It has been shown that spindles are dynamic cell organelles, and their formation and morphological changes depend on the activity of MTs and MFs, and motor proteins associated with chromosomes and MTs. The assembly and disassembly of the cytoskeleton, in particular MTs and MFs, play key roles in the regulation of spindle formation, chromosome alignment and segregation, movement of nuclear material from a central position to the cortical area, and emission of 1PB and 2PB (Li et al., 2005). Shinozawa et al. (2004) showed the importance of microtubule organization for embryo developmental block, where development did not progress after two-cell stage in rats when microtubules presented abnormal organization. So with repolymerization and adequate microtubule organization after culturing treated oocytes in demecolcine-free medium, embryos recovered from the chemically assisted enucleation technique probably will not present disorders linked to microtubule disorganization and its consequent embryo developmental block.

Efficiency of the demecolcine-assisted enucleation technique and embryo development after the nuclear transfer

The removal of maternal chromosomes from matured oocytes is a decisive factor that may influence cloning efficiency. Chemically induced enucleation has been attempted in experimental and domestic animals since the early 90s (Elsheikh et al., 1997, 1998; Fulka and Moor, 1993; Gasparrini et al., 2003; Ibáñez et al., 2003). However, the overall efficiency of nuclear transfer procedures based on these methods remained low, mainly in domestic animals. Such as we observed in bovine oocytes, induced chemical enucleation did not provide satisfactory results, with only around 16% of enucleation rate. Therefore, in these conditions, the chemical enucleation of bovine oocytes exposured to demecolcine in MI was an inefficient technique, and the poor rates obtained turns impracticable the use of these oocytes as recipient cytoplasts in NT. However, herein the chemically assisted enucleation of MII oocytes resulted in a mean value of 90.6% enucleated oocytes which was close to other studies (96%, Li et al., 2004; and 98%, Vajta et al., 2005). Also, the demecolcine-assisted enucleation technique on bovine oocytes was highly reliable, avoiding the need for DNA specific stains that may cause oocyte damage (Smith, 1993), and compromise embryo survival (Dominko et al., 2000).

During NT, we were able to obtain 42.5% of oocytes with formed protrusions, therefore available for enucleation. Experiment I resulted in 55.1% of oocytes with protrusion, which could be explained by the difference between demecolcine exposure moments, since oocytes matured for 21 h were used in Experiment I, whereas for NT oocyte maturation was allowed for only 19 h. This earlier treatment was made due to fast metaphasic plate displacement from the polar body, which could compromise enucleation efficiency. We had previously verified that treated oocytes with 21 h maturation formed protrusions away from the PB, thus difficulting enucleation (data not shown). Because the formation of cytoplasmic protrusions is undoubtedly a physiological process, the ability of an oocyte to form the protrusion could be used as a marker for oocyte quality. In our experiments no previous selection based on the presence of polar bodies was performed, as reported by Li et al. (2004) that obtained more than 70% of oocytes with protrusion.

Vajta et al. (2005) reported an efficient chemically assisted procedure for enucleation for handmade cloning (HMC) technique in bovine oocytes. After the treatment of oocytes with demecolcine, the zonae pellucidae were digested with pronase, and 94% of oocytes had a detectable extrusion cone and could be bisected using a microblade. Nonetheless, the high efficiency achieved by the authors was not only the consequence of incubation with demecolcine, but was due to the combined effect of demecolcine and subsequent pronase digestion. Demecolcine incubation without pronase digestion also induced extrusion, but the proportion of oocytes with protrusions was smaller, and reached only 64% after the removal of the zona which was close to the values obtained for us in Experiment I.

We obtained a mean of 27.6% blastocysts after NT with bovine oocytes submitted to demecolcine-assisted enucleation. These results are lower than Vajta et al. (2005), which achieved 48% using the chemically assisted procedure in association with HMC technique, and similar to Li et al. (2004) that reported up to 29.6% blastocyst using the demecolcine-assisted enucleation. However, the last authors did not compare the chemically assisted enucleation to the traditional technique.

Kato and Tsunoda (1992) verified that prolonged demecolcine treatment (12.5 to 14.5 h) of mouse embryos had a negative effect on development. However, we found no detrimental effect of the drug as cleavage and blastocyst development rates were similar to those achieved with traditional NT. Tani et al. (2006) also did not verify differences in reconstructed embryo development potential after demecolcine-assisted enucleation, and these authors obtained a healthy calf after transferring four NT embryos, which had been produced using demecolcine-assisted chemical enucleation.

Activation of MPF, a complex formed from two proteins (cyclin B and $p34^{cdc2}$) occurs when the cell enters the M phase with induction of nuclear envelope breakdown (NEBD), chromosome condensation, cytoskeleton reorganization, and other changes in cell morphology (Campbell et al., 1993). Tani et al. (2006) recently verified that MPF activity increases in bovine oocytes exposed to demecolcine in MII by up to 30%. This complex is also extremely important for adequate donor cell remodeling and reprogramming, presenting high activity levels in reconstructed embryos after demecolcine-assisted enucleation.

The best measure of blastocyst quality is its ability to establish pregnancy and produce a live offspring (Lonergan et al., 2003). Therefore, in addition to the potential of embryo development to the blastocyst stage, the quality of embryos reconstituted by this chemically assisted enucleation technique using demecolcine still requires further elucidation.

Acknowledgments

The authors are grateful to Roberta Vantini for technical assistance. This work was supported by the Foundation for the Support of Research of the State of São Paulo (FAPESP), Brazil.

Author Disclosure Statement

The author declare that no competing financial interests exist.

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