Developmental competence of gametes reconstructed by germinal vesicle transplantation from fresh and cryopreserved bovine oocytes


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Objective: To evaluate the use of fresh or frozen bovine oocytes as an animal model for reconstructing artificial gametes by germinal vesicle transplantation (GVT), to study nucleocytoplasmic interaction and define clinical procedures for ooplasm donation in humans.

Design: Prospective experimental study.

Setting: University-based experimental laboratory.

Animal(s): Bovine oocytes from slaughterhouse ovaries.

Intervention(s): A total of 446 gametes were reconstructed from fresh immature oocytes; nuclear and cytoplasmic competencies were analyzed through the assessment of meiotic progression and cytoskeleton reorganization; embryonic developmental capability was evaluated after parthenogenetic activation of metaphase II (MII) reconstructed oocytes. Furthermore, the distribution of mitochondria in karyoplast and cytoplasm in grafted oocytes was studied. Finally, meiotic and developmental competencies were determined in 199 gametes reconstructed from vitrified immature oocytes.

Main Outcome Measure(s): Maturational and developmental rate of reconstructed oocytes, cytoskeleton organization, and mitochondrial distribution.

Result(s): Gametes reconstructed from either fresh or cryopreserved immature oocytes showed similar meiotic competence (41.6% vs. 37.7%, respectively). All reconstituted oocytes that reached MII displayed a normal distribution of cytoskeletal elements. Embryonic developmental capability was higher in oocytes derived from fresh than from cryopreserved gametes (30.8% vs. 8.1%, respectively). Finally, oocyte centrifugation was effective in obtaining karyoplasts with <5% of mitochondria.

Conclusion(s): Cows can provide a suitable organism model to develop GVT technique in both research and clinical settings as well as in fertility preservation programs. (Fertil Steril® 2010;93:229–38. ©2010 by American Society for Reproductive Medicine.)

Germinal vesicle transplantation (GVT) technique could represent a useful tool for studying the interaction between nucleus and cytoplasm in the oocyte maturation process in mammals (1–7). Using micromanipulation and electrofusion procedures, it is possible to reconstruct an artificial oocyte by transplanting the nucleus of an immature oocyte (karyoplast) into another oocyte previously enucleated (cytoplast) at the same developmental stage. In a series of well designed studies in mice, it has been demonstrated that both nuclear and cytoplasmic deficiencies are responsible for poor oocyte quality, because they contribute to meiotic defects and subsequent impaired embryo development (4, 8–11). In addition, GVT offers the opportunity to investigate the role of maternal epigenetic modifications during oocyte growth. The use of such a technique demonstrated that epigenetic modifications correlate with extended parthenogenetic development in mice and that epigenetic modifications necessary for postimplantation development occur throughout a specific phase of oocyte growth (1, 12, 13). This indicates that GVT offers the ability to identify precisely when, during oocyte development, genomic imprinting takes place and to investigate the genomic imprinting of gametes.

Furthermore, heterologous GVT has been suggested as a cell model for studying interspecific nuclear and cytoplasmic factors which regulate oocyte meiotic cell cycle progression (3, 14).

Several studies have been conducted with different animal models (3, 5, 6). However, most of them are limited to the evaluation of the in vitro maturation (IVM) capability of reconstructed oocytes.

The embryonic developmental capability of reconstructed oocytes was analyzed in a few recent studies (15–19). In cows, developmental capability was assessed by transferring metaphase II (MII) plate from a mature reconstructed oocyte into an ooplasm obtained from an enucleated oocyte matured in vitro (7), failing to demonstrate whether the cytoplasmic
compartment derived from GVT could also sustain embryonic development of the newly reconstructed oocyte.

Oocyte reconstruction can be an attractive cell model in the study of basic biologic processes; it may also represent an innovative approach to correcting some specific fertility problems in humans. GVT has been indicated as a possible treatment to correct age-related aneuploidy caused by dysfunctional ooplasm (15, 20) and as a therapeutic advancement which could help prevent the transmission of mutated mitochondrial DNA from mother to offspring (21–23).

However, in either research or clinical procedures, GVT presents a major logistic problem due to the difficulty of obtaining donor and recipient oocytes at the same developmental stage. For this reason, immature oocyte cryopreservation could provide a solution for determining the proper combination of karyoplast and cytoplast from different sources (24–27).

Starting from these observations, the aim of the present study was to establish an efficient and reliable protocol for GVT in a cow model that can be primarily used to elucidate the cytoplasmic and nuclear interplay underlying the onset and progression of meiosis, as well as to determine the effect of GVT on the developmental capability of oocytes. In addition, the efficiency of gamete reconstruction when both karyoplast and cytoplast were derived from cryopreserved GV-stage oocytes was assessed, to establish whether oocyte banking can be successfully combined with GVT technique. Finally, additional research was performed to assess the extent to which mitochondria are carried over during the GVT procedure.

MATERIALS AND METHODS
Chemicals and Reagents
All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Oocyte Collection and Preparation
Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory within 2 h in sterile saline (9 g NaCl/L) maintained at 26°C. Cumulus-oocyte complexes (COCs) were retrieved from 2–6 mm middle antral follicles with a 16-gauge needle mounted on an aspiration pump (Cook-IVF, Brisbane, Australia) with a vacuum pressure of –28 mm/Hg. All subsequent procedures, unless otherwise specified, were performed at 35°C–38°C.

The COCs were washed in M199 supplemented with 20 mmol/L HEPES, 1,790 U/L heparin, and 0.4% bovine serum albumin (HM199) and examined under a stereomicroscope. Only COCs medium-brown in color, with five or more layers of cumulus cells and homogeneous ooplasm were used. Oocytes were denuded as previously described (27). In brief, denuded oocytes (DOs) were obtained by mechanically removing cumulus cells from selected COCs by the use of vortex agitation (2 min, 35 Hz) in M199 supplemented with 20 mmol/L HEPES and 5% calf serum (Gibco Invitrogen, Milan, Italy).

To prevent spontaneous germinal vesicle breakdown (GVBD) before and during GVT procedures, the oocytes were incubated in M199 supplemented with 10% calf serum and 0.2 mmol/L 3-isobutyl-methylxanthine (IBMX). Because of the presence of lipid droplets in bovine oocytes, starting from 40 min before each micromanipulation session, a group of 10–15 oocytes were centrifuged for 5 min at 14,000 rpm to visualize the nuclear envelope (Figs. 1A and 1B). Centrifuged oocytes were exposed to M199 supplemented with 10% calf serum and 7.5 μg/mL cytochalasin B (CB) for 30 min.

Cytoplast and Karyoplast Preparation
Micromanipulation was performed in a glass Petri dish in 20-μL droplets of M199 supplemented with 0.2 mmol/L IBMX, 20 mmol/L HEPES, and 10% calf serum. All micromanipulation tools were made from borosilicate glass capillaries (Clark Electro Medical Instruments, Reading, U.K.) drawn on a horizontal micropuller (PN-30; Narishige, Tokyo, Japan) and calibrated, cut, and fire-polished on a microforge (MF-900; Narishige). Transfer pipettes were beveled on a beveler (EG-40 Narishige). Micromanipulation procedures were carried out on an inverted fluorescence microscope with Nomarsky optics (Diaphot; Nikon Corp., Tokyo, Japan) equipped with two hydraulic micromanipulators (MO188; Narishige), and tools were controlled by two injectors (holding pipette microinjector: IM-188; transfer pipette microinjector: IM-9b; Narishige).

Micromanipulation steps are illustrated in Figure 1. In particular, the GV envelope was identified by carefully rotating the oocyte as shown in Figure 1A. The zona pellucida was lanced immediately overlying the GV (Fig. 1C) with a sharp-tipped pipette, and the GV was pressure-removed from oocytes with a small amount of cytoplasm (Fig. 1D), encapsulated by part of the oocyte membrane, thus forming a karyoplast of 40–45 μm in diameter (Figs. 1E and 1F). The rest of the oocyte cytoplasm, membrane and zona pellucida formed a cytoplast.

To follow the GV during manipulation, in a preliminary study some DOs were washed two times in HM199, then DNA was stained in M199 containing 1 μg/mL Hoechst 33342 for 5 min in the dark and transferred to a glass Petri dish in the micromanipulation drop (28). Oocytes were exposed to fluorescence irradiation for no more than 3 s to visualize the chromatin in the GVs (Figs. 1B, 1F, and 1J).

Oocytes were processed and manipulated in groups of 10–15. All the gametes in each group were first enucleated and the karyoplast membrane was allowed to settle for 10 min following the enucleation procedure. During the time needed for enucleation, karyoplasts and cytoplasts obtained from the same oocytes were placed adjacent to each other and in serial order to avoid autotransplantation.

GVT and Electrofusion
Each karyoplast (Fig. 1G) was aspirated in the transfer pipette (inner diameter 35–40 μm) in a solution of 40% polyvinylpyrrolidone (PVP) and inserted into the perivitelline space...
of a heterologous cytoplast, through the incision previously made in the zona pellucida (Fig. 1H). As depicted in Figures 1I and 1J, a “grafted oocyte” constitutes a manipulated gamete in which a karyoplast and a cytoplast, isolated from two different oocytes, are still distinct entities (5).

The grafted oocytes were conditioned for 30 min in M199 supplemented with 10% calf serum, 0.68 mmol/L L-glutamine, 25 mmol/L NaHCO3, and 0.2 mmol/L sodium pyruvate at 38.5°C under 5% CO2 in humidified air before electrofusion.

The electrofusion was carried out in the fusion chamber of the Zot Cell Fusion 2000 (LPS-Electronics, Cremona, Italy) filled with 300 μL of a nonelectrolytic medium: a solution of sucrose 0.25 mol/L, as previously described (5). Each grafted oocyte was manually aligned between the two platinum filaments of the chamber. The alignment consisted in orienting the axis along the adjacent cell membranes perpendicular to the electric current vector (5). To induce fusion, a 1.0 kV/cm direct current pulse was delivered for 70 μs. A second pulse was delivered after an interval of 30 min if fusion did not occur.

Assessment of Meiotic Competence and Cytoskeleton Organization of Reconstructed Oocytes After in Vitro Maturation

Oocytes reconstructed as described above were matured in 500 μL of medium, in four-well dishes (NUNC; VWR International, Milan, Italy) and incubated for 24 h at 38.5°C in humidified air and in the presence of an equal number of intact COCs, as previously described to sustain denuded oocyte maturation (29). The maturation medium was M199, supplemented with 10% calf serum, 0.68 mmol/L L-glutamine, 25 mmol/L NaHCO3, 0.2 mmol/L sodium pyruvate, and 0.1 IU/mL of recombinant human FSH (rhFSH: Gonal-F; Serono, Rome, Italy).

The stage of nuclear maturation was assessed by fluorescence microscopy following fixation in 60% methanol in phosphate-buffered saline (PBS) and staining with 0.5 mg/mL propidium iodide (30).

Oocytes were classified as follows: GV: oocytes that did not develop beyond the germinal vesicle stage; intermediate: oocytes at GVBD and metaphase I stage; mature: oocytes from anaphase I to MII stage. Oocytes which could not be identified as being at any of these stages were classified as not matured.

To assess whether manipulation procedures affected maturation capability, two experimental groups of GV oocytes were constituted as follows: 1) IBMX: DOs incubated with IBMX for 3 h but not micromanipulated before in vitro maturation; and 2) IBMX+CB: DOs incubated with IBMX for 3 h, centrifuged for 5 min at 14,000 rpm, and incubated with CB for 30 min, but not micromanipulated before in vitro maturation. A group of untreated DOs were used as a control. The stage of meiotic progression was assessed as described above.

IVM. In short, DOs were fixed after 24 h of IVM in 0.3% Triton X-100 and 2% paraformaldehyde in Dulbecco’s PBS (DPBS) at 37°C for 1 h. After incubation for 30 min at room temperature in DPBS containing 1% bovine serum albumin (BSA), 10% Normal Donkey Serum, and 0.3% Triton X-100, the oocytes were incubated overnight at 4°C with an antitubulin monoclonal antibody diluted 1:200 in DPBS. Next, they were incubated with a fluorescein isothiocyanate–conjugated antimouse IgG antibody raised in donkey (Jackson Immunoresearch Lab, West Grove, PA) for 1 h at room temperature. To detect the distribution of microfilaments, the oocytes were exposed to tetramethylrhodamine B isothiocyanate–labeled phalloidin (1 μM) for 1 h at room temperature. As a control, some DOs were treated with unlabeled phalloidin before incubation with a labeled filaments marker and incubated directly with the secondary antibody, without pretreatment. All samples were mounted with an antifade medium (Vectashield Vector Lab, Burlingame, CA) and observed with a conventional epifluorescence microscope (Eclipse E600; Nikon Corp., Tokyo, Japan).

Immature Oocyte Vitrification and Warming

To obtain vitrified/warmed gametes for GVT procedure, oocyte vitrification was carried out following the method of open pulled straws (OPS; Minitube, Tiefenbach, Germany) (31), modified for denuded oocytes as previously described (27). Briefly, groups of three to five DOs were initially equilibrated for 1 min in M199 supplemented with 20 mmol/L Hepes and 20% calf serum (HM). After equilibration, oocytes were incubated in HM supplemented with 10% of ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO) for 30 sec and then transferred into HM supplemented with 20% EG, 20% DMSO, and 1 mol/L sucrose for 25 s. Oocytes were loaded in straws and immediately submerged into liquid nitrogen.

Warming was performed by placing the end of the straw directly into the HM supplemented with 0.3 mol/L sucrose. The vitrified medium became liquid within 1–2 s, whereupon the medium entered the straw. The oocytes flowed out of the straw into the HM where they remained for 1 min and were then transferred and washed for 5 min in 0.2 mol/L sucrose–supplemented HM.

After this, oocytes were washed in HM and equilibrated for 15–30 min in M199 supplemented with 10% calf serum, 0.68 mmol/L L-glutamine, 25 mmol/L NaHCO3, and 0.2 mmol/L sodium pyruvate at 38.5°C under 5% CO2 in humidified air. After equilibration, oocytes were processed for GVT to reconstruct gametes entirely composed of frozen/thawed oocytes.

Meiotic and Developmental Competence of Reconstructed Oocytes Obtained From Fresh and Cryopreserved Gametes After Parthenogenetic Activation

Oocytes reconstructed entirely from fresh or from vitrified/warmed oocytes were matured in vitro for 24 h as described above. After maturation, some replicates were fixed and stained to assess the stage of meiotic progression. In other replicates, oocytes displaying polar body were activated with 10 μmol/L calcium ionophore A23187 for 5 min followed by exposure to 2 mmol/L 6-DMAP for 4 h (32). Subsequently, the oocytes were washed three times in a modified synthetic oviduct fluid (SOF) (33) supplemented with 0.3% (w/v) BSA fraction V, fatty acid–free, MEM essential and nonessential aminoacids, and 0.72 mmol/L sodium pyruvate and buffered with 10 mmol/L HEPES and 5 mmol/L NaHCO3 (29). Activated oocytes were rinsed two times and then transferred to 400 μL embryo culture medium. The embryo culture medium was SOF buffered with 25 mmol/L NaHCO3 supplemented with MEM essential and nonessential aminoacids, 0.72 mmol/L sodium pyruvate, 2.74 mmol/L myo-inositol, 0.34 mmol/L sodium citrate, and 5% calf serum. Incubation was performed at 38.5°C under 5% CO2, 5% O2, and 90% N2 in humidified air as previously described (29). In each experiment, two groups of nonmanipulated DOs, thawed and fresh, were used as control groups (Control-V and Control-F, respectively).

At the end of the culture period (168 h after activation), embryos were fixed in 60% methanol in PBS. Cleavage and blastocyst rates were assessed after staining with 0.5 mg/mL propidium iodide, as previously described (30), and cell nuclei were counted under fluorescence microscopy.

Effect of Manipulation Procedures on Mitochondrial Distribution in Grafted Oocytes

To assess to what extent the centrifugation and manipulation procedure affects which mitochondria were carried over during GV removal, we labeled total mitochondria in grafted oocytes before membrane fusion. For this purpose, in each experiment a group of three or four grafted oocytes reconstituted as described above was incubated with 300 nmol/L MitoTracker GreenFM (Molecular Probes, Eugene, OR) for 30 min in M199 supplemented with 10% calf serum, 0.68 mmol/L L-glutamine, 25 mmol/L NaHCO3, and 0.2 mmol/L sodium pyruvate at 38.5°C under 5% CO2 in humidified air. After incubation, grafted oocytes were washed several times then mounted on slides and observed by conventional epifluorescence microscopy (Eclipse E600). The proportion of mitochondria in each karyoplast and cytoplast was estimated according to Liu et al. (11, 34). Digitalized images of grafted oocytes after staining were taken, and the relative fluorescence intensity was assessed using NIH ImageJ 1.38 software (Rasband WS, ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

Statistical Analysis

All experiments were repeated at least 5 times. Differences were analyzed by the chi-squared test. Probabilities of less than 0.05 were considered to be statistically significant.

RESULTS

In total, 645 GV oocytes (446 fresh and 199 vitrified) that underwent micromanipulation were included in the present study.
Assessment of Meiotic Competence and Cytoskeleton Organization of Reconstructed Oocytes After in Vitro Maturation

The effects of IBMX, CB, and centrifugation treatments that preceded the oocyte manipulation were assessed to evaluate whether they affect the maturation capability. As presented in Table 1, in both of the experimental groups, IBMX (DOs incubated with IBMX for 3 h) and IBMX+CB (DOs incubated with IBMX for 3 h, centrifuged, and incubated with CB for 30 min), the maturation rate was slightly, but significantly, lower compared with the control oocytes.

The efficiency of the reconstruction procedures was judged by the capability of grafted oocytes to undergo fusion between cytoplast and karyoplast membranes and to resume and complete the first meiotic division after IVM. An analysis of the data, reported in Table 2, indicated a high survival rate in reconstructed oocytes. In particular, fusion was detected in more than 70% of grafted oocytes, and over 50% resumed meiosis. Importantly, 41.7% of the reconstructed oocytes were able to mature in vitro. However, the reconstruction procedures significantly affect maturational competence, because the percentage of matured reconstructed oocytes was lower than in the control group.

The cytoskeleton organization of reconstituted oocytes after IVM was analyzed to assess the effectiveness of gamete reconstruction procedures on nucleocytoplasmic cooperation after karyoplast and cytoplast fusion. The immunocytochemical study demonstrated that the normal distribution of microfilaments and microtubules was associated with the proper progression of meiotic division through MII. In fact, oocytes that did not correctly reach MII showed macroscopic alterations under epifluorescence microscopy, with tubulin diffuse in the ooplasm, sometimes organized in clusters, with crater formations, and showing discontinuity in the cytoskeletal actin microfilaments.

An example of an oocyte showing abnormal distribution of microfilaments and microtubules after reconstruction and subsequent maturation is shown in Figures 2A–2C. Conversely, all mature oocytes showed a correct localization and distribution of microfilaments and microtubules. Microfilaments were localized mainly in the cortex, overlying the metaphase chromatin and the polar body (Fig. 2D). Microtubules were detected both in the spindle and in the polar body (Fig. 2E). The same distribution of cytoskeletal elements was also observed in matured oocytes that did not undergo manipulation (control group).

Meiotic and Developmental Competence of Reconstructed Oocytes Obtained From Fresh and Cryopreserved Gametes

Fusion efficiency and maturational competence were not significantly different between artificial gametes reconstructed from either fresh or cryopreserved GV-stage oocytes. Specifically, as shown in Table 3, no significant differences were observed in the reconstruction efficiency after fusion between the two groups (69.6% and 77.8%, respectively). The maturational competence of artificial gametes in which both karyoplast and cytoplast were derived from cryopreserved oocytes was not different from that of gametes reconstructed entirely with fresh oocytes (37.7% and 41.6%, respectively). However, compared with the respective control samples, the maturation rate of reconstructed oocytes was significantly reduced, as shown in Table 3.

On the other hand, blastocyst yield was affected by vitrification and warming procedures. As indicated in Table 4, after parthenogenetic activation, 66.7% of reconstructed gametes originated from fresh oocytes cleaved and 30.8% developed up to the blastocyst stage (Fig. 3). This percentage was similar to the blastocyst rate of fresh denuded oocytes (Control-F) after parthenogenetic activation (33.3%). In contrast, the developmental capability of oocytes reconstituted from vitrified/warmed denuded oocytes appeared significantly lower than that of oocytes reconstructed from fresh gametes (8.1%). However, a decrease of developmental capability was also observed in vitrified/warmed DOs, even in the absence of manipulation (Control-V). The embryo quality, as judged by the number of cells composing each blastocyst, was similar in all experimental groups.

Effect of Manipulation Procedures on Mitochondrial Distribution in Grafted Oocytes

The relative amounts of mitochondria that were carried over during GV removal were assessed to establish whether centrifugation treatment before manipulation, which is necessary to visualize the nuclear envelope in bovine oocytes, could
also be an effective means of obtaining karyoplasts with minimal contaminating mitochondria. The relative fluorescence intensity analysis, as assessed by using NIH ImageJ software on digitized images, indicated that the proportion of mitochondria in the karyoplasts of grafted oocytes was an average 4.3 ± 0.5% (mean ± SE) of total mitochondria. Figure 4 clearly shows that the GV karyoplast displayed a slight green signal, indicating a negligible content of mitochondria, and almost all of these organelles were confined to the enucleated cytoplasm. Importantly, all of the grafted oocytes examined showed a similar mitochondrial ratio between karyoplast and cytoplast, indicating that centrifugation was effective in drastically reducing the transmission of mitochondria through the karyoplast.

**DISCUSSION**

Currently, understanding of the nucleocytoplasmic interaction necessary for normal oocyte differentiation (and ultimately for regular meiotic progression) is limited by the lack of appropriate cell models for analyzing the relative contributions of each intracellular compartment. Several studies have demonstrated that nuclear transplantation at GV stage offers the prospect of creating reconstituted immature oocytes for studies on nucleocytoplasmic interaction during gametogenesis.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oocytes, n</th>
<th>Fused, n (%)</th>
<th>GV, n (%)</th>
<th>Intermediate, n (%)</th>
<th>Mature, n (%)</th>
<th>Not mature, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVT</td>
<td>181</td>
<td>132 (72.9)</td>
<td>4 (3.1)</td>
<td>13 (9.8)</td>
<td>55 (41.7&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>60 (45.4&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Control</td>
<td>197</td>
<td>—</td>
<td>1 (0.6)</td>
<td>13 (6.6)</td>
<td>164 (83.2&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>19 (9.6&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

Note: GV = germinal vesicle; GVT = germinal vesicle transplantation.  
<sup>a,b</sup> Within columns, different superscripts indicate significant differences (P < .05).


**FIGURE 2**

Fluorescent images of reconstructed oocytes after in vitro maturation, showing microfilaments in red, microtubules in green, and DNA in blue. (Top row) Representative images of improperly matured oocytes. (A, B) Microfilaments and microtubules diffused irregularly in the ooplasm and partially condensed in small clusters. (C) DNA condensed into a single clump. (Bottom row) Representative images of metaphase II–stage oocyte. (D, E) Microfilaments and microtubules disposed beneath the oolemma and overlying the metaphase plate and the polar body. (F) Metaphase plate and polar body. Control denuded oocytes showed the pattern and distribution of cytoskeleton elements presented in the lower row. Bar represents 50 μm.

The present research shows that cows can provide an appropriate animal model to study the above-mentioned issues, also demonstrating for the first time in this species that artificial oocytes reconstructed by GVT can successfully sustain both the meiotic process and embryonic development, without needing fresh mature ooplasm and MII oocyte reconstruction (7). In particular, more than 50% of reconstructed oocytes resumed meiosis and 42% matured in vitro. However, reconstituted oocytes showed a lower maturation rate compared with the control group (86%). The effect of pre-manipulation treatments and the invasive procedure, which are essential but negatively affect the outcome of this technique, clearly account for this decrease.

In mice, it has been shown that treatment with CB to induce microfilament disruption is required for enucleation and nuclear transfer in GV oocytes (35).

Because of the presence of lipids, ooplasm centrifugation is necessary to visualize the nuclear envelope in bovine oocytes (36) as an alternative to nuclear staining with Hoechst 33342 and ultraviolet light exposure during micromanipulation (28), which are detrimental to oocyte viability (37, 38).

Throughout the GVT procedure, oocytes are maintained in meiotic arrest. An earlier study on mice demonstrated that the removal of cumulus cells and IBMX treatment before GVT did not cause abnormalities in meiotic progression (11). However, bovine oocytes could be sensitive to the phosphodiesterase inhibitor during meiotic arrest in a different way (39).

One of the main factors limiting the early development of reconstituted oocytes could be the absence of cumulus cells during oocyte maturation (40). However, as we previously demonstrated, the presence of intact oocyte-cumulus complexes in coculture during IVM of reconstructed gametes can overcome the reduced developmental capacity of denuded bovine oocytes and restore the original embryonic developmental competence (28, 29).

The immunocytochemical analysis on microtubules and microfilaments demonstrated that when properly allocated, cytoskeleton elements contribute to normal meiotic resumption and completion. This corresponds with earlier observations using both confocal and epifluorescence microscopy (27, 41, 42). The present analysis indicated that after micromanipulation and electrofusion procedures, artificial oocytes are still able to reorganize the ooplasmic components directly involved in meiotic division.

To our knowledge, the present work demonstrated for the first time that a bovine oocyte could sustain maturation after

### TABLE 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oocytes, n Fused, n (%)</th>
<th>GV, n (%)</th>
<th>Intermediate, n (%)</th>
<th>Mature, n (%)</th>
<th>Not mature, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified</td>
<td>99 77 (77.8) 4 (5.2)</td>
<td>10 (13)</td>
<td>29 (37.7)</td>
<td>34 (44.1)</td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>145 101 (69.6) 3 (3.0)</td>
<td>11 (10.9)</td>
<td>42 (41.6)</td>
<td>45 (44.5)</td>
<td></td>
</tr>
<tr>
<td>Control-V</td>
<td>142 — 2 (1.4)</td>
<td>11 (7.7)</td>
<td>111 (78.2)</td>
<td>18 (12.7)</td>
<td></td>
</tr>
<tr>
<td>Control-F</td>
<td>149 — 1 (0.7)</td>
<td>9 (6)</td>
<td>124 (83.2)</td>
<td>15 (10.1)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Control-V, Control-F: nonmanipulated vitrified/thawed and fresh donor oocytes, respectively; GV = germinal vesicle. a,b Within columns, different superscripts indicate significant differences (P < .05).


### TABLE 4

<table>
<thead>
<tr>
<th>Development to:</th>
<th>Treatment</th>
<th>Oocytes, n</th>
<th>2–4 cells, n (%)</th>
<th>Blastocyst, n (%)</th>
<th>Cell number, (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitrified</td>
<td>37</td>
<td>12 (32.4)</td>
<td>3 (8.1)</td>
<td>55.1 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
<td>39</td>
<td>26 (66.7)</td>
<td>12 (30.8)</td>
<td>73.8 ± 16.5</td>
</tr>
<tr>
<td></td>
<td>Control-V</td>
<td>28</td>
<td>20 (71.4)</td>
<td>3 (10.7)</td>
<td>57.2 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>Control-F</td>
<td>102</td>
<td>78 (76.5)</td>
<td>34 (33.3)</td>
<td>76.3 ± 6.8</td>
</tr>
</tbody>
</table>

Note: Abbreviations as in Table 4. a,b Within columns, different superscripts indicate significant differences (P < .05).

reconstitution even when both nuclear and cytoplasmic components are derived from cryopreserved immature oocytes. Moreover, the fusion rate and the maturational capability of oocytes entirely reconstituted from thawed material did not differ from those of artificial gametes reconstructed with fresh oocytes, a finding that has also been reported in mouse models (43). In Moffa et al.’s study (43) the maturation rate of reconstructed GV oocytes was not affected by freezing, because no appreciable differences were observed in the maturation rate when comparing oocytes reconstructed using fresh or thawed cytoplast and/or karyoplast. In fact, Moffa et al. observed a slight significant increase when oocytes were reconstructed with both components derived from cryopreserved gametes. For this reason, in the present work we tested the meiotic and developmental competencies from all-fresh and all-cryopreserved reconstructed oocytes. However, in our experiments we did not observe this trend, because the maturation rates of the two experimental groups were similar.

After parthenogenetic activation, oocytes reconstructed from fresh gametes developed up to the blastocyst stage similarly to the control group (30.8% and 33.3%, respectively), demonstrating that both cytoplasmic and nuclear components of the GVT-reconstructed oocyte are capable of early embryonic development.

However, in the present study it was the vitrification process rather than manipulation procedures that dramatically reduced both early segmentation and embryonic developmental competence, as shown in Table 4. Although these results demonstrate that changes occurring in the oocyte during vitrification/warming are critical for embryonic developmental competence but not for maturational capacity, further studies are needed to identify which component(s) is(are) affected by the cryopreservation procedure. In perspective, this would be useful to identify a strategy of donation using fresh or cryopreserved karyoplasts/cytoplasts.
Therefore, optimization of GVT technique with frozen/thawed oocytes, using either cryopreserved karyoplast or the donor’s cytoplast, might be an option not just for research purposes but also to preserve fertility in young cancer patients or individuals with a history of premature ovarian failure. Combining immature oocyte cryopreservation and GVT technique could have a strong impact in the application of assisted reproduction in cases where fertilizable oocytes are lacking, such as infertility, animal breeding and fertility preservation programs. This option could increase the number of oocytes rescued from a single ovary because of the large number of oocytes collected from growing follicles, stored and successively used as GV donors.

Germlinal vesicle transplantation has also been suggested as a means to overcome problems associated with aged oocytes, whereby the GV is transferred into an enucleated recipient oocyte from the same stage of development as the donor nucleus. In aged oocytes, there is likely to be a higher proportion of mutant mitochondrial DNA (44). Furthermore, it has been proposed that aberrant mitochondrial function in aged oocytes may play a role in oocyte aneuploidy (45), because mitochondria are known to be involved in chromosome organization and movement. In fact, the induction of mitochondrial damage in mouse oocytes prevented oocyte maturation, chromosomal segregation, and spindle formation (15). This damage was overcome by transplanting the karyoplast derived from a damaged oocyte into the ooplasm of a healthy enucleated oocyte. Therefore, by means of GVT the nuclear component of an aged oocyte can be transferred into the enucleated ooplasm of a younger and healthier oocyte.

Germlinal vesicle transplantation has been proposed as a technique for treating mitochondrial DNA disease (46). However, potential problems could arise because the transferred GV is still surrounded by defective mitochondria, which would also be carried over into the donor ooplasm. The most important issue would be heteroplasmy resulting from a mixture of donor and recipient mitochondria (47). This may be solved eventually by mitochondria-free karyoplasts. In the present study, oocyte centrifugation before manipulation led to the isolation of karyoplasts with a negligible amount of mitochondria (4.3%), lower than that approximated in previous reports (11, 34), therefore preventing the heteroplasmy which would result from the mixture of karyoplast and cytoplast mitochondria, as recently suggested (21, 47).

However, given the potential for following a clinical approach of ooplasm donation and GVT in humans (48, 49), efforts still need to be made to evaluate such treatments for both efficacy and safety in an appropriate animal model.

During oocyte growth, epigenetic modifications have a dramatic effect on subsequent development after fertilization or parthenogenetic activation (12, 50). Maternal imprinting is established during oogenesis and is associated with allele-specific modification through DNA methylation. In the oocyte GV, chromatin remodeling occurs in a stage-specific manner during oocyte differentiation, and DNA modifications confer meiotic and developmental competence on growing oocytes. By using GVT, earlier studies demonstrated that chromatins from nongrowing primary oocytes can support embryonic development only up to implantation, whereas development beyond this stage requires epigenetic changes, which are completed during the final process of oocyte growth (1, 12, 13).

Therefore, GVT technique provides an exciting way to investigate the molecular mechanisms governing the genomic imprinting in the female gamete, and to study the role of specific patterns of DNA modification on embryonic development.

In conclusion, the present results demonstrate that cows are a suitable organism model for developing GVT technique, and that both nuclear and cytoplasmic components derived from fresh or cryopreserved immature bovine oocytes are suitable for GVT procedure, because they generate oocytes able to progress to MII and sustain embryonic development after parthenogenetic activation. In particular, when not compromised by GVT procedures, cytoskeleton elements appear properly allocated and contribute to normal meiotic resumption and completion, assisting progress through early embryonic development.

Finally, this technique could provide a powerful tool for studying the mechanisms involved in the interplay between the nucleus-cytoplasm and epigenetic establishment during oocyte growth and differentiation. Moreover it could offer the possibility of using cryopreserved immature oocytes as a source of nuclei and ooplasm, and it could help in applying GVT procedure in research and clinical settings as well as fertility preservation programs.

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