

## Preliminary observations in in vitro development of equine embryo after ICSI

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**Abstract** — The objective of this study was to perform intracytoplasmic sperm injection (ICSI) on in vitro matured equine oocytes and to improve in vitro embryonic development on Vero cells after activation of the microinjected oocytes with calcium ionophore. After maturation (23 or 40 h, 38.5 °C, 5 % CO<sub>2</sub>), the cumulus-oocyte complexes were denuded, centrifuged and all oocytes exhibiting the first polar body were microinjected. ICSI was performed using fresh semen from three fertile stallions. Microinjected oocytes were activated with calcium ionophore A23187 (10 min, 10 μM) and cultured individually for 7 days on Vero cells in microdrops. In seven trials, 353 cumulus-oocyte complexes were matured and 103 oocytes were microinjected. Eight oocytes were sham microinjected. After ICSI, 85 oocytes (82.5 %) survived the sperm injection procedure. Among the 76 successfully microinjected oocytes, 52 (68 %) were fertilized (two pronuclei, syngamy stage and cleaved ova). Sham microinjected oocytes were not activated. After in vitro culture, 35 ova (46 %) were cleaved 2 days after ICSI and early embryonic development was obtained (three embryos of 23 cells, 50 cells and more than 80 cells) 5 to 7 days after ICSI. © Inra/Elsevier, Paris.

### ICSI / equine / oocyte activation / co-culture / embryo development

**Résumé** — Développement embryonnaire in vitro après ICSI dans l'espèce équine : observations préliminaires. L'objectif de ce travail était d'étudier, après microinjection d'un spermatozoïde dans le cytoplasme d'ovocytes de jument (ICSI) et activation par un ionophore calcique des ovocytes microinjectés, le développement in vitro des embryons ainsi obtenus sur un tapis de cellules Véro. Après maturation in vitro (23 h ou 40 h, 38,5 °C, 5 % CO<sub>2</sub>), les complexes ovocyte – cumulus ont été décoronés, centrifugés, et tous les ovocytes présentant un globule polaire ont été microinjectés. Le sperme frais de trois étalons fertiles a été utilisé. Les ovocytes microinjectés ont été activés à l'ionophore calcique A23187 (10 min, 10 μM) puis cultivés individuellement pendant 7 jours dans des microgouttes, sur un tapis de cellules Véro. Au cours de sept essais, 353 complexes ovocyte – cumulus ont été maturés et 103 ovocytes microinjectés. Huit ovocytes ont été microinjectés sans spermatozoïde (témoins). Après microinjection, 85 ovocytes (82,5 %) ne présentaient aucune lésion

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apparente. Sur 76 ovocytes microinjectés avec succès, 52 (68 %) ont été fécondés (deux pronuclei, syngamie et clivage). Les ovocytes témoins n'ont pas été activés. Après 2 j de culture in vitro post ICSI, 35 ovocytes microinjectés (46 %) se sont divisés et, 5 à 7 j post-ICSI, trois jeunes embryons ont été obtenus, 2 de 23 et 50 cellules, et un de plus de 80 cellules. © Inra/Elsevier, Paris.

## ICSI / équin / activation / coculture / développement embryonnaire

### 1. INTRODUCTION

To date, limited success has been achieved with conventional in vitro maturation/in vitro fertilization (IVM/IVF) systems using equine oocytes, whereas it is now widely used in other domestic animals. The main problems in this species are 1) the in vitro zona pellucida hardening (probably induced by the oocyte IVM) which has detrimental effects on in vitro sperm penetration [16, 26] and 2) an efficient in vitro capacitation method for stallion spermatozoa in conventional IVF which is necessary to accomplish binding and then fusion with the oocyte [3].

In human IVF, several assisted fertilization techniques have been used to overcome severe forms of male infertility. In particular, the intracytoplasmic sperm injection (ICSI) has been introduced with large success [35, 36, 45, 46]. This technique bypasses the different barriers such as the zona pellucida and does not require human sperm capacitation or acrosome reaction [27, 38] even if some form of membrane modification occurring during capacitation seems to be required for achieving fertilization after ICSI in species such as the rabbit [23], cow [14] and mouse [25]. In the equine species, the ICSI technique has been shown in recent studies to be an alternative solution to bypass the in vitro barriers of the conventional IVF [5, 18, 19, 31, 40]. This technique has been demonstrated to significantly increase the fertilization rate of in vitro matured oocytes in comparison to conventional IVF ([7]: 44.7 versus 22.2 %). The first pregnancy was reported by Squires et al. [40]. After transfer of one cleaved egg obtained after

ICSI of four IVM equine oocytes, one normal foal was born. Recently, new pregnancies and two foal births were reported by Cochran et al. [6] and McKinnon et al. [30] respectively, after microinjection of in vitro [6] and in vivo [30] matured oocytes and embryo transfer 48 h post-ICSI.

There are still two major problems in equine ICSI, however. First, the higher fertilization rates obtained after ICSI in equine species were not paralleled by increased cleavage, thus questioning the quality of the oocytes in terms of completeness of maturation and activation ([8]: 52 % fertilized and 13 % cleaved; [17]: 50 and 16 %; Guignot, unpublished results: 67 and 24 %). Recently, Dell'Aquila et al. [9] increased the cleavage rate after ICSI by maturing oocytes in equine follicular fluid versus oestrous mare serum (48.4 % of cleaved ova and 4.2 %, respectively). Some constituents of the follicular fluid such as high concentrations of steroids or FSH could enhance cytoplasmic maturation resulting in a higher cleavage rate. In bovine, the activation of oocytes with calcium regulating agents such as calcium ionophore A23187 significantly enhanced the rate of cleavage after ICSI ([24]: 38 versus 8 %). Second, in equine species, the further embryonic development (> 16 cells) of the cleaved ova obtained after ICSI is limited in vitro [9, 31], questioning the in vitro culture (IVC) conditions. Little work has been carried out on in vitro culture of IVF embryos in horses. Only one study reported a glucose concentration effect on in vitro development of embryos to the morula stage after assisted fertilization (partial zona removal) in a chemically defined medium under a low oxygen concentration

[1]. When comparing co-culture with mare oviductal cells and culture medium alone, Ball and Miller [2] found a positive effect of co-culture on in vitro development of equine embryos (four to eight cells) recovered in vivo from the oviduct after fertilization. In other domestic species and in humans, various co-culture systems such as cellular monolayers were designed to overcome the low rate of in vitro embryonic development to blastocyst stage after IVF. Co-culture on monolayers of Vero cells (kidney cells, Green African monkey) has improved the in vitro early embryonic development in humans [34, 39, 43], in mice [4] and in bovine [32]. Moreover, co-cultured embryos yield viable pregnancies in humans [34, 43]. In equine species, Dell'Aquila et al. [8] demonstrated that co-culture with Vero cell monolayers was more efficient than granulosa cell monolayers or mare oviductal epithelial cells after ICSI in order to improve embryo cleavage rate. No data were given about co-culture effects on later in vitro embryonic development.

The aim of our study was to perform ICSI on in vitro matured equine oocytes and to try to improve the cleavage rate with activation of the microinjected oocytes with calcium ionophore and to observe further in vitro embryonic development on Vero cells.

## 2. MATERIALS AND METHODS

### 2.1. Oocyte preparation

Unless otherwise stated, during all manipulations ovaries and cumulus-oocyte complexes (COCs) were kept at 37 °C. Ovaries of mares slaughtered between April and June were transported to the laboratory within 9 h of slaughter in 0.9 % NaCl. At the laboratory, a controlled aspiration technique was used to puncture follicles of 2 to 30 mm in diameter. COCs were distributed among four classes: with a compact or partial cumulus (cc), surrounded by corona radiata without cumulus (cr), with a totally or partially expanded cumulus (exp) and denuded, cumulus and corona radiata completely absent (d). This

latter class (d) was eliminated. The other classes of COCs (cc, cr, exp) were matured in vitro in the medium described by Goudet et al. [15]. It consisted of tissue culture medium 199 (TCM199, M 4530; Sigma, La Verpillère, France), supplemented with inactivated foetal calf serum (FCS, 20 %; Gibco, Eragny, France), antibiotics (100 IU·mL<sup>-1</sup> penicillin, 100 µg·mL<sup>-1</sup> streptomycin and 0.25 µg·mL<sup>-1</sup> Fungizone; Gibco), epidermal growth factor (EGF, 50 ng·mL<sup>-1</sup>; Sigma) and estradiol 17 β (1 µg·mL<sup>-1</sup>; Sigma). Within 11 to 12 h after mare slaughter, COCs were matured in groups, 5 to 30 COCs of the same class per 500 µL, for 23 h (cr and exp classes) or 40 h (cc class) at 38.5 °C in humidified air containing 5 % CO<sub>2</sub>. At the end of the maturation period, COCs were treated with hyaluronidase (87.5 IU·mL<sup>-1</sup>, type I-S; Sigma) for 2 min, then rinsed in a saline buffer solution (PBS Dulbecco 'A'; Unipath, Dardilly, France) with 3 % FCS. All oocytes with an intact cytoplasmic membrane were centrifuged in PBS (8 min, 1 500 g) to condense the lipid droplets to one sector of the oocytes. Only oocytes exhibiting the first polar body (PB), i.e. presumed at matured nuclear stage, were microinjected. Oocytes of the exp and cr classes without PB after 23 h of maturation were matured for a further 17 h as cited earlier.

### 2.2. Sperm preparation

Three fertile stallions of proved fertility were used. After collection, semen was diluted to 20·10<sup>6</sup> spermatozoa·mL<sup>-1</sup> in modified Hank's salts supplemented with 67 mM glucose and 126 mM lactose (HGLL, [29]) at pH 7.1, centrifuged (5 min, 500 g) to remove seminal plasma, resuspended in 10 mL of HGLL with BSA (1 %, Sigma, fraction V) and divided into two aliquots. The first one was immediately used to microinject oocytes of the cr and exp classes, whereas the second was stored at 15 °C for 17 h and used to microinject oocytes of the cc class.

### 2.3. Intracytoplasmic sperm injection procedure

The intracytoplasmic sperm injection procedure was performed basically as described by Van Steirteghem et al. for humans [46]. Briefly, the glass pipettes were pulled using a horizontal microelectrode puller (Type 773; Campden Instruments Ltd., London, England). The holding

pipette was cut and fire-polished (outer and inner diameters of 90 and 20  $\mu\text{m}$ , respectively) on a with a microforge (Microforge of De Fonbrune, 86 950; Alcatel, Annecy, France). For the injection pipette, the tip of the pulled capillary was ground to outer and inner diameters of 7 and 5  $\mu\text{m}$ , respectively, on a grinding wheel. The bevel angle was 40–45°.

The ICSI procedures were carried out on the heated stage of an inverted microscope (CK-2; Olympus, 400 $\times$ ) using phase contrast optics and Narishige micromanipulators (MO188; Narishige, Tokyo, Japan). A row of 2.5  $\mu\text{L}$  PBS droplets were placed near a row of 5  $\mu\text{L}$  HGLL droplets with 8 % polyvinylpyrrolidone (PVP 360 000; Sigma) in a Petri dish (Falcon type 1 016) under sterile light white oil (Sigma). One oocyte was placed in each PBS droplet and 0.5  $\mu\text{L}$  of sperm were added to an HGLL-PVP droplet. The concentration of sperm in the HGLL-PVP droplet was approximately  $2 \cdot 10^6$  spermatozoa  $\cdot \text{mL}^{-1}$ . A single motile spermatozoon was selected and immobilized by crushing the tail against the bottom of the dish with the injection pipette. It was then aspirated tail first into the injection pipette and positioned close to the pipette opening. After the polar body of the oocyte was orientated at 12 or 6 o'clock, the injection pipette was pushed into the ooplasm at 3 o'clock (*figure 1a*). A small amount of ooplasm was aspirated into the injection pipette to ensure that the oolemma was penetrated (*figure 1b*). Then, it was gently pushed back into the centre of the oocyte with the spermatozoon and a minimal volume of HGLL-PVP (1–2 pL) (*figure 1c*). The oocytes controlled as microinjected with one spermatozoon into the ooplasm and not damaged during ICSI were considered as successfully microinjected oocytes. Eight oocytes were sham microinjected (without spermatozoon, but with injection medium). All microinjected oocytes were observed 5 to 15 min later to estimate if they had, by morphology only, survived the injection traumatism.

#### 2.4. Ionophore treatment and embryo culture

After ICSI, oocytes were activated for 10 min in culture medium containing 10  $\mu\text{M}$  calcium ionophore A23187 dissolved in 0.02 % DMSO (Sigma), at 38.5 °C in humidified air containing 5 %  $\text{CO}_2$ . The culture medium consisted of TCM199 (M 4530; Sigma), supplemented with

inactivated FCS (20 %; Gibco) and antibiotics (100 IU  $\cdot \text{mL}^{-1}$  penicillin, 100  $\mu\text{g} \cdot \text{mL}^{-1}$  streptomycin and 0.25  $\mu\text{g} \cdot \text{mL}^{-1}$  Fungizone; Gibco). After ionophore treatment, the oocytes were carefully rinsed (four times) in fresh culture medium.

Vero cells were seeded from frozen samples at a concentration of  $1 \times 10^5$  cells per microdrop of 20  $\mu\text{L}$  under sterile oil in the culture medium (38.5 °C, 5 %  $\text{CO}_2$ ). At confluence, the microdrops were ready for culture of microinjected oocytes. Half of the culture medium in each microdrop was renewed 1 day before starting culture, the day of starting culture and every 48 h. All microinjected oocytes were individually cultured for 7 days on Vero cells in microdrops.

#### 2.5. Embryonic assessment

Two days after ICSI, ova were observed on a light microscope. Culture of uncleaved ova was stopped, while all cleaved ova were cultured another 5 days before embryonic assessment.

The ova were labelled with Hoechst 33342 (2.5  $\mu\text{g} \cdot \text{mL}^{-1}$ ; Sigma) and observed under an epifluorescence microscope (Olympus IMT-2, 400 $\times$ ) to visualize fertilization in the uncleaved ova (swollen sperm head, pronuclei or syngamy) 2 days after ICSI and to confirm cleavage by the presence of a nucleus in each cell 7 days after ICSI.

#### 2.6. Statistical analysis

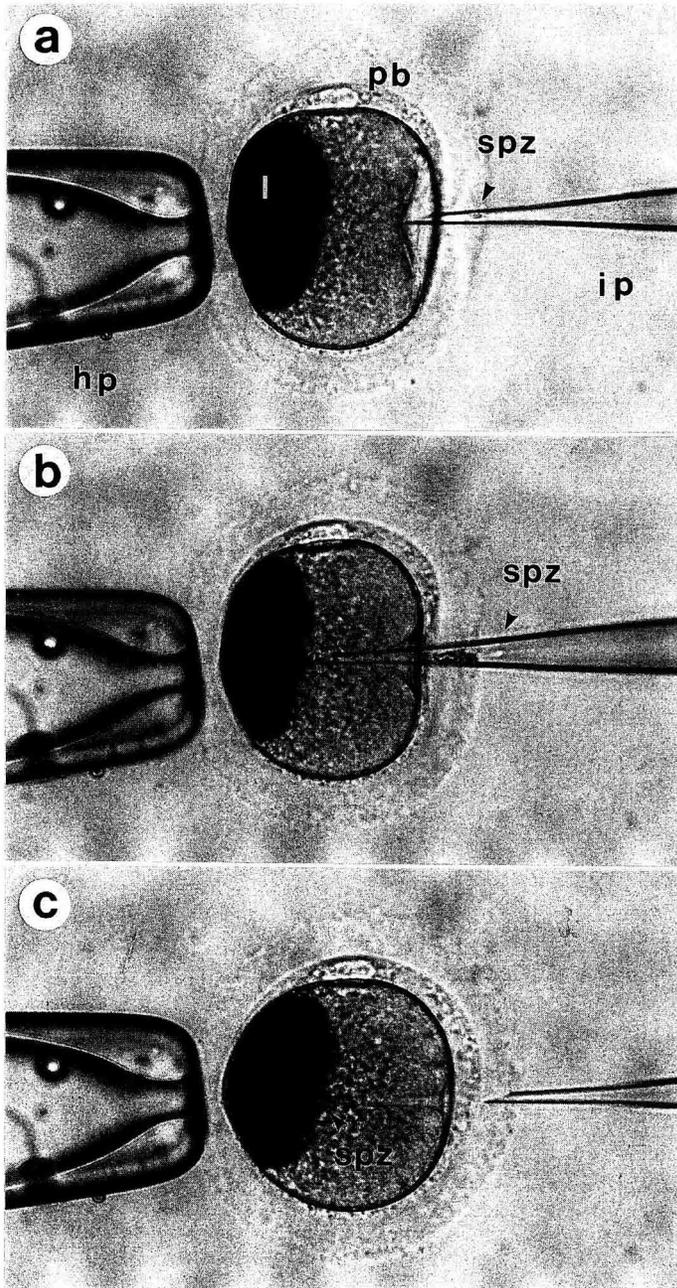
The cleavage rate was calculated among the successfully microinjected oocytes. The data were analysed by the Chi-square test. A probability (*P*) value less than or equal to 5 % was considered significant.

### 3. RESULTS

*Table 1* shows the results of maturation, microinjection, cleavage and embryonic development.

#### 3.1. COC maturation

After collection, 353 COCs (63, 23 and 14 % of cc, cr and exp class, respectively)



**Figure 1.** Microinjection procedure. (a) Introduction of the injection pipette into the ooplasm at 3 o'clock. (b) Aspiration of a small amount of ooplasm into the injection pipette. (c) The spermatozoon is deposited in the ooplasm. hp: holding pipette; ip: injection pipette; pb: polar body; l: lipids; spz: spermatozoon ( $\times 400$ ).

**Table I.** Results of microinjection after in vitro culture on Vero cells in the three classes of equine matured in vitro cumulus-oocyte complexes.

Oocytes	Number (%) of oocytes in each class			Total (%)
	cc	cr	exp	
Matured in vitro	222	80	51	353
Microinjected (%) <sup>a</sup>	62 (27.9) <sup>g</sup>	24 (30.0) <sup>g</sup>	17 (33.3) <sup>g</sup>	103
sham microinjected	5	3	0	8
with one sperm cell	57	21	17	95
Damaged during ICSI	10	2 <sup>f</sup>	6	18 <sup>f</sup>
Spermatozoa found in PVS <sup>b</sup>	0	1	1	2
Successful ICSI	47	19	10	76 (80.0)
Fertilized ova <sup>c</sup>	39	16	9	64
Cleaved ov <sup>c</sup> (%) <sup>d</sup>	25 (53.2) <sup>g</sup>	6 (31.6) <sup>g</sup>	4 (40.0) <sup>g</sup>	35 (46.1)
2–8 cells <sup>e</sup>	19	6	2	27
9–16 cells <sup>e</sup>	3	0	2	5
> 16 cells <sup>e</sup>	3	0	0	3

Total of seven trials. <sup>a</sup> As a percentage of matured oocytes; <sup>b</sup> oocytes with sperm cells found in the perivitelline space (PVS); <sup>c</sup> observation 2 days after intracytoplasmic sperm injection (ICSI); <sup>d</sup> as a percentage of successfully microinjected oocytes; <sup>e</sup> embryonic assessment realized at the end of in vitro culture (7 days after ICSI); <sup>f</sup> including one sham microinjected oocyte; <sup>g</sup> values do not differ significantly ( $P > 0.05$ ); cc: a compact or partial cumulus; cr: surrounded by corona radiata without cumulus; exp: with a totally or partially expanded cumulus.

were matured in vitro in seven consecutive trials. After IVM, 103 oocytes survived denudation and had condensed lipids, an intact cytoplasmic membrane and visible PB (i.e. were presumed metaphase II oocytes) and were microinjected. Among them, four oocytes of the cr class and one of the exp class were matured for a further 17 h. The rate of presumed metaphase II oocytes was similar for the three classes of COCs ( $P > 0.05$ ).

### 3.2. Intracytoplasmic injection

Among presumed metaphase II oocytes, 95 oocytes were microinjected with one spermatozoon and eight oocytes were sham microinjected. After ICSI, 85 oocytes (82.5 %) survived the injection procedure. The others were damaged: they presented

either disrupted cytoplasmic membrane or extruded ooplasm just after ICSI, or swollen ooplasm shortly thereafter. Only two oocytes were found with spermatozoon in the perivitelline space.

### 3.3. Fertilization and cleavage rate

Sixty-four oocytes were fertilized after ICSI; namely seven oocytes were found with a swollen sperm head into the ooplasm, 13 with 2 PN, five with 3 PN, four at the syngamy stage and 35 cleaved ova. Only oocytes with 2 PN, at the syngamy stage and cleaved ova were considered as being normally fertilized, i.e. 52 oocytes (68.4 % of the successfully microinjected oocytes).

About 46 % of the successfully microinjected oocytes were cleaved 2 days after

ICSI. This cleavage rate did not differ between the three classes of COCs ( $P > 0.05$ ). Calcium ionophore did not activate the sham microinjected oocytes (no cleavage, no pronucleus).

### 3.4. Embryonic development

After IVC on Vero cells, some early embryonic development was achieved: one embryo of 23 cells 5 days post-ICSI (the IVC was voluntarily stopped) (*figure 2a, b*), and two embryos of, respectively, 50 cells and more than 80 cells, 7 days post-ICSI (*figure 2d, e*).

Some blastomeres were without nuclei (see *figure 2d*): this could be attributed to signs of fragmentation or to cell degeneration. The embryo in *figure 2e* could be considered as a collapsed blastocyst. At the time of observation, the blastocoele was not visible.

## 4. DISCUSSION

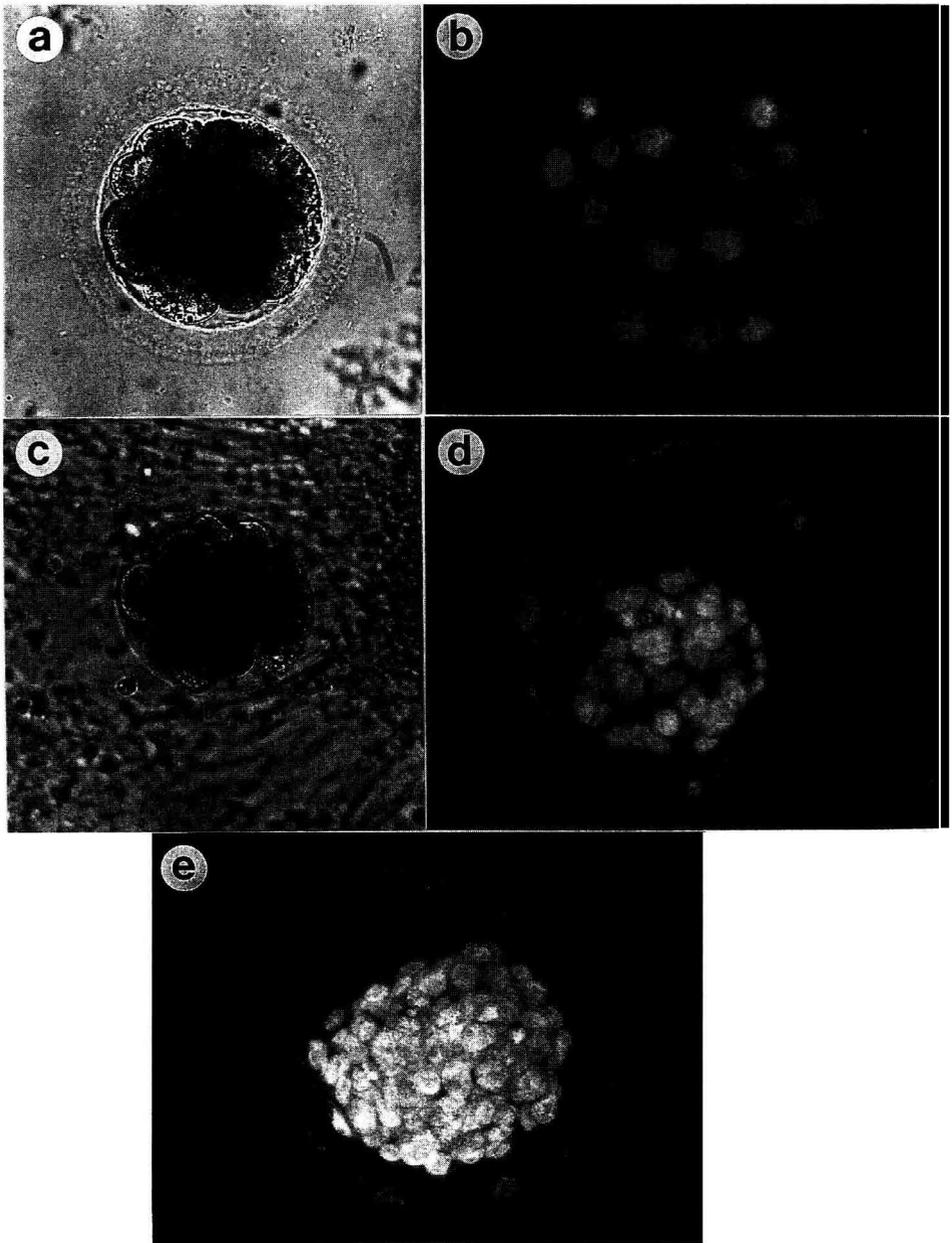
In the present study, we examined oocyte activation by a calcium ionophore after the ICSI procedure and IVC on Vero cells in order to improve the cleavage rate and early in vitro embryonic development.

When compared to our previous results (unpublished data: 24 %), and those from Dell'Aquila et al. ([8]: 13 %) and Grondahl et al. ([17]: 16 %), oocyte activation seems to increase the cleavage rate. Our very first results are corroborated by the recent study of Kato et al. [22] in equine species. Two days after ICSI, more oocytes treated with the calcium ionophore (5 min, 10  $\mu$ M) cleaved than oocytes without activation treatment (21 %,  $n = 24$  versus 5 %,  $n = 44$ ). The difference in the cleavage rate between the two experiments could be explained by the different incubation durations which were applied. Such improvement was shown in bovine ([24]: 38 % of cleaved ova ver-

sus 8 %) and in humans [42] by triggering  $\text{Ca}^{2+}$  oscillation in oocytes previously injected with spermatozoa [41]. Moreover, in our study, calcium ionophore incubation without spermatozoon injection did not activate the oocytes (no PN, no cleavage) as already reported by Meintjes et al. [31] and in our laboratory in previous experiments (Guignot, unpublished data). Thus, in equine species, it seems that the activation of the oocytes depends on the presence of a spermatozoon in the ooplasm. The same result was found in mouse and human oocytes (for a review, see [11]). Effectively, in humans a soluble sperm factor, termed oscillin, was recently identified as being responsible for oocyte activation by inducing  $\text{Ca}^{2+}$  oscillations [37].

In our study, some early embryonic development was obtained after IVC on Vero cells 5 to 7 days after ICSI. Our preliminary results are confirmed by recent data of Dell'Aquila et al. [9] in equine species. In spite of the species barrier, Vero cells can support the equine in vitro embryonic development, as they do in humans [34, 39, 43], in mice [4] and in bovine [32]. They could have several functions. 1) An embryotrophic effect through the secretion of soluble factors [4, 28, 32]. Chen et al. [4] demonstrated that Vero cells secreted small peptides (6.5–35.9 kDa) which could be important in the hatching of mouse embryos. Recently, Desai and Goldfarb [10] identified growth factors and cytokines presents in Vero cell conditioned medium. 2) They could have a function of detoxification by removing toxic metabolites secreted by the embryos into the culture medium or by reducing the high oxygen metabolite levels to which the embryos are exposed [21, 33]. Similarly, the expression of transcripts for antioxidant agents was found during embryo culture on bovine oviductal cell monolayers [20].

As compared to epithelial cell feeder obtained from the oviduct or uterus, Vero cells are a cell line, the cryostorage of which allows better standardization of culture con-



**Figure 2.** Photomicrographs of three embryos after in vitro culture (IVC) on Vero cells. Embryo number 1: 5 days after intracytoplasmic sperm injection (ICSI), (a) transmitted light microscopy, (b) transmitted fluorescence microscopy (after squashing, 23 nuclei are visible). Embryo number 2: (c) 5 days after ICSI, transmitted light microscopy, (d) 7 days after ICSI, transmitted fluorescence microscopy (50 nuclei are visible). Embryo number 3: (e) 7 days after ICSI, transmitted fluorescence microscopy (more than 80 nuclei are visible). ( $\times 400$ : a, b, d, e;  $\times 300$ : c).

ditions: the variability between replicate trials is reduced, as the same source of cells can be used over a long period [32]. Moreover, it is a cell line controlled for virus and other contaminants [33].

Even without any treatments of sperm capacitation, as it was required in conventional IVF [3], fresh ejaculated sperm, microinjected immediately after collection or after 17 h of storage at 15 °C, could fertilize oocytes after ICSI in equine species. It is supposed that the immobilization of the spermatozoon by crushing its tail before ICSI, which enhanced the fertilization rate in humans [13, 44], was sufficient enough in equine species to allow fertilization. Dozortev et al. [12] and Van den Bergh et al. [44] supposed that this type of spermatozoon immobilization in humans may contribute to damage the spermatozoon plasma membrane and allow the release of the sperm-associated oocyte-activating factor and accessibility of the sperm head to sperm nucleus decondensing factor.

In conclusion, it seems that the association 'oocyte activation with calcium ionophore-IVC of microinjected oocytes on Vero cells' applied in this study after ICSI had a positive effect on cleavage rate and on further embryonic development in the equine species as compared with our previous results. The best demonstrations of the respective effects of these factors should have been to compare oocyte activation to non activation, and IVC in the presence or absence of Vero cells. However, the limited number of available slaughtered mares and consequently the limited number of recovered oocytes did not allow us to do this comparison. The next step will be to improve IVC efficiency and then to transfer the ICSI embryos into recipient mares.

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