# Sperm Immobilization Prior to Intracytoplasmic Sperm Injection (ICSI) and Oocyte Activation Improves Early Development of Microfertilized Goat Oocytes

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*ABSTRACT.* **A preliminary goat oocyte activation and intracytoplasmic sperm injection was evaluated in this study. Immature goat oocytes were cultured in TCM-199 supplemented with 10% goat serum for 27 hours for maturation at 38.5°C in an atmosphere of 5% $CO_2$ in humidified air. In vitro matured oocytes were used for activation and intracytoplasmic sperm injection. In the first experiments, calcium ionophore were used at final concentration 0, 1, 50, 100 and 200 μM to evaluate sham-injected goat oocyte activation. In the second experiment, the effect of immobilized of goat sperm cell on the cleavage and the development of sperm-injected goat oocyte was evaluated. Results indicated that in vitro matured goat oocyte did not activate spontaneously. Exogenous stimuli such as Calcium ionophore A23187 at certain level (in this experiment > 50 μM) can be used to activate in vitro matured goat oocytes. In this study, results indicated that sperm injected as well as sham injected goat oocyte can initiate first several cycles of cleavage after activation with calcium ionophore. Results in the current study also indicated that immobilization of goat sperm cells increase the percentage of early embryo development to cleavage after intracytoplasmic sperm injection.**

**Key words:** ICSI, sperm immobilization, oocyte activation, embryo development

## *Introduction*

Over the years, live births have been reported with injection of sperm cells into the cytoplasm of oocytes (ICSI) in mice (Roknabadi et al, 1994), rabbits (Hosoi et al, 1988), cows (Gòto et al, 1990) as well as in humans (Palermo et al, 1992). Now ICSI is being used successfully as an effective technique for treating couples with severe male factor infertility (Kuramoto et al, 2000). One advantage of ICSI over other assisted fertilization techniques (zona drilling and subzonal fertilization) is that sperm cells are need not to be motile with ICSI (Goto et al, 1990; Palermo et al, 1992). ICSI may be especially useful in farm animals in cases where a premium male has a physical injury so testicle sperm can be collected and used for sperm injection.

Hosoi et al, (1988) reported the birth of live rabbit offspring following transfer of ova fertilized by injection of a live sperm cell into the ooplasm without exogenous activation. Goto et al, (1990) reported that the birth of live calves after intracytoplasmic injection of frozen-thawed bovine sperm cells followed by subsequent activation of oocytes with exogenous calcium ionophore A23187. These results indicated that species difference in the needed for exogenous stimuli after sperm are injected into cytoplasm of the oocyte to activate the oocyte.

During fertilization, oocyte activation is initiated after the sperm cell membrane fuses with the membrane of the egg. The sperm receptor on the oocyte surface interacts with an oocyte-binding protein located on the sperm surface to form a complex resulting in species-specific adhesions between the sperm cell and the oocyte (Kinsey et al, 1980). However intracytoplasmic sperm injection (ICSI) bypasses the normal sequence of the fertilization event.

It has been suggested that oocytes may be activated by the injection of high-calcium medium during intracytoplasmic sperm injection (Edwards and Van Steirteghem, 1993). However, recent data showed that oocyte activation is started after a considerable lag period following sperm injection, and activation is probably caused by a soluble factor released from the exogenous sperm cell (Tesarik et al, 1994). Tesarik and Sousa (1995) reported that the absence of human oocyte activation is the cause of fertilization failure in most cases when intracytoplasmic sperm injection fails, and

exogenous calcium A23187 can activate sperm-injected human oocytes.

Immotile sperm cells from humans, bulls and rabbits can fertilize oocytes when sperm cells are injected into the ooplasm of oocytes, which suggest that immotile caprine sperm cells also could fertilize oocyte once injected into the oocyte cytoplasm. Unfortunately, there is no information on goat intracytoplasmic sperm injection. Nor is it clear whether goat oocytes need exogenous stimulation to be activated after sperm are injected into oocyte cytoplasm.

The objectives of this study were to evaluate sham-injected goat oocyte activation and to evaluate the injection of immobilized goat sperm cells into oocytes to activate oocytes and to evaluate subsequent cleavage.

## *Materials and Methods*

### *Oocyte collection and maturation*

The goat ovaries were harvested from a local slaughterhouse. Following collection, the ovaries were transported in a thermos containing 0.9% saline solution (20 to 25°C) supplemented with 100 IU/L penicillin and 0.2 mg/L streptomycin to laboratory within 4 hours. The ovaries were then washed thoroughly with phosphate-buffered saline (PBS) and oocytes were aspirated using a 20-gauge needle attached to a 5-ml sterile syringe. Oocytes were collected from follicles with diameter between 2 and 5 mm and washed twice in PBS containing 5% (v/v) goat serum (Sigma, Cat. No. G-6767). Cumulus-oocyte complexes were evaluated based on the layer of cumulus cells and the consistency of ooplasm. Only good quality oocytes with intact, compact cumulus cells and homogenous ooplasm were selected for the experiments. Oocytes were washed twice and matured in TCM-199 (GIBCO BRL, Cat. No. A-1114–D) supplemented with 10% heat-inactivated goat serum, 0.01 mg/ml follicle stimulating hormone (FSH; Sigma, Cat. No. F-4520), 10 mg/ml luteinizing hormone (LH; Sigma, Cat. No. L-9773), 1 mg/ml estradiol (E2; Sigma, Cat. No. E-2257) and 50 mg/ml gentamicin sulphate (GIBCO BRL, Cat. No. 15750-060). Groups of 20 to 30 oocytes were placed in 500µl maturation medium in each individual well of 4–well tissue culture plates (Nunc, France) covered with mineral oil (Sigma, Cat. No. M-8410) at 39°C in an atn osphere of 5% $CO_2$ in humidified air for 27 hours.

### *Preparation of oocytes for injection*

Matured cumulus-oocyte complexes were briefly treated with 1 mg/ml hyaluronidase (Sigma, Cat. No. H-2251) and the cumulus cells surrounding the oocytes were partially removed by pipetting, the oocytes were then washed in m-PBS (PBS supplemented with 10% goat serum). The injection window was set at 27 to 33 hours after starting maturation of oocytes.

### *Sperm preparation*

Frozen semen from a fertile goat was used during the experiment. French straws (0.25 ml each) of sperm cells were thawed for 1 min at 37°C, then the extender was removed from frozen-thawed semen by washing twice with Bracket-Oliphant (BO) medium (Bracket and Oliphant, 1975) and centrifuging at 500 x g for 5 min. Sperm cell concentration was adjusted to 1 x $10^7$ sperm/ml. For induction of acrosome reaction, calcium ionophore A23187 was added to the sperm mixture to a final concentration of 1 µM and incubation at 37° C for 30 min to induce the acrosome reaction (Watson et al, 1991).

### *Preparation of oocyte-holding and sperm-injection pipettes*

The holding and injection pipettes were made by drawing glass capillary tubes with a pipette puller and were further processed on a microgrinder and a microforge. The outer and inner diameters of the pipettes were 100 and 20 µm and 10 and 6 µm for the holding and injection pipettes respectively. The injection pipettes had a bevel angle of 30 degrees (Figure 1).

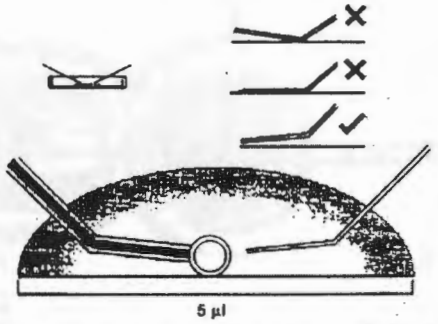


Fig.1. Position of micromanipulation tools for intracyto-plasmic sperm injection (ICSI). The angle tip of the tool is almost parallel to the bottom of the dish, which is necessary to perform sperm immobiliza-tion.

*Intracytoplasmic sperm injection (ICSI)*

Intracytoplasmic sperm injection was conducted on the heated stage of an inverted microscope (Nikon, Japan). The pipettes were mounted to micro-manipulators (Narishige, Japan). The dishes used for injection were cell culture chamber slides (Falcon 1006, USA). Five micro liters of m-PBS drops were arranged vertically for performing the sperm injection containing one oocyte per drop in left side. In right side, two elongated drops of 10 µl BO medium containing sperm were made and one elongated drop of 10 µl of 5% polyvynil pyrrolydone (PVP, Sigma, Cat. No. P-0930). These drops were covered with mineral oil to prevent evaporation during injection (Figure 2).

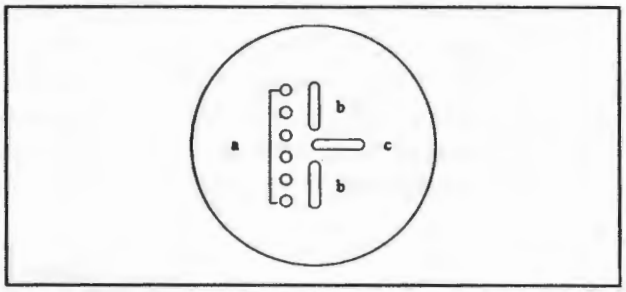


**Fig.2.** Arrangement of micromanipulation drops for intracytoplasmic sperm injection (ICSI). a) ICSI drops (5 µl) containing oocytes, b) sperm drops (10 µl), c) sperm immobilization drop (10 µl) supplemented with 5% PVP.

Sperm immobilization was done by positioning the spermatozoa at 90° to the tip of the pipette, which was then lowered gently, compressing the sperm flagellum and causing that section to adhere to the bottom of the dish. If during the immobilization process, the sperm tail was inadvertently damaged or kinked, that spermatozoon was discarded and the procedure was repeated with another spermatozoon (Figure 3.A).

Immobilized sperm cell was aspirated into the injection pipette with tail first, the injection pipette was raised and the stage was moved to locate the oocyte drop. Suction was applied to hold the oocyte onto the holding pipette, the injection pipette was then pushed through the zona pellucida and the oolemma at the opposite pole to the holding pipette. A single sperm was injected into the cytoplasm on oocyte (Figure 3.B). Sham-injection was performed using the same procedure as for sperm injection, but without injection sperm of the sperm cell. Once all oocytes were injected, oocytes were washed in PBS and activated in calcium ionophore A23187 at a final concentration according to each treatment. After activation for 10 min, oocytes were washed in PBS containing 0.3% BSA to stop the action of A23187. Then oocytes were cultured on a monolayer of cumulus cells for further development observation (Boediono et al, 1994).

*Experimental design*

Experiment 1. Calcium ionophore A23187 activation of sham-injected goat oocytes.

This experiment was designed to evaluate if the act of injection is enough to activate the goat oocyte, and to titrate the concentration of calcium ionophore A23187 required to activate goat oocyte. After oocytes were matured for 27 hours, sham-injection was performed using the same procedure as for sperm injection, but without injection of a sperm cell. After injection, goat oocytes were activated with different levels of Calcium ionophore A23187 (0, 1,

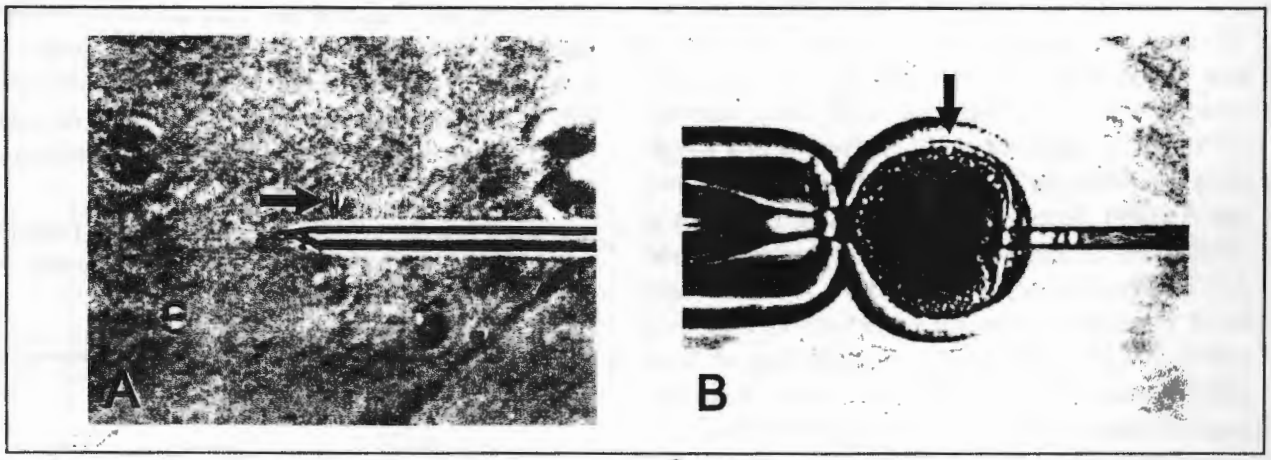


**Fig.3.** Immobilization of the spermatozoon prior intracytoplasmic sperm injection (ICSI). A). Immobilization of the spermatozoon (arrow: spermatozoon), B). Intracytoplasmic sperm injection (ICSI) with the oocyte in 12 o'clock position (arrow: first polar body)

50, 100 and 200 mM). Activated oocytes were further cultured on cumulus cell monolayer and cleavage of injected oocytes was evaluated 48 h after injection.

Experiment 2. Intracytoplasmic injection of goat oocytes with immobilized sperm cells.

This experiment was designed to evaluate early development to cleavage after injection of immobilized goat sperm cells, to evaluate the effect of immobilization sperm cells related to oocyte activation (50 μM Calcium ionophore A23187) and early cleavage.

*Statistical analysis*

The criteria for evaluation included the percentage of oocyte degeneration and cleavage. The results were analyzed with SAS ANOVA procedure (SAS institute, Gary, NC). A probability (P) value of <0.05 was regarded as statistically significant. The differences among individual treatments were assayed by developing 95% confidence intervals.

## Results

*Experiment 1*

In this experiment, the effect of exogenous calcium ionophore on activation of sham-injected goat oocytes was evaluated. Oocyte degeneration and cleavage results are shown in Table 1. In the study, goat oocytes were not activated by sham-injection action. Furthermore, low levels of A23187 (0.1 to 1 μM) did not activate sham-injected oocytes. When the concentration of calcium ionophore was raised to 50μM to activate the oocytes, a 17.5% of sham-injected goat oocytes was activated and performed the first cycle of cleavage. The highest cleavage rate (69.2%) was achieved when 200 μM A23187 was used to activate sham-injected goat oocytes. In this experiment, about 12% of the oocyte degeneration among different levels of calcium ionophore.

Table 1. Development of sham-injected caprine oocytes after activation with calcium ionophore A23187 (48 hours after injection).

| Level of Calcium Ionophore (μM) | No. of Oocytes Sham-injected | Developed to | | Degenerated (%) |
|---|---|---|---|---|
| | | 1-cell (%) | Cleavage (%) | |
| 0 | 38 | 34 (89.5) | 0 [a] | 4 (13.2) |
| 1 | 40 | 36 (90.0) | 0 [a] | 4 (10.0) |
| 50 | 40 | 27 (67.5) | 7 (17.5) [a] | 6 (15.0) |
| 100 | 38 | 21 (55.3) | 13 (34.2) [b] | 4 (10.5) |
| 200 | 39 | 7 (17.9) | 27 (69.2) [c] | 5 (12.8) |

Different superscripts within a column are significantly different (a-b-c, P<0.05)

*Experiment 2*

Immobilization effect on sperm cells related to oocyte cleavage was evaluated. The oocyte degeneration and cleavage rates of each treatment are shown in Table 2. Cleavage was observed in all treatments with injected sperm cells or sham control without injected sperm cells after activation with calcium ionophore. In this study, cleavage was not significantly different between sperm-injected oocytes and sham-injected oocytes after oocytes were activated at 50 μM of calcium ionophore. There was a substantial difference between cleavage of immobilized sperm injected oocytes and sham-injected oocytes. When oocytes were activated with 50 μM calcium ionophore, cleavage was also substantially higher (P<0.05) in immobilized sperm injected oocytes (58.7%) than in sham controls (18.2%). The cleavage rate in oocytes with immobilization treated sperm cells was substantially higher than that in sperm injected oocytes (P<0.05).

**Table 2.** Intracytoplasmic sperm injection of caprine oocytes with immobilized sperm

| Sperm Treatment | No. of Oocytes Injected | Developed to | | Degenerated (%) |
|---|---|---|---|---|
| | | 1-cell (%) | Cleavage (%) | |
| Motile sperm | 44 | 36 (8.18) | 3 (6.8) [a] | 5 (11.4) |
| Immobilized sperm | 46 | 13 (28.3) | 27 (58.7) [b] | 6 (13.0) |
| Sham (control) | 44 | 33 (75.0) | 8 (18.2) [a] | 3 (6.8) |

Different superscripts within a column are significantly different (a-b, P<0.05)

## Discussion

It is well known that for successful fertilization, sperm must activate the oocyte. In all mammalian species studied so far, sperm trigger repetitive rises in the $Ca^{2+}$, which occur at regular intervals for several hours in the mouse (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992), the Hamster (Igusa and Miyazaki, 1986; Miyazaki et al, 1990), cattle (Fissore et al, 1992; Sun et al, 1994), pig (Sun et al, 1992), and human (Taylor et al, 1992). It has been suggested that these oscillations of intracellular free calcium $Ca^{2+}$ lead to oocyte activation, DNA synthesis and further embryonic development. It has been further suggested that the activation factors originate from sperm cells. Swann (1990) demonstrated that injection of sperm extract causes repetitive transients of free calcium $Ca^{2+}$ in hamster oocytes.

Mammalian oocytes can also be activated with exogenous stimuli. Injecting $Ca^{2+}$ directly into oocytes, electropermeabilization in the presence of $Ca^{2+}$, addition of calcium ionophore A23187 all trigger exocytosis of cortical granules, completion of meiosis and early development in mouse, hamster and pig oocytes (Steinhardt et al, 1974; Fulton and Whittingham, 1978; Sun et al, 1992). In the present study, sham injected goat oocytes were also activated with A23187. These results agree with most other activation studies (Steinhardt et al, 1974; Fulton and Whittingham, 1978; Sun et al, 1992). Results indicate that a certain level of A23187 is required to activate sham injected goat oocytes. Fifty μM A23187 activated in some oocytes (17.5%) while maximum cleavage was achieved with 200 μM of A23187 (69.2%).

In mice, Kimura and Yangimachi (1995) demonstrated that mouse oocytes injected with testicular and epididymal sperm cells did not need exogenous stimuli for oocyte activation while spermatids or spermatocytes were unable to activate oocytes. Furthermore oocytes containing injected spermatids or spermatocytes had to be activated artificially to support normal fertilization and full term development. These results demonstrate that sperm factors exist in mouse sperm cells and these factors are formed during sperm maturation, and that artificial stimulation can support full development of mouse oocytes injected with spermatids or spermatocytes.

Tesarik et al (1994) sowed that treatment of sperm-injected human oocytes with A23187 supported the development of calcium oscillations. It is known that calcium oscillations can only be found in fertilized oocytes and parthenogenetic activation never produces such oscillations (Cuthbertson et al, 1981). In the present study, 50 μM A23187 was applied for 10 min to activate sperm-injected goat oocytes. Even though we could not distinguish normally fertilized oocytes from possibly parthenogenetic cleaved oocytes, the results show that a higher cleavage rate was achieved with oocytes injected with immobilized sperm cells than with motile sperm cells or sham control. This indicated that some factors from sperm cells could be released more easily after immobilization, and that these factors could synergize with calcium ionophore to activate oocytes.

In the present study, the combination of immobilization of sperm cells with 50 μM A23187 activation of sperm injected goat oocytes resulted a 58.7% cleavage rate. This further supports the idea that some factor(s) from sperm cells can synergize with exogenous stimuli such as A23187 to activate sperm injected oocytes and perform normal fertilization and early development. Whether these cleaved goat embryos could develop to full term remains to be determined.

In this study, immobilized goat sperm cells were used for intracytoplasmic sperm injection. Results indicate that goat sperm motility was not required under the ICSI procedure. This result agrees with results from the human (Palermo et al, 1992) and the cow (Goto et al, 1990). It was reported that motile mouse sperm are required to fertilize the ova while immotile sperm fail to fertilize when the sperm are injected under the zona (Kobayashi et al, 1992), suggesting that "motile" function was required for sperm penetration of zona and fusion with ovum cytoplasm membrane. It was noticed that if motile mouse sperm cells were injected into the cytoplasm of the oocyte, they kept moving within the ooplasm for a few minutes and sometimes for as long as 20 min before they became motionless (Kimura and Yanagimachi, 1995). Results further indicated that continuous movement of sperm cells within the cytoplasm may disorganize the oocyte's structural elements and/or disturb the "oolemma's wound healing" (Kimura and Yanagimachi, 1995).

In summary, a preliminary goat intracytoplasmic sperm injection procedure with immobilized sperm cell was established in this study. Overall, 58.7% cleavage rate was achieved with immobilization of sperm cells prior to microinjection and goat oocytes activated with 50 μM calcium ionophore A23187 for 10 min.

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