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The impaired development of sheep ICSI derived embryos is not related to centriole dysfunction

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ABSTRACT

While intracytoplasmic sperm injection (ICSI) is an asset in human Assisted Reproduction Technologies (ART), its outcomes, in terms of blastocyst, is still unacceptably low in ruminants. The picture typically found in ICSI derived bovine and ovine embryos is an asymmetry between a high activation rate, marked by a pronuclear development, and a low first cleavage rate. Abnormal centriole function has been indicated as a possible factor which undermines embryonic development following ICSI, especially when Freeze Dried spermatozoa (FD) are used. In order to verify the hypothesis that centriole dysfunction might be responsible for low ICSI outcomes in sheep, we have investigated micro-tubular dynamics, markedly aster nucleation, in fertilized sheep zygotes by ICSI with frozen/thawed (FT) and FD spermatozoa; In Vitro Fertilized (IVF) sheep oocytes were used as control. The spermatozoa aster nucleation was assessed at different time points following ICSI and IVF by immune-detection of α-tubulin. Pronuclear stage, syngamy and embryo development were assessed. No difference was noticed in the timing of aster nucleation and microtubule elongation in ICSI-FT derived embryos with control IVF ones, while a delay was recorded in ICSI-FD ones. The proportion of 2-pronuclear stage zygotes was similar in ICSI-FT and ICSI-FD (47% and 53%, respectively), both much lower comparing the IVF ones (73%). Likewise, syngamy was observed in a minority of both ICSI groups (28.5% vs 12.5% in ICSI-FT/FD respectively) comparing to IVF controls (50%), with a high number of zygotes blocked at the 2-pronuclear stage (71.5% vs 87.5% respectively). While no significant differences were noticed in the cleavage rate between ICSI-FD, ICSI-FT and IVF groups (31%, 34% and 44%) respectively, development to blastocyst stage was markedly compromised in both ICSI groups, especially with FD spermatozoa (10% in ICIS-FD and 19% in ICSI-FT vs 33% in IVF ($P < 0.005$, ICSI-FD vs IVF and $P < 0.05$, IVF vs ICSI-FT, respectively). Hence, here we have demonstrated that the reduced cleavage, and the ensuing impaired development to blastocysts stage of ICSI derived sheep embryos is not related to centriole dysfunction, as suggested by other authors. The major recorded problem is the lack of syngamy in ICSI derived zygotes, an issue that should be addressed in further studies to improve ICSI procedure in sheep embryos.

1. Introduction

ICSI was introduced to reduce *in vitro* fertilization failures and mainly to treat severe male infertility. The technique was originally mastered in human [1] and has been successfully applied in other species such as cow [2], sheep [3], horse [4] and goat [5]. ICSI has been widely used as a standard fertilization technique, but also as a relevant tool to understand fundamental biological questions like cell cycle control, molecular mechanisms of oocyte activation, male pronucleus transformation as well as early embryo development events [6]. Despite the increased use of the technique and its potential advantages, severe limitations are still encountered in terms of its application in many species, particularly in ruminants. As a matter of fact, ICSI derived embryos in ruminants display a considerably lower developmental rate compared to IVF ones; and several factors need to be taken into account in order to justify this failure. For instance, the persistence of the spermatozoa membrane, and consequently the acrosome, are thought to be the major determinants in compromising embryo development, as they were found to cause ineffective sperm oocyte-activation [7,8]. Likewise, atypical and incomplete calcium (Ca^{2+}) oscillations were described in ICSI bovine embryos using frozen bull semen [9–12]. The same

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findings have been reported in sheep as well [13–15]. To overcome these limitations, while partially mimicking natural fertilization, chemical treatments using some detergents were proposed to remove the spermatozoa membranes prior ICSI. Along with this line, it has been also found that mechanically damaged ram spermatozoa prior ICSI enhance embryonic development until the blastocyst stage [15]. However, despite these expedients, *in vitro* embryonic development following ICSI remains still low in sheep and others ruminants [13]. Previous studies in our laboratory have revealed an asymmetry between the activation, based on the pronuclear detection (80% in average), and the cleavage rate, lower (30% in average). In the same study, freeze-dried spermatozoa were suggested to be used in ICSI procedure as an eventual procedure for fertility preservation [17]. In human, most ICSI failures were associated to post-ICSI events and the related molecular mediators [18]. Sperm aster abnormalities and irregular microtubule organization, in particular, were identified as causal factors of developmental restrain following ICSI [19,20]. In fact, sperm aster nucleation and microtubule dynamics are crucial post-ICSI events [21,22], however, their involvement in ICSI failure in ruminant embryos is insufficiently documented [18]. In this work we set up to investigate centriole, microtubule dynamics and developmental potential in zygotes obtained by ICSI-fertilized sheep eggs. Given that ICSI outcomes are particularly negative using freeze-dried spermatozoa, we decided to use them to exacerbate the problem, rendering it easier to detect.

2. Material and methods

All chemicals were purchased from Sigma (Milano, Italy), unless otherwise stated.

2.1. Sperm preparation

Semen was collected from adult fertile Sardinian rams using an Artificial Vagina. Ejaculates were selected for further processing based on their concentration and motility.

2.1.1. Spermatozoa cryopreservation

Initially, basic medium was prepared by dissolving 2.42 g TRIS base, 1.36 g citric acid, 1 g fructose, 100.000 IU penicillin G, 0.1 g streptomycin in 67.20 ml of bi-distilled water (ddH20) (pH = 6.7–6.8), then equally divided into 2 vol of 33.60 ml each. Medium A (30 °C medium) and Medium B (4 °C medium) were prepared separately in each tube by respectively adding 10 ml of egg yolk and 6.40 ml of ddH20 and 10 ml of egg yolk plus 6.40 ml of glycerol. Each tube was kept at 30 and 4 °C, respectively, before use. Shortly after sperm collection, Medium A was poured to the ejaculate and transferred immediately to a cold room (4 °C) for 2 h to enable a controlled cooling - from 30 to 4 °C. Then, Medium B was gently poured to the suspension which was kept for additional 2 h in the cold room. Equal volumes of both mediums were added to ensure a final dilution of the ejaculate to a concentration of 400×10^6 spermatozoa/ml. Tubes were gently mixed each 30 min by rotating the plugged flask 180°. The suspension was divided into 250 μl plastic straws which were sealed with polyvinyl alcohol (PVA) and placed on a metallic grid to stabilize for 2 h at 4 °C. Finally, the sealed straws were exposed to Liquid Nitrogen (LN) vapors (−80 °C) in a Dewar flask initially for 6 min, then plunged into LN to be stored in filled tanks with LN until use.

2.1.2. Sperm freeze-drying

Freeze-dying spermatozoa was performed as previously described [17]. Briefly, semen was transferred into a polypropylene tube which contained 1 ml of CZB medium supplemented with 10% fetal bovine serum. Semen was incubated for 30 min at 37.5 °C, then the upper 0.3–0.5 ml of the sperm suspension was discarded. Semen was diluted in freeze-drying medium [1 ml 0.5 M TRIS (in water), 5 ml 0.5 M EGTA (in water), 2.5 ml 1 M NaCl (in water)], after that an aliquot of 100 μL of the sperm suspension was put inside a 2 ml ampule which was directly plunged into liquid nitrogen for 10 min. After semen freezing, the vials were placed into Freeze-Dry apparatus (SP Scientific-VirTis, 2.0 BenchTop) with the condenser at a temperature of −58 °C and the freeze-drying chamber at −12 °C.

After 12 h approximatively, each ampule was sealed under vacuum condition (pressure 15 μBar). Vials were individually wrapped with aluminum foil and stored at room temperature until use.

2.2. Oocyte recovery and in vitro maturation (IVM)

Sheep ovaries were collected from local slaughterhouses and transferred within 1–2 h at 37 °C to the laboratory. Aspiration of the oocytes from the follicles was done in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered TCM-199 medium with 0.005% heparin using 21G needles. In Vitro Maturation (IVM) medium is composed by MEM-199 containing 2 mM glutamine, 0.3 mM sodium pyruvate, 100 μM cysteamine, 10% fetal bovine serum (FBS), 5 μg/ml follicle stimulating hormone FSH, 5 μg/ml luteinizing hormone (LH), and 1 μg/ml estradiol. Only oocytes with at least 2 or 3 layers of compact cumulus cells were selected for maturation. After 2 washes in the IVM medium, oocytes underwent maturation in 0.5 ml of IVM medium in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 24 h.

2.3. In vitro fertilization (IVF)

Twenty-four hours post IVM, only mature oocytes (MII) with expanded cumulus and normal morphology were selected for *in vitro* fertilization (IVF). Expanded Cumulus-Oocytes-Complexes (COCs) were mechanically deprived from the majority of the cumulus cells by repeated pipetting in 300 U/mL hyaluronidase dissolved in Hepes buffered M199. A total of 287 oocytes were used for IVF, 220 oocytes were dedicated for the sperm aster immunofluorescence and 67 oocytes were maintained in culture for embryo development assessment. Groups of 10 oocytes were put in 50 μL drops of IVF medium composed by Synthetic Oviductal Fluid (SOF) medium, plus 20% estrus sheep serum and 16 μM isoproterenol and covered by mineral oil. In parallel, frozen ram semen was rapidly thawed in 35 °C water and centrifuged in bicarbonate-buffered SOF containing 0.4% BSA at 112 × g for 5 min. Then, supernatant was discarded and 1.3–2 μL of pellet ($\pm 5\times 10^6$ spermatozoa/ml) were aspirated and transferred into oocytes containing drops. Gametes were co-incubated in a humidified atmosphere at 38.5 °C, 5% CO_2, and 7% O_2, at least for 3.5 h to allow fertilization. IVF has been stopped at different time points and processed for immune-detection of α-tubulin, in line with the experimental design.

2.4. Intracytoplasmic sperm injection (ICSI)

At 24 h from IVM, a total of 290 matured (MII) oocytes were completely denuded from the cumulus cells by pipetting in 300 U/mL hyaluronidase dissolved in Hepes buffered M199. One part of the oocytes (157 oocytes), were injected with FT and FD semen (87 oocytes and 70 oocytes, respectively) and allocated to sperm aster immunofluorescence detection. Second part of the oocytes (133 oocytes) were allocated to embryo development as described below. ICSI with FT (ICSI-FT) and ICIS with FD spermatozoa (ICSI-FD) were performed as described by Ref. [15–17] and briefly below. FT were fast-thawed by immersing the straw in 35 °C water for a

few seconds and FD were rehydrated by adding 100 μL of bi-distilled water. For ICSI-FT and ICSI-FD, a volume of 5 μL of semen was diluted in 100 μL of M199 medium, then the mixture was diluted 1:1 with 12% PolyVinylPyrrolidone (PVP, 360 kDa). The injection was performed under an inverted microscope (Nikon Eclipse E−800) connected to a micromanipulation system (Narishige, Tokyo, Japan), using a piezo-driven device (PiezoXpert, Eppendorf, Milan, Italy. Immediately after ICSI, oocytes were artificially activated by culturing them in 5 μM ionomycin dissolved in M199 plus 0.4% BSA for 5 min; then washed 5 min and placed in embryo culture dish.

2.5. Embryo culture

Presumptive zygotes, resulting from ICSI and IVF, were cultured in groups of 5 in 20 μL drops of SOF medium with 2% basal medium Eagle (BME)-essential amino acids (EAA), 1% minimum essential medium (MEM)-nonessential amino acids (NEAA) (Gibco), 1 mM glutamine, and 8 mg/ml fatty acid-free BSA, covered by mineral oil in a humidified atmosphere at 38.5 °C, 5% CO_2, and 7% O_2.

For ICSI-FT, ICSI-FD and IVF derived embryos which were maintained in culture, the medium was refreshed at day 3 (SOF-supplemented with 0.27 mg/ml glucose (SOF+), 2% EAA, 1% NEAA), day 5 (SOF+ with 10% of charcoal stripped FBS (cs-FBS), 2% EAA, 1% NEAA); and day 6 (1:1 MEM/M199 enriched with 10% cs-FBS, 2.5 μg/ml gentamicin and 1% sodium pyruvate) until day 7 or 8 of culture. Embryo development was assessed 24-h post-culture for the cleavage (2 cell-stage) and at day 7 or 8 post-culture for blastocyst formation.

2.6. Immunofluorescent detection of tubulin

Presumptive zygotes were fixed at different time points after sperm-egg co-culture starting from 1 to 2 h till 9-h post-fertilization (pf), for IVF derived embryos, or post-activation (pa), for ICSI derived zygotes. Zygotes were treated with acid Tyrode's solution (pH 2.4) for 30 s followed by 30 s incubation in 0.5% Pronase to dissolve the ZP. Zone free zygotes were fixed in 4% paraformaldehyde (PFA) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM MgSO 4 ·7H 2 O, pH 6.9) [23] for 20 min then washed in PBS with 0.4% PVP, and next permeabilized for 15 min with 0.5% Triton X-100 in PHEM. After four washes (5 min each) in PHEM with 0.05% Tween 20, zygotes were blocked in 1% BSA in PHEM with 100 mM glycine at room temperature for 1 h. Zygotes were incubated in mouse monoclonal IgG anti-α-tubulin from Santa Cruz (sc-23949 Lot #C0718) (1:200 in 1% BSA/PHEM with 100 mM glycine) overnight at 4 °C. After four washes in PHEM with 0.05% Tween 20, the zygotes were incubated with FITC-conjugated goat-*anti*-mouse IgG (Sigma F5262 Lot # 057M4883V) and/or rabbit anti-goat IgG-PF from Santa Cruz (sc-3755, Lot #I2503) (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min at room temperature. DNA was counterstained for 5 min in 1 μg Hoechst 33,342 in 10 ml of PBS at room temperature. Then slides were mounted, and images were captured using a confocal microscope (Nikon Eclipse Ti-E).

2.7. Pronuclear detection

The formation of 2 pronuclei (2 PN) was assessed in all experimental groups (ICSI-FT, ICSI-FD and IVF). IVF group was considered as control, which is supposed to show 2 PN at 10 h *pf* the syngamy at 22 h *pf*. To this extend, a total of additional 90 fertilized oocytes (30 ICSI-FT, 30 ICSI-FD and 30 IVF) were carefully cleaned from cumulus cells and incubated in 5 min 1 μg Hoechst 33,342 in 10 ml of embryo culture medium. Oocytes were observed one by one under inverted microscope connected with a micromanipulator system, at 10 h pf/pa first, for the 2 PN assessment, then later at 22 h pf/pa, for syngamy stage assessment.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 software. Kruskal-Wallis test was applied to compare *in vitro* embryo development on a minimum of 5 replicates for each group (ICSI-FT, ICSI-FD and IVF). The considered data in each group were the number of the cleaved embryos and the blastocyst number in each group, a multiple comparison was done between groups. Statistical significance threshold has been set with $P < 0.05$. Chi-square test was applied to compare 2 PN formation and early syngamy event. The accurate number of oocytes showing 2 PN and syngamy events were the considered data. ICSI groups were compared to IVF. ICSI-FT and ICSI-FD groups were also compared between each other. Statistical significance threshold has been set with $P < 0.01$.

3. Results

3.1. Sperm evaluation

After rehydration, freeze-dried spermatozoa exhibited the 58.5% of external membrane damaged (valuated by Pisum sativum agglutinin-PSA). The percentage of intact DNA was similar as reported in our previously work [24] with an estimation of 9.9%, analyzed by Comet-assay.

3.2. Sperm aster nucleation and microtubular dynamics

ICSI-FT zygotes completed the II meiosis by 3 h pa and simultaneously the proximal centriole initiated aster nucleation (Fig. 1A, arrow; Fig. 2A). A further microtubular growth was observed from 4 to 5-h pa (Fig. 1B, arrow; Fig. 2B) with a marked increase by 8 to 9-h pa, with microtubules radiating from the decondensed male pronucleus (Fig. 1C, arrow; Fig. 2C; Fig. 1D, arrow; Fig. 2D). Aster formation was delayed 1 h in the ICSI-FD derived zygotes (Fig. 1E, arrow; Fig. 2E) with nucleation starting only by 4 h pa (Fig. 1F, arrow; Fig. 2F). However, once the nucleation started, the sperm aster followed the same dynamics from the 5 h onward, as previously described (Fig. 1G–H, arrow; Fig. 2G–H).

Overall, the aster formation and microtubular dynamics in ICSI-FT overlapped those detected in IVF control zygotes (Fig. 1I-J-K-L, arrows; Fig. 2I-J-K-L).

3.3. Pronuclear and syngamy assessment

Zygotes were checked for the 2 PN and syngamy at 10-h pf/pa and 22-h pf/pa respectively. At 10-h pf/pa, the percentage of pronuclear stage embryos was quite similar in ICSI-FT and ICSI-FD, respectively 47% (14/30 zygotes) and 53% (16/30 zygotes) while the majority of IVF zygotes, 73% (22/30) were at the 2 PN stage (Table 1) (IVF vs ICSI-FT, $P < 0.001$). Syngamy was detected at 22-h pa in only 28.5% (4/14) of ICSI-FT and 12.5% (2/16) of ICSI-FD zygotes, while most of them were arrested at 2 PN stage (71.5% (10/14) and 87.5% (14/16), respectively). The picture radically changed in IVF zygotes, where half 50% (11/22) completed syngamy, and half 50% (11/22) were found blocked at 2 PN stage (IVF vs ICSI-FD, $P < 0.001$).

3.4. Embryo development

Embryo development is summarized in Table 2. No significant differences were found in the cleavage rate between the different

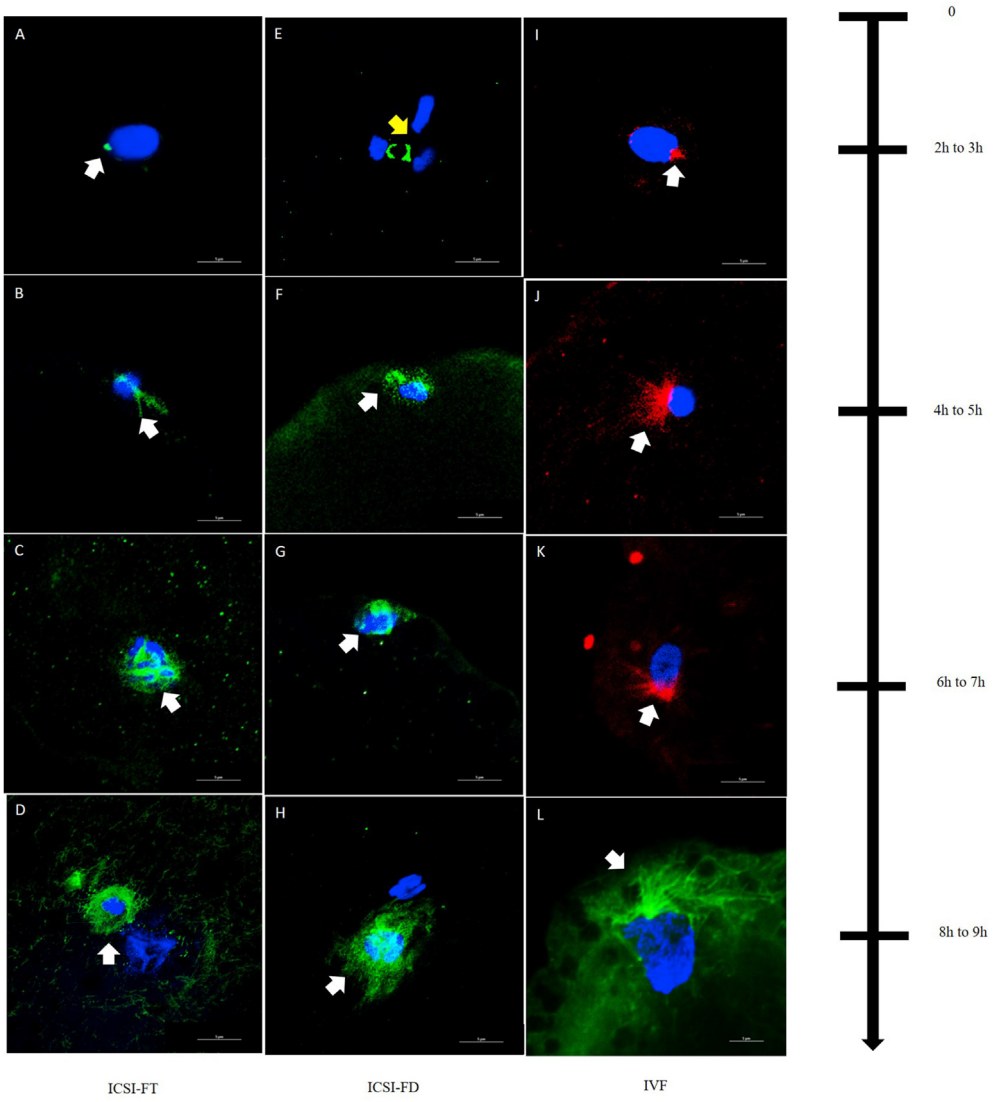

Fig. 1. Sperm aster nucleation dynamics in ICSI-FT (A-B-C-D); ICSI-FD (E-F-G-H) and IVF (I-J-K-L) presumptive zygotes; 2 to 3-h pf/pa (A-E-I), 4 to 5-h *pf/pa* (B–F-J), 6 to 7-h *pf/pa* (C-G-K), 8 to 9-h *pf/pa* (D-H-L). Arrows: sperm aster nucleation and microtubule elongation; yellow arrow: absence of sperm aster nucleation. Red/green: α– tubulin; blue – nucleus/ Hoechst.

groups, while blastocyst rate significantly differed between ICSI embryos and IVF control ones (IV^a vs ICSI-FT, $P < 0.05$; IVF vs ICSI-F^b, $P < 0.005$).

4. Discussion

The current low efficiency of ICSI assisted fertilization in ruminants, and the puzzling asymmetry between the activation and the cleavage rates, prompted us to investigate whether centriole dysfunction might be the cause. Our findings on spermatozoa aster nucleation and microtubule organization in normal IVF and ICSI sheep oocytes using frozen/thawed and freeze-dried ram spermatozoa ruled out this hypothesis. We have found that aster nucleation and microtubular dynamics in control IVF and ICSI with FT spermatozoa take place almost synchronously, while a delay in aster organization was instead noticed when FD spermatozoa were used for ICSI. In these latter zygotes, the aster started to assemble around 4-h pa, means 1 h later than the previous groups. In fact, the sperm centrosome must be exposed to the oocyte's reducing cytoplasm to be activated [25]. It is likely that the structural changes induced by drying in the peri-centrosomal area might have slowed down its "priming" to acquire microtubule nucleating capacity.

A similar study carried out in ICSI derived bovine embryos injected with FD bull spermatozoa partially disagrees with our findings [26]. The paper reports a reduced microtubular nucleating activity in ICSI embryos derived from FT and FD semen, without however any difference in centrosome function in FD spermatozoa. It is likely that differences in the drying technique, like the residual water, and length of de-hydration might account for the differences between our and their study. However, despite the recovery of microtubular nucleation in ICSI-FD embryos, their development to blastocyst stage was significantly lower comparing to the other groups, very likely owing to other factors, like DNA damage in freeze dry spermatozoa [24]. Therefore, our data have demonstrated that centriole dysfunction is not the major cause which hamper the development of ICSI derived embryos in sheep, as suggested by other authors [19,20]. The puzzling observation made in this study is that pronuclear formation in the majority of the two ICSI groups (71.5% and 87.5% respectively in ICSI-FT and ICSI-FD)

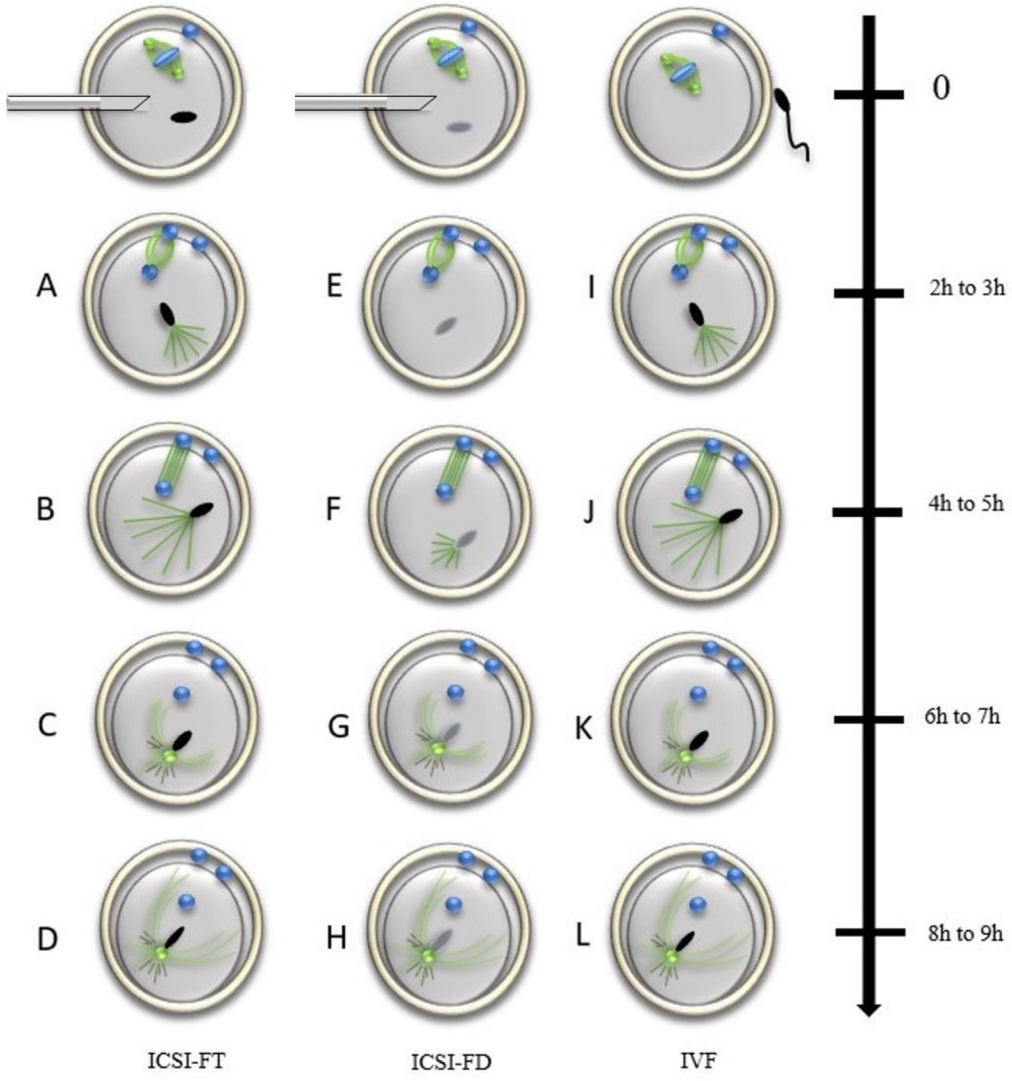

Fig. 2. Schematic representation of the sperm aster nucleation dynamics in ICSI-FT (A-B-C-D); ICSI-FD (E-F-G-H) and IVF (I-J-K-L) presumptive zygotes; Fertilization beginning (0), 2 to 3-h pf/pa (A-E-I), 4 to 5-h pf/pa (B–F-J), 6 to 7-h pf/pa (C-G-K), 8 to 9-h pf/pa (D-H-L).

was not followed by syngamy. It is thus likely that the absence of syngamy in ICSI derived zygotes might trigger the G2/M block, leading to the lack of mitosis [5]. Pronuclear apposition and the fusion of nuclear membrane in preparation for the first mitosis are poorly understood phenomenon. Apparently, nuclear membrane fusion promotes pronuclear apposition [27] and membrane fusion is typically promoted by Ca^{2+} ions. A series of Ca^{2+} spikes induced by sperm soluble factor, identified as PLCzeta (zeta) [28], affects several post-fertilization events [29] and very likely the pronuclear membrane fusion too. While we do not have elements to understand the absence of syngamy in ICSI derived embryos, the abnormal calcium spiking reported in ICSI zygotes, essentially a shorter duration of Ca^{2+} oscillations than IVF, might be responsible for the reduced pronuclear fusion [30,31]. The issue certainly needs further investigation in order to finally optimize embryonic development following ICSI in ruminant embryos.

CRediT authorship contribution statement

Yosra Ressaissi: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Debora Agata**

Table 1
Pronuclei stage and syngamy assessment in IVF, ICSI-FT and ICSI-FD embryos.

Groups	No. oocytes	2 PN (%)	Syngamy (%)
ICSI-FT	30	14/30 (4[b]%)[a]	4/14 (28.5%)
ICSI-FD	30	16/30 (53%)	2/16 (12.5%)[b]
IVF	30	22/30 (73%)	11/22 (50%)

[a] IVF vs ICSI-FT, $P < 0.001$.
[b] IVF vs ICSI-FD, $P < 0.001$.

Table 2
Embryo deve[a]opment outcomes from IVF, ICSI-FT and ICSI-FD.

Groups	No. oocytes	2-Cells (%)	Blastocysts (%)
ICSI-FT	56	19/56 (34%)	11/56 (19%)[a]
ICSI-FD	77	24/77 (31%)	8/77 (10%)[b]
IVF	67	30/67 (44%)	22/67 (33%)

[a] IVF vs ICSI-FT, $P < 0.05$.
[b] IVF vs ICSI-FD, $P < 0.005$.

Anzalone: Conceptualization, Methodology, Validation, Writing - review & editing. **Luca Palazzese:** Methodology, Data curation, Writing - review & editing. **Marta Czernik:** Conceptualization, Methodology, Validation, Writing - review & editing. **Pasqualino Loi:** Conceptualization, Methodology, Project administration, Writing - review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors have no conflicts of interest.

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