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Effect of cumulus cells removal and sperm preincubation with progesterone on in vitro fertilization of equine gametes in the presence of oviductal fluid or cells.

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Effect of cumulus cells removal and sperm preincubation with progesterone on in vitro fertilization of equine gametes in the presence of oviductal fluid or cells.

Running title: effect of cumulus cells and progesterone on equine IVF.

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KEYWORDS

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ABSTRACT

In spite of many attempts to establish an in vitro fertilization (IVF) technique in the equine, no efficient conventional IVF technique is available. The presence of oviductal fluid or oviductal cells during IVF help to improve embryo production in vitro but is not sufficient to reach high fertilization rates. Thus, our aim was to perform equine IVF either after sperm preincubation with oviductal fluid or in the presence of oviductal cells, and to evaluate the effect of cumulus removal from the oocyte or sperm preincubation with progesterone. In experiment 1 and 2, IVF was performed in the presence of porcine oviduct epithelial cells. The removal of cumulus cells from equine oocytes after in vitro maturation tended to increase the percentage of fertilization when fresh sperm was used (1/33 vs 4/31, $p > 0.05$) but had no effect when frozen sperm was used (1/32 vs 1/32). Equine sperm preincubation with progesterone did not significantly influence the fertilization rate when fresh or frozen sperm was used (2/14 vs 2/18 for fresh, 1/29 vs 1/25 for frozen). In experiment 3 and 4, IVF was performed after preincubation of sperm with porcine oviductal fluid. The removal of cumulus cells tented to increase the percentage of fertilization when fresh sperm was used (1/24 vs 3/26, $p>0.05$). Sperm preincubation with progesterone did not significantly influence the fertilization rate when fresh or frozen sperm was used (2/39 vs 2/36 for fresh, 2/37 vs 1/46 for frozen), but two 3-4 cells stage zygotes were obtained with fresh sperm preincubated with progesterone. This is an encouraging result for the setting up of an efficient IVF procedure in equine.

INTRODUCTION

In spite of many attempts to establish an in vitro fertilization (IVF) technique in the equine, no efficient conventional IVF technique is available. The low success rate of in vitro fertlization in horses was probably due to the poor ability of in vitro capacitated stallion spermatozoa to penetrate the cumuIus cells and zona pellucida of oocytes (Blue et al. 1989). The first and only two IVF-produced foals were obtained 30 years ago using preovulatory oocytes and fresh sperm treated with calcium ionophore (Bézard et al. 1989). However, IVF rates remained low (Alm et al. 2001, Palmer et al. 1991, Hinrichs et al. 2002, Mugnier et al. 2009b). A 32% equine IVF rate was reported after incubation of in vitro matured oocytes with frozen sperm treated with heparin but these results couldn't be improved (Alm et al. 2001, Dell'Aquila et al. 1997a, Dell'Aquila et al. 1997b, Dell'Aquila et al. 1996, Roasa et al. 2007). Disappointing success rates were obtained using caffeine (Mugnier et al. 2009a). A 37 to 60% fertilization rate was reported after treatment of fresh spermatozoa with procaine (Ambruosi et al. 2013, McPartlin et al. 2009), but equine IVF embryos fail to develop beyond the 8-16 cell stage (Leemans et al. 2015). Fertilization rates reaching 50% were obtained using fresh semen incubated with preovulatory follicular fluid or progesterone (Lange-Consiglio et al. 2016). However, conventional IVF for equine embryo production is far from being a well-established assisted reproductive technique as observed in human, bovine or porcine.

In mammals, the oviductal environment is of crucial importance for oocyte preparation for fertilization and development (Aviles et al. 2010, Coy et al. 2012). Moreover, several studies have shown the key role played by the oviduct in the preparation of equine gametes for fertilization (Goudet 2011, Leemans et al. 2016). The development of in vitro culture conditions able to mimic the maturation of the ovulated oocyte in the oviduct help to improve embryo production in vitro. For example, pre-incubation of equine oocytes with oviductal fluid or oviductal cells increases fertilization rate (Mugnier et al. 2009b, Ambruosi et al. 2013). However, the presence of oviductal fluid or oviductal cells during IVF is not sufficient to reach high fertilization rates. Previous studies showed that the removal of cumulus cells after equine oocyte maturation tended to increase the IVF rate (Mugnier et al. 2009b, Dell'Aquila et al. 1996). Moreover, progesterone has been proposed to be involved in equine sperm capacitation (Meyers et al. 1995, Cheng et al. 1998b). Thus, our aim was to perform IVF either after sperm preincubation with oviductal fluid or in the presence of oviductal cells, and to evaluate the effect of cumulus removal from the oocyte or sperm preincubation with progesterone.

MATERIAL AND METHODS

All chemicals were purchased from Sigma-Aldrich (St Quentin Fallavier, France) unless otherwise

indicated.

Collection and preparation of porcine oviductal fluid and epithelial cells

Collection of porcine oviducts

Genital tracts from sows were obtained from a commercial slaughterhouse and transported to the laboratory at room temperature for oviductal fluid collection or at 37ºC in 0.9% (w/v) NaCl for oviductal epithelial cells obtaining. Oviducts were classified as pre-ovulatory or post-ovulatory on the basis of ovarian morphology on both ovaries from the same female as described previously (Carrasco et al. 2008).

Collection of porcine oviductal fluid

Oviducts at pre-ovulatory or post-ovulatory stages were dissected free from surrounding tissues. The oviductal fluid from the ampulla was expelled by gentle squeezing using a sterile microscope slide from the isthmus to the ampulla. It was collected by aspiration with an automatic pipette by introducing the tip into the ampulla (Carrasco et al. 2008). After centrifugation at 10 000 g for 15 min at 4°C, the supernatant containing secreted and intracellular components was immediately stored at -20 ºC until use.

Preparation of porcine oviduct epithelial cells (OEC)

Oviducts at pre-ovulatory stage were separated from the tracts, washed twice with 0.5% v/v in phosphate buffered saline (PBS, Dulbecco A, Oxoid, France) of a solution containing 1% w/v Hexadecyltrimethylammonium bromide (ref H-5882) in 5% ethanol and twice with PBS and dissected free from surrounding tissues. The oviductal fluid and cells from the ampulla were expelled by gentle squeezing using a sterile microscope slide. Epithelial cells were then washed three times and recovered after passive sedimentation in Medium 199 with Earle's salts supplemented with 12% (v/v) fetal calf serum (FCS) and 25 µg/ml gentamycin. Cells were cultured in 500 µl of media in 4 well-dishes in an atmosphere of 5% CO_2 in air at 38.5°C and 100% humidity for 4 to 8 days until confluence. The day before IVF the culture medium was changed for DMEM-F12 with 2.5mM L-glutamine and 15mM HEPES (ref D8900) supplemented with 1.2 g/l $NaHCO_3$, 10% FCS and 25 µg/ml gentamycin.

Collection and *in vitro* maturation (IVM) of equine immature oocytes

Collection of equine immature oocytes

Equine immature cumulus-oocyte complexes (COCs) were collected during the breeding season from slaughtered mares in commercial abattoirs. Ovaries were transported to the laboratory within 2 hours in 0.9% (w/v) NaCl at 32-38°C. COCs were collected using the aspiration procedure previously described (Goudet et al. 2000). Briefly, the tunica albuginea was removed and all follicles larger than

5 mm were aspirated with an 18 gauge needle at 100 mm Hg of vacuum pressure. Follicular fluids were examined under a stereomicroscope for COCs recovery. Oocytes denuded of cumulus cells and degenerated oocytes showing dense or fragmented cytoplasm were discarded.

IVM of equine immature oocytes

Just after collection, COCs were washed in Medium 199 with Earle's Salts, 25 mM HEPES and $NaHCO_3$ supplemented with 20% (v/v) FCS and 25 µg/ml gentamycin. They were then cultured in group of 10 to 30 oocytes for 27-30 hours in Medium 199 with Earle's salts supplemented with 20% (v/v) FCS and 50 ng/ml Epidermal Growth Factor in an atmosphere of 5% CO_2 in air at 38.5°C and 100% humidity (Goudet et al. 2000).

In vitro fertilization (IVF) procedure

Stallion semen collection

The experiment included three mature Welsh stallions of proven fertility from our experimental stud. Semen was collected from these stallions on a regular basis by using a closed artificial vagina (INRA model) in accordance with the "guiding principles for the care and use of animals in research facilities" from the French Government and with the approval of the ethical committee "Comité d'Ethique en Expérimentation Animale Val de Loire n°19". After collection, raw semen was filtered through gauze to exclude the gel fraction of the ejaculate. Sperm motility was visually evaluated under light microscopy on a heated stage and sperm concentration was assessed using a spectrophotometer.

Sperm freezing procedure

The freezing procedure was performed as previously described (Pillet et al. 2012). Filtered semen of each ejaculate was diluted in INRA96® extender (IMV-Technologies, France) at 37°C. Ejaculates were then cooled (22°C for 10 min) and centrifuged (600 g x 10 min). The pellet was re-suspended in INRA Freeze® (IMV-Technologies) to obtain 100 x 10^6 sperm cells/ml. Diluted semen was cooled to 4°C and maintained at this temperature over 75 min. The cooled semen was loaded into 0.5 mL polyvinyl chloride straws (IMV-Technologies) sealed with polyvinyl alcohol sealing powder. Freezing was performed with a programmable freezer (Nitrogen freezer, automatic Mini-Digitcool, IMV-Technologies) (60°C/min until -140°C), then plunged and stored in liquid nitrogen.

Preparation of fresh and frozen equine sperm for IVF

Fresh equine semen was collected as previously described and immediately diluted at 20 x 10^6 spermatozoa/ml in INRA96® previously warmed at 37ºC. For frozen sperm, straws were thawed for 30 seconds in a water bath at 37ºC. The content was diluted at 20 x 10^6 spermatozoa/ml in INRA96® previously warmed at 37ºC. Diluted sperm was centrifuged at 500 g for 5 min at 37°C and the pellet

was re-suspended at 20 x 10^6 spermatozoa/ml in INRA96®. Motility was evaluated visually under a microscope (Olympus, IMT-2, Paris, France).

Pre-incubation of sperm with oviductal fluid

Before IVF, sperm was incubated or not with oviductal fluid at pre-ovulatory (preOF) or post-ovulatory (postOF) stages at a final concentration of 10% or 20% in INRA96® for 10 minutes at 37ºC.

Pre-incubation of sperm with progesterone

Before IVF, sperm was incubated or not with 1µg/ml progesterone (P4) (ref P0130) in INRA96® for 2 minutes at 37ºC.

Preparation of equine oocytes and IVF

Mature COCs were removed or not from their cumulus cells and washed in DMEM-F12 with 2.5mM L-glutamine and 15mM HEPES (ref D8900) supplemented with 1.2 g/l $NaHCO_3$, 10% FCS and 25 µg/ml gentamycin. They were then transferred to droplets of 85 µl of this media covered with mineral oil or 4 well-dishes containing epithelial cells at confluence covered by 500µl of the same media. Oocytes from both groups were inseminated with 2 x 10^6 spermatozoa/ml and co-incubated for 18 hours in an atmosphere of 5% CO_2 in air at 38.5°C and 100% humidity.

Assessment of nuclear status

Nuclear status was assessed after 18h in vitro fertilization. Oocytes and zygotes were washed in PBS by aspiration in and out of a pipette, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, washed in PBS and processed for DNA and nuclear membrane staining. They were incubated for 30 min at room temperature in 0.2% Triton X-100 in PBS. Nonspecific reactions were blocked by incubation for 1 hour at room temperature in 10% goat serum in PBS. Oocytes and zygotes were incubated for 2 hours at room temperature with an anti-lamin A/C antibody (Thermo scientific) diluted 1:100 in PBS containing 0.2% Bovine Serum Albumin (BSA) and 0.1% Tween. After 4 washings for 5 min in PBS containing 0.2% BSA and 0.1% Tween, they were incubated for 1 hour at room temperature with an AlexaFluor 594-conjugated-anti-mouse antibody (Life technologies) diluted 1:400 in PBS. They were then washed 5 times for 5 min in PBS containing 0.1% Tween and 2 times for 5 min in PBS. They were incubated with 1 µg/ml bis-benzimide (Hoechst 33342) in PBS for 5 min and mounted on microscope slides in Mowiol V4-88 (133 mg/ml; Hoechst, Frankfurt, Germany) and n-propyl gallate (5 mg/ml). The slides were kept at 4°C in darkness until observation. Oocytes were observed under an epifluorescence microscope (Zeiss). Controls were performed using no primary antibodies to ascertain the absence of non-specific binding or no secondary antibodies to ascertain the absence of auto-fluorescence.

Statistical analysis

The percentages of fertilized oocytes were compared between groups using chi-square test with Yates correction when necessary. Differences were considered statistically significant at $p < 0.05$.

RESULTS

In experiment 1, our aim was to analyse the influence of cumulus cells during IVF performed in the presence of porcine oviduct epithelial cells (OEC). Thus, mature COCs were removed or not from their cumulus cells after IVM and processed for IVF in the presence of OEC, with fresh and frozen sperm. The results are presented in table 1. When fresh sperm was used, the removal of cumulus cells tended to increase the percentage of fertilization: 1/33 intact COC vs 4/31 oocytes without cumulus cells were penetrated ($p > 0.05$). All penetrated oocytes contained 2 pronuclei (Figure 1). When frozen sperm was used, the removal of cumulus cells did not influence the fertilization rate: 1/32 oocyte was penetrated, whether the cumulus was present or not. Both penetrated oocytes were polyspermic.

In experiment 2, our aim was to analyse the influence of preincubation of sperm with progesterone before IVF performed in the presence of OEC. Mature COCs were removed from their cumulus cells after IVM, according to the results of the first experiment. They were processed for IVF with fresh and frozen sperm preincubated or not with progesterone. The results are presented in table 2. When fresh sperm was used, its preincubation with progesterone did not significantly influence the fertilization rate: 2/14 oocytes were penetrated when progesterone was used vs 2/18. When frozen sperm was used, similar data were obtained: 1/29 oocytes was penetrated when progesterone was used vs 1/25. All penetrated oocytes contained 2 pronuclei.

In experiment 3, our aim was to analyse the influence of cumulus cells during IVF performed after preincubation of sperm with oviductal fluid. Thus, mature COCs were removed or not from their cumulus cells after IVM and processed for IVF with fresh sperm preincubated with 10% pre-ovulatory oviductal fluid (preOF). Due to some technical problems, frozen sperm could not be tested. The results are presented in table 3. The removal of cumulus cells tended to increase the percentage of fertilization: 1/24 intact COC vs 3/26 oocytes without cumulus cells were penetrated ($p > 0.05$). Penetrated oocytes contained 2 pronuclei, except one denuded oocyte that was polyspermic.

In experiment 4, our aim was to analyse the influence of preincubation of sperm with progesterone combined with preincubation with oviductal fluid before IVF. Thus, mature COCs were removed from

their cumulus cells after IVM according to the results of experiment 3. They were processed for IVF, with fresh and frozen sperm preincubated with 10% pre-ovulatory oviductal fluid (preOF) and preincubated or not with progesterone. The results are presented in table 4. When fresh sperm was used, its preincubation with progesterone did not significantly influence the fertilization rate: 2/39 oocytes were penetrated (2 zygotes with 3 and 4 cells; Figure 1) when progesterone was used vs 2/36 (both contained 2 pronuclei). When frozen sperm was used, similar data were obtained: 2/37 oocytes was penetrated when progesterone was used vs 1/46, all were polyspermic.

In experiment 5, our aim was to analyse the influence of sperm preincubated with 10% or 20% post-ovulatory oviductal fluid before IVF. Thus, mature COCs were removed from their cumulus cells after IVM and processed for IVF with fresh sperm preincubated with 10% or 20% post-ovulatory oviductal fluid (postOF). The results are presented in table 5. No fertilization was observed whether sperm was incubated with 10% or with 20% post-ovulatory oviductal fluid.

Overall, the removal of cumulus cells tended to increase the penetration rate after IVF performed in the presence of OEC or after sperm preincubation with oviductal fluid. Moreover, fresh sperm tended to give higher penetration rates than frozen sperm after IVF performed in the presence of OEC. Finally, pre-ovulatory and post-ovulatory oviductal fluid may not have the same ability to prepare sperm for oocyte fertilization.

DISCUSSION

Our results show that zygotes with 3-4 cells were obtained after IVF of oocytes without cumulus cells and fresh sperm preincubated with pre-ovulatory oviductal fluid and progesterone before IVF. This is encouraging results for the setting up of an efficient IVF procedure in equine.

The removal of cumulus cells after oocyte IVM tended to increase the percentage of fertilization when IVF was performed in the presence of porcine oviduct epithelial cells or after preincubation of fresh sperm with oviductal fluid. In a previous study, the removal of cumulus cells after equine oocyte IVM tended to increase the percentage of fertilization when oocytes were pre-incubated with oviduct epithelial cells and fresh sperm was treated with calcium ionophore (Mugnier et al. 2009b). Moreover, higher in vitro fertilization rates were obtained after partial cumulus removal compared with equine oocytes with an intact cumulus (Dell'Aquila et al. 1996). Thus, the removal of cumulus cells from oocytes before IVF seems to increase the in vitro fertilization rate in several conditions, leading us to advice of removing the cumulus during IVF procedures, especially when fresh sperm was used.

When IVF was performed with denuded oocytes in the presence of OEC, the percentage of penetrated oocytes tended to be higher for fresh sperm (11-14%) than frozen sperm (3-4%). When IVF was performed with denuded oocytes and sperm preincubated with oviductal fluid, the percentage of penetrated oocytes tended to be slightly higher for fresh sperm (5-12%) than frozen sperm (2-5%). Thus, fresh sperm seemed to be more competent for IVF in our conditions. In a heterologous oocyte-binding assay, significantly more fresh stallion spermatozoa were found attached to the bovine oocytes compared with frozen-thawed spermatozoa (Clulow et al. 2010). Similar data were obtained in in vivo conditions, when equine oocytes were transferred into the oviduct, embryo development rates were higher when fresh spermatozoa were placed in the oviduct than when frozen sperm was used (Coutinho da Silva et al. 2004). However, in a previous study, fresh sperm treated with calcium ionophore showed a higher penetration rate than cryopreserved sperm, whereas fresh sperm treated with heparin showed a slightly lower penetration rate than cryopreserved sperm (Alm et al. 2001). Thus, fresh and frozen sperm require different treatment to acquire the ability to fertilize the oocyte.

Sperm preincubation with oviductal fluid leaded to the production of zygotes with 3-4 cells in our conditions. Previous studies have shown that exposure to oviduct secretions triggers sperm capacitation in vivo (Leemans et al. 2016, Goudet 2011). Moreover, oviductal fluid induces tyrosine phosphorylation and acrosome reaction in bull spermatozoa (Kumaresan et al. 2019, Kumaresan et al. 2012), acrosome reaction in humans (De Jonge et al. 1993) and pigs (Kim et al. 1997) and sperm capacitation in dogs (Kawakami et al. 1998). Thus, exposure of spermatozoa to oviductal fluid plays a role in their preparation for fertilisation. Oocyte preincubation with oviductal fluid also have a positive role on oocyte preparation for fertilisation since it increased the fertilisation rate in equine (Ambruosi et al. 2013) and porcine (Batista et al. 2016). Moreover, in the present study, the highest penetration rates were obtained when IVF was performed in the presence of OEC, and previous studies showed an increased IVF rate in the presence of oviductal cells (Mugnier et al. 2009b). Thus, oviductal cells and secretions seem to play a crucial role in both the preparation for fertilisation of male and female gametes and fertilisation itself.

Sperm preincubation with progesterone did not significantly increase the fertilization rate in our conditions whether fresh or frozen sperm was used. Progesterone is involved in equine sperm capacitation, induction of stallion sperm acrosome reaction by binding to its receptor on the sperm plasma membrane and spermatozoa binding to the zona pellucida (Cheng et al. 1998b, Cheng et al. 1998a, Rathi et al. 2003, Meyers et al. 1995). However, the capacitation-related modifications observed in equine sperm in these studies do not seem to be sufficient to increase the fertilization rate, since we did not observe any effect of progesterone on the IVF rates. Several studies have shown the actions of progesterone on spermatozoa. Progesterone stimulates in vitro capacitation and acrosome

reaction in human (Yamano et al. 2004, Kay et al. 1994) and induces acrosome reaction in mouse (Roldan et al. 1994), pig (Melendrez et al. 1994), goat (Somanath et al. 2000) and ovine (Gimeno-Martos et al. 2017). In the present study, two zygotes were obtained after IVF with fresh sperm preincubated with progesterone, thus, a positive effect of progesterone can not be ruled out.

In conclusion, our study shows encouraging results for the setting up of an efficient IVF procedure in equine, with the production of zygotes. Further studies are in progress to increase the IVF rates and establish conditions for development of IVF embryos.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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TABLES

Table 1 : nuclear stage for oocytes with or without cumulus cells, fresh and frozen sperm and IVF in the presence of OEC. Oocytes containing one pronucleus were considered activated, oocytes containing two or more pronucleus were considered penetrated, oocytes without plasma membrane or showing fragmented cytoplasm were considered degenerated. The percentages of penetrated oocytes were not significantly different between groups.

		Nuclear stage after IVF				
Sperm	**Oocytes**	**Metaphase 2**	**activated**	**penetrated**	**degenerated**	**penetrated/total**
Fresh	With cumulus cells	12	7	1	13	1/33 (3%)
	Without cumulus cells	17	3	4	7	4/31 (13%)
Frozen	With cumulus cells	15	5	1	11	1/32 (3%)
	Without cumulus cells	20	0	1	11	1/32 (3%)

Table 2 : nuclear stage for oocytes without cumulus cells, fresh and frozen sperm preincubated or not with progesterone and IVF in the presence of OEC. Oocytes containing one pronucleus were considered activated, oocytes containing two or more pronucleus were considered penetrated, oocytes without plasma membrane or showing fragmented cytoplasm were considered degenerated. The percentages of penetrated oocytes were not significantly different between groups.

		Nuclear stage after IVF				
Sperm	**Preincubation of sperm**	**Metaphase 2**	**activated**	**penetrated**	**degenerated**	**penetrated/total**
Fresh	With progesterone	5	1	2	6	2/14 (14%)
	Without progesterone	9	3	2	4	2/18 (11%)
Frozen	With progesterone	18	3	1	7	1/29 (3%)
	Without progesterone	14	4	1	6	1/25 (4%)

Table 3 : nuclear stage for oocytes with or without cumulus cells, fresh sperm preincubated with 10% pre-ovulatory oviductal fluid and IVF. Oocytes containing one pronucleus were considered activated, oocytes containing two or more pronucleus were considered penetrated, oocytes without plasma membrane or showing fragmented cytoplasm were considered degenerated. The percentages of penetrated oocytes were not significantly different between groups.

		Nuclear stage after IVF				
Sperm	**Oocytes**	**Metaphase 2**	**activated**	**penetrated**	**degenerated**	**penetrated/total**
Fresh	With cumulus cells	19	0	1	4	1/24 (4%)
	Without cumulus cells	17	2	3	4	3/26 (12%)

Table 4 : nuclear stage for oocytes without cumulus cells, fresh and frozen sperm preincubated with 10% pre-ovulatory oviductal fluid and preincubated or not with progesterone and IVF. Oocytes containing one pronucleus were considered activated, oocytes containing two or more pronucleus were considered penetrated, oocytes without plasma membrane or showing fragmented cytoplasm were considered degenerated. The percentages of penetrated oocytes were not significantly different between groups. * both are zygotes with 3 and 4 cells.

		Nuclear stage after IVF				
Sperm	**Preincubation of sperm**	**Metaphase 2**	**activated**	**penetrated**	**degenerated**	**penetrated/total**
Fresh	With progesterone	18	2	2*	17	2/39 (5%)
	Without progesterone	25	1	2	8	2/36 (6%)
Frozen	With progesterone	26	3	2	6	2/37 (5%)
	Without progesterone	36	1	1	8	1/46 (2%)

Table 5 : nuclear stage for oocytes without cumulus cells, fresh sperm preincubated with 10% or 20% post-ovulatory oviductal fluid (postOF) and IVF. Oocytes containing one pronucleus were considered activated, oocytes containing two or more pronucleus were considered penetrated, oocytes without plasma membrane or showing fragmented cytoplasm were considered degenerated.

		Nuclear stage after IVF				
Sperm	**Préincubation of sperm**	**Metaphase 2**	**activated**	**penetrated**	**degenerated**	**penetrated/total**
Fresh	With 10% postOF	21	1	0	8	0/30 (0%)
	With 20% postOF	18	1	0	10	0/29 (0%)

FIGURE LEGEND

Figure 1: Nuclear status assessed by staining of chromatin with Hoechst stain (a,c) and nuclear envelope by lamin A/C antibody (b,d); a,b: an oocyte with two pronuclei with chromatin (blue) within the nuclear envelope (red); c,d: a zygote with 4 cells and 4 nuclei (one is in another focal plan). Scale bar represent 60 µm.

Figure 1