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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1743319 since 2020-07-07T16:47:29Z

Published version:

DOI:10.1017/S096719941700048X

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doi:10.1017/S096719941700048X

Exposure to follicular fluid during oocyte maturation and oviductal fluid during post-maturation does not improve *in vitro* embryo production in the horse

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Date submitted: 13.12.2016. Date revised/accepted: 25.07.2017

Summary

Most wild equids and many domestic horse breeds are at risk of extinction, so there is an urgent need for genome resource banking. Embryos cryopreservation allows the preservation of genetics from male and female and is the fastest method to restore a breed. In the equine, embryo production *in vitro* would allow the production of several embryos per cycle. Intracytoplasmic sperm injection (ICSI) is used to generate horse embryos, but it requires expensive equipment and expertise in micromanipulation, and blastocyst development rates remain low. No conventional *in vitro* fertilization (IVF) technique for equine embryo production is available. The development of culture conditions able to mimic the maturation of the oocyte in preovulatory follicular fluid (pFF) and the post-maturation in oviductal fluid (OF) may improve embryo production *in vitro*. Our aim was to analyse the effect of *in vitro* maturation in pFF and incubation in OF on *in vitro* maturation of equine oocytes, fertilization using conventional IVF or ICSI, and embryo development after culture in synthetic oviductal fluid (SOF) or DMEM-F12. Oocytes collected from slaughtered mares or by ovum pick up were matured *in vitro* in pFF or semi-synthetic maturation medium (MM). The *in vitro* maturation, fertilization and development rates were not statistically different between pFF and MM. After *in vitro* maturation, oocytes were incubated with or without OF. Post-maturation in OF did not significantly improve the fertilization and development rates. Thus, in our study, exposure to physiological fluids for oocyte maturation and post-maturation does not improve *in vitro* embryo production in the horse.

Keywords: Equine, IVM, IVF, Oocyte, Spermatozoa

Introduction

Most wild equids are currently endangered or threatened in the wild, such as the Asiatic wild ass or Grevy's zebra, as mentioned in the IUCN Red List of endangered animal species of the International Union for the Conservation of Nature (Adams *et al.*, 2009). Moreover, many domestic horse breeds are at risk of

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extinction, with less than 300 active breeding mares, and several breeds that are nearly extinct with fewer than 100 active breeding mares, such as the Faer Island pony in Denmark, Estonian draught horse in Estonia, Landais pony and Grand Noir du Berry donkey in France (Smits *et al*., 2012b). The actions that are currently undertaken to preserve endangered horse breeds include the creation of a Genome Resource Bank.

Genome resource banking requires cryopreservation of semen, oocytes and/or embryos. Embryo cryopreservation allows the preservation of genetics from both male and female and is the fastest method to restore a breed. In equids, embryo production *in vivo* is limited, as experimental induction of multiple ovulations has a low efficiency (Meyers-Brown *et al*., 2011) and routine induction of multiple ovulations is still ineffective (Smits *et al*., 2012b). Embryo production *in vitro* allows the production of several embryos per cycle (Hinrichs, 2012). Intracytoplasmic sperm injection (ICSI) has been widely adopted to generate horse embryos *in vitro*, both for scientific purposes and in the horse breeding industry (Choi *et al*., 2011; Hinrichs, 2012). This method allows the investigation of specific aspects of fertilization in the horse, such as sperm chromatin–ooplasm interactions, overcoming limited efficiency of *in vitro* sperm penetration through oocyte barriers. However ICSI requires expensive equipment and expertise in micromanipulation, and very few laboratories worldwide routinely produce equine embryos following ICSI. Moreover, blastocyst development rates following ICSI do not exceed 40% per cleaved oocyte (Hinrichs, 2012). Over the last decades, several attempts to establish an efficient *in vitro* fertilization (IVF) technique in the equine were performed. Palmer and collaborators published the first paper on IVF of equine oocytes (Bézard *et al*., 1989) and obtained the first and only two IVF-produced foals (Palmer *et al*., 1991) using preovulatory oocytes and fresh sperm treated with calcium ionophore. However, this technique did not yield IVF rates higher than 36% (Palmer *et al*., 1991; Alm *et al*., 2001) and, although low IVF rates have been reported sporadically (Hinrichs *et al*., 2002), this technique was not repeatable (Mugnier *et al*., 2009). Dell'Aquila and collaborators reported a 32% rate of equine IVF after incubation of *in vitro* matured oocytes subjected to partial cumulus removal, with frozen sperm treated with heparin (Dell'Aquila *et al*., 1996) but these results could not be replicated (Dell'Aquila *et al*., 1997a, b). Low rates of IVF (0–36%) were obtained by Alm and collaborators (Alm *et al*., 2001) after exposure of spermatozoa to calcium ionophore or heparin. Since then, no reports showing the efficiency of one of these IVF techniques have been published. In 2009, a 60% rate of fertilized oocytes was reported after treatment of fresh spermatozoa with procaine to induce hyperactivated motility (McPartlin *et al*., 2009). Using the same technique, we obtained 37% of fertilized oocytes (Ambruosi *et al*., 2013), but Leemans and collaborators showed that equine IVF embryos fail to develop beyond the 8–16-cell stage (Leemans *et al*., 2015). Thus, to date, no efficient conventional IVF technique for equine embryo production *in vitro* is available.

During its journey in the follicle and the oviduct, the oocyte acquires factors necessary for fertilization and early development. The somatic environment is of crucial importance for oocyte preparation for fertilization and development (Coy *et al*., 2012; Aviles *et al*., 2010). The development of *in vitro* culture conditions able to mimic the maturation of the oocyte in follicular fluid and the post-maturation of the ovulated oocyte in OF may help to improve embryo production *in vitro*. For example, addition of follicular fluid to maturation medium of equine oocytes increases fertilization and cleavage rate (Dell'Aquila *et al*., 1997b). Pre-incubation of equine oocytes with OF or oviductal cells increases fertilization rate (Mugnier *et al*., 2009) (Ambruosi *et al*., 2013). Moreover, several studies have shown the crucial role played by the oviduct in the preparation of equine gametes for fertilization (Goudet, 2011).

Several media have been used for *in vitro* culture of equine zygotes in different labs: Synthetic OF (SOF) medium (Tremoleda *et al*., 2003), DMEM-F12–51445C medium (in ME Dell'Aquila laboratory), DMEM-F12-D8437 medium (in G Goudet lab), DMEM-F12-D8900 medium (Choi *et al*., 2011; Martino *et al*., 2016). However, these medium have never been used in the same laboratory and conditions.

Our aim was to analyse the effect of *in vitro* maturation in follicular fluid and pre-incubation in OF on *in vitro* maturation, fertilization using conventional IVF or ICSI, and development of equine oocytes. For this purpose: (1) we compared a semi-synthetic *in vitro* maturation medium with preovulatory follicular fluid, in which maturation naturally occurs; (2) we analysed the influence of pre-incubation of oocytes with OF before fertilization, which occurs in vivo after ovulation of the oocyte into the oviduct; (3) we tested four culture media for the *in vitro* development of fertilized oocytes using conventional IVF or ICSI.

Materials and Methods

All procedures on animals were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the French Ministry of Agriculture and with the approval of the ethical review

committee (Comité d'Ethique en Expérimentation Animale Val de Loire) under numbers 2011/6 and 02701.01. The study was conducted in France and in Italy (48° and 41° North parallel) during two subsequent breeding seasons.

All chemicals were purchased from Sigma-Aldrich (Milano, Italy and St Quentin Fallavier, France) unless otherwise indicated.

Collection of equine immature oocytes

Equine immature cumulus–oocyte complexes (COCs) were collected during the breeding season either from slaughtered mares in commercial abattoirs in France and Italy or by transvaginal ultrasound-guided aspiration on experimental mares in France.

For COCs collection from slaughtered mares, ovaries from females of unknown reproductive history were obtained at local commercial abattoirs immediately after females were slaughtered. They were transported to the laboratory within 2 h in 0.9% (w/v) NaCl at 32–38°C. In the French laboratory, COCs were collected using the aspiration procedure previously described by Goudet and collaborators (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 mmHg of vacuum pressure, the ovaries were cut into thick sections with a scalpel blade to find other follicles within the ovarian stroma. Follicular fluids were examined under a stereomicroscope for COCs recovery. In the Italian laboratory, COCs were collected using the scraping procedure as previously described (Dell'Aquila *et al.*, 2001). All follicles from 5 to 25 mm in diameter were opened with a scalpel blade and the granulosa cells layer scraped with a curette, COCs were identified in the collected mural granulosa cells by using a dissection microscope. In both laboratories, oocytes denuded of cumulus and degenerated oocytes showing shrunken, dense or fragmented cytoplasm, were discarded.

For COCs collection by transvaginal ultrasound-guided aspiration (ovum pick up: OPU), adult cyclic pony mares from our experimental stud were used. Ovarian activity was assessed by routine rectal ultrasound scanning to choose mares with several follicles from 5 to 25 mm. Follicles were punctured by transvaginal ultrasound-guided aspiration with a double-lumen needle (length 700 mm, outer diameter 2.3 mm, internal diameter 1.35 mm, Casmed, Cheam, Surrey, England) and a sectorial probe (Aloka SSD900) as previously described (Goudet *et al.*, 1997). After follicular fluid aspiration, the follicle was flushed with PBS (Phosphate Buffered Saline, Dulbecco A, Oxoid, Basingstoke, Hampshire, England) and heparin (Choay, Sanofi Aventis 5000 IU/ml) at 38°C. All aspirated fluids were examined for oocyte recovery, and oocytes denuded of cumulus or degenerated oocytes showing shrunken, dense or fragmented cytoplasm were discarded. During the collection procedure, mares were injected with detomidine (Medesedan®, 0.25 ml/animal i.v., 10 mg/ml detomidine, Centravet, Plancoet, France) and butorphanol (Dolorex®, 0.6 ml/animal i.v., 10 mg/ml butorphanol tartrate and 0.1 mg/ml benzethonium chloride, Centravet) for sedation and analgesia, dipyrone and butylscopolamine (Estocelan®, 15 ml/animal i.v., 4 mg/ml butylscopolamine and 500 mg sodique metamizole, Centravet) for analgesia and antispasmodia. After puncture, the mares received a preventive antibiotic injection (Depocilline, 20 ml/animal i.m., benzylpenicillin 170.41 mg/ml Intervet, Beaucouze, France).

In vitro maturation (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) fetal calf serum (FCS) and 25 μg/ml gentamycin. They were then cultured in group of 10 to 30 for 27 h in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity in 500 μl of maturation medium (MM) or 100% equine preovulatory follicular fluid (pFF). The maturation medium was Medium 199 with Earle's salts supplemented with 20% (v/v) FCS and 50 ng/ml epidermal growth factor (Goudet *et al.*, 2000). The equine pFF was collected by transvaginal ultrasound-guided aspiration on three adult cyclic pony mares from our experimental stud. Ovarian activity was assessed by routine rectal ultrasound scanning. At the emergence of a follicle larger than 33 mm in diameter, the mare was injected with 1500 IU human chorionic gonadotropin (hCG, i.v., Chorulon, Intervet). The preovulatory follicle was punctured 35 h after hCG injection, just before ovulation, by transvaginal ultrasound-guided aspiration with a single-lumen needle (length 600 mm, outer diameter 1.8 mm, Thiebaud Freres, Jouvernex Margencel, France) and a sectorial probe as previously described. The presence of a metaphase II oocyte was ascertained using nuclear chromatin configuration analysis as described below. The pFF was centrifuged at 1500 *g* for 10 min at 4°C. The supernatants were pooled and kept at −20°C.

Post-maturation of equine oocytes in OF

After IVM, equine COCs were partially denuded and incubated in droplets of 30 μl of porcine OF for 30 min in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity. For porcine OF collection, genital tracts from gilts were obtained at a commercial abattoir and transported to the laboratory at room temperature.

Genital tracts with both ovaries containing several follicles larger than 5 mm were used. The oviducts were dissected free from surrounding tissues. The oviductal content from the ampulla was expelled by gentle squeezing using a sterile microscope slide and collected by introducing the tip of a pipette into the ampulla and aspirating while making a manual ascendant pressure from the isthmus to the ampulla (Carrasco *et al*., 2008). After centrifugation at 10,000 *g* for 15 min, the supernatant containing secreted and intracellular components was immediately stored at −20°C until use as 'OF'.

In vitro fertilization (IVF) procedure

The IVF procedure was performed in France, using oocytes collected from slaughtered mares or by OPU.

Preparation of equine sperm and IVF

Fresh equine semen was collected with a closed artificial vagina from three adult Welsh pony stallions of proven fertility from our experimental stud. Semen was filtered through gauze, sperm motility was visually evaluated under light microscopy on a heated stage and sperm concentration was assessed using a spectrophotometer (Ciba-Geigy). Then, 1 ml of semen was diluted in 2 ml of pre-warmed modified Whitten's Medium (MW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt, 1 mM pyruvic acid) supplemented with 5.5 mM glucose (anhydrous), pH 7.25 (McPartlin *et al*., 2009). Diluted sperm was transported to the laboratory within a few minutes at 37°C and centrifuged in 15 ml conical tubes at 100 *g* for 1 min at 37°C to remove particulate matter and dead sperm. The supernatant was then transferred to a 14 ml round-bottom tube and centrifuged at 600 *g* for 5 min at 37°C. The pellet was re-suspended in 1.5 ml of pre-warmed MW supplemented with glucose, and the concentration was determined by counting on a Thoma chamber under a microscope (Olympus, IMT-2, Paris, France). Spermatozoa were then diluted at 10×10^6/ml in pre-warmed MW supplemented with 5.5 mM glucose, 25 mM $NaHCO_3$ and 7 mg/ml BSA, pH 7.25 (capacitating MW) (McPartlin *et al*., 2009). Spermatozoa were incubated in 500 µl aliquots in polyvinyl alcohol-coated 5 ml round-bottom tubes at 37°C in a humidified atmosphere during 6 h. The motility was visually evaluated under a microscope (Olympus, IMT-2, Paris, France) at the beginning and at the end of the incubation period. Spermatozoa were then diluted at 1×10^6/ml in capacitating MW supplemented with 5 mM procaine to induce hyperactivated motility. Droplets of 100 µl of spermatozoa suspension were laid down onto culture dishes and covered with mineral oil.

Equine oocytes were washed in capacitating MW and groups of 10 were transferred to droplets of 100 µl of spermatozoa suspension and co-incubated for 18 h in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity.

Control of parthenogenetic activation

Equine oocytes were washed in capacitating MW and transferred to droplets of 100 µl of capacitating MW supplemented with 5 mM procaine without spermatozoa and incubated for 18 h in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity.

In vitro *culture of equine zygotes*

After 18 h co-incubation with spermatozoa, equine zygotes were washed in the culture medium and flushed to remove attached spermatozoa. Groups of 10 were transferred to droplets of 30 µl of culture medium for 30 h or 54 h (48 h or 72 h post IVF) in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C in 100% humidity.

Four culture media were tested. Synthetic OF (SOF) medium was SOF (Minitub) supplemented with 2% Basal Medium Eagle amino acids solution, 1% Minimum Essential Medium non-essential amino acids solution, 0.33 mg/ml sodium pyruvate, 6 mg/ml BSA fatty acid free, 5% FCS and 25 µg/ml gentamycin. DMEM-F12–51445C medium was Dulbecco's Modified Eagle's Medium Ham's Nutrient Mixture F12 with 3151 mg/l dextrose, 2.5 mM L-glutamine, 15 mM HEPES, 55 mg/l sodium pyruvate (ref. 51445C) supplemented with 10% FCS and 25 µg/ml gentamycin. DMEM-F12-D8437 medium was DMEM-F12 with 2.5 mM L-glutamine, 15 mM HEPES, 1.2 g/l sodium bicarbonate (ref D8437) supplemented with 10% FCS and 25 µg/ml gentamycin. DMEM-F12-D8900 medium was DMEM-F12 with 2.5 mM L-glutamine and 15 mM HEPES (ref D8900) supplemented with 1.2 g/l $NaHCO_3$, 10% FCS and 25 µg/ml gentamycin. The three references of DMEM-F12 had identical composition but different forms (liquid or powder, sodium bicarbonate included or added subsequently).

Assessment of nuclear status

Nuclear status was assessed either after 27 h IVM, after 18 h IVF or after 30 h or 54 h *in vitro* development. Oocytes and zygotes were washed by aspiration in and out of a pipette in PBS, 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. Non-specific reactions were blocked by incubation for 1 h at room temperature in 10% goat serum in PBS. Oocytes and zygotes were incubated overnight at 4°C or 2 h at room temperature with an anti-lamin A/C antibody (ThermoScientific) diluted 1:100 in PBS containing

0.2% BSA and 0.1% Tween. After four washings for 5 min in PBS containing 0.2% BSA and 0.1% Tween, they were incubated for 1 h at room temperature with an AlexaFluor 594-conjugated-anti-mouse antibody (Life Technologies) diluted 1:400 in PBS. They were then washed five times for 5 min in PBS containing 0.1% Tween and two 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 the dark 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.

Intracytoplasmic sperm injection (ICSI) procedure

The ICSI procedure was performed in Italy, using oocytes collected from slaughtered mares and matured *in vitro* with the procedure described above.

Preparation of equine sperm and ICSI procedure

Fresh semen samples from three mature stallions with a reproductive history of normal fertility were used. The stallions were located in the reproductive centre Pegasus (Veterinary Clinics and Animal Productions Unit – DETO, Polo di Valenzano, University of Bari Aldo Moro, Valenzano, Bari, Italy) and were routinely used in artificial insemination programs. Semen was collected with a Missouri artificial vagina with an in-line gel filter, and was extended with INRA 96 (IMV Technologies, Piacenza, Italy) at a concentration of 20 to 25 × 10^6 sperm cells/ml and used immediately. Sperm cells for ICSI were prepared by the swim-up procedure in Earle's balanced salt solution supplemented with 0.4% BSA and 50 μg/ml gentamicin as previously described (Dell'Aquila *et al.*, 2001, 2003; Ambruosi *et al.*, 2009).

Intracytoplasmic sperm injection was carried out as previously reported (Ambruosi *et al.*, 2009; Dell'Aquila *et al.*, 2001, 2003). All procedures were performed at 38.5°C in Quinn's Advantage Fertilization HTF Universal medium (Cooper Surgical, Trumbull, CT, USA) supplemented with 5 mg/ml human serum albumin (HSA).

In vitro *culture of embryos*

In a first embryo culture experiment, aimed to test the influence of IVM medium (MM versus pFF) on fertilization and early embryonic development, injected oocytes were put immediately after ICSI in one of the two embryo culture medium (either DMEM-F12–51445C or DMEM-F12-D8900 supplemented with 1.2 g/l $NaHCO_3$) plus 10% FCS with 25 μg/ml gentamycin under mineral oil in droplets of 10 μl and cultured individually for 72 h in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. At the end of the culture period, early embryos and uncleaved ova were removed from culture, fixed and evaluated using the procedures as described below. In a second embryo culture experiment, aimed to test the influence of pre-incubation with OF on embryo development, injected oocytes were cultured in DMEM-F12 D8900 for up to 10 days. At day 10 of culture, embryos (developed, delayed and degenerated embryos and uncleaved ova) were morphologically evaluated, fixed and stained for assessing nuclear chromatin, as described below.

Nuclear chromatin evaluation

Nuclear chromatin configuration of embryos and uncleaved ova was evaluated under an epifluorescence microscope (Nikon Eclipse 600 equipped with B-2 A, 346 nm excitation/460 nm emission filter) after staining with Hoechst 33258, as previously described (Dell'Aquila *et al.*, 2001; Hinrichs *et al.*, 2005; Lange Consiglio *et al.*, 2009). Normally cleaved embryos were defined by the presence of nuclei of regular morphology within each blastomere. The number of morphologically normal nuclei was counted for each embryo. Embryos were classified as morulae when they had more than 32 nuclei. Embryos with more than 64 nuclei and having an outer layer of apparent differentiating trophoblast cells were considered to be blastocysts (Choi *et al.*, 2006). In the group of uncleaved ova, normal fertilization was defined by the presence of two polar bodies with two pronuclei (PN). Oocytes showing one PN with intact sperm cell were classified as activated oocytes. Oocytes showing a metaphase plate and one polar body with an intact sperm cell were classified as unfertilized; oocytes having degenerated, irregularly clustered or faint chromatin were classified as degenerating.

Statistical analysis

The percentages of mature oocytes, fertilized oocytes, cleaved embryos, morulae and blastocysts were compared between groups using chi-square analysis. Differences were considered statistically significant at $P < 0.05$.

Results

Maturation rates after IVM

After 27 h IVM in MM or pFF, nuclear status of the oocytes was assessed. Oocytes with metaphase II and one polar body were considered mature (Fig. 1a).

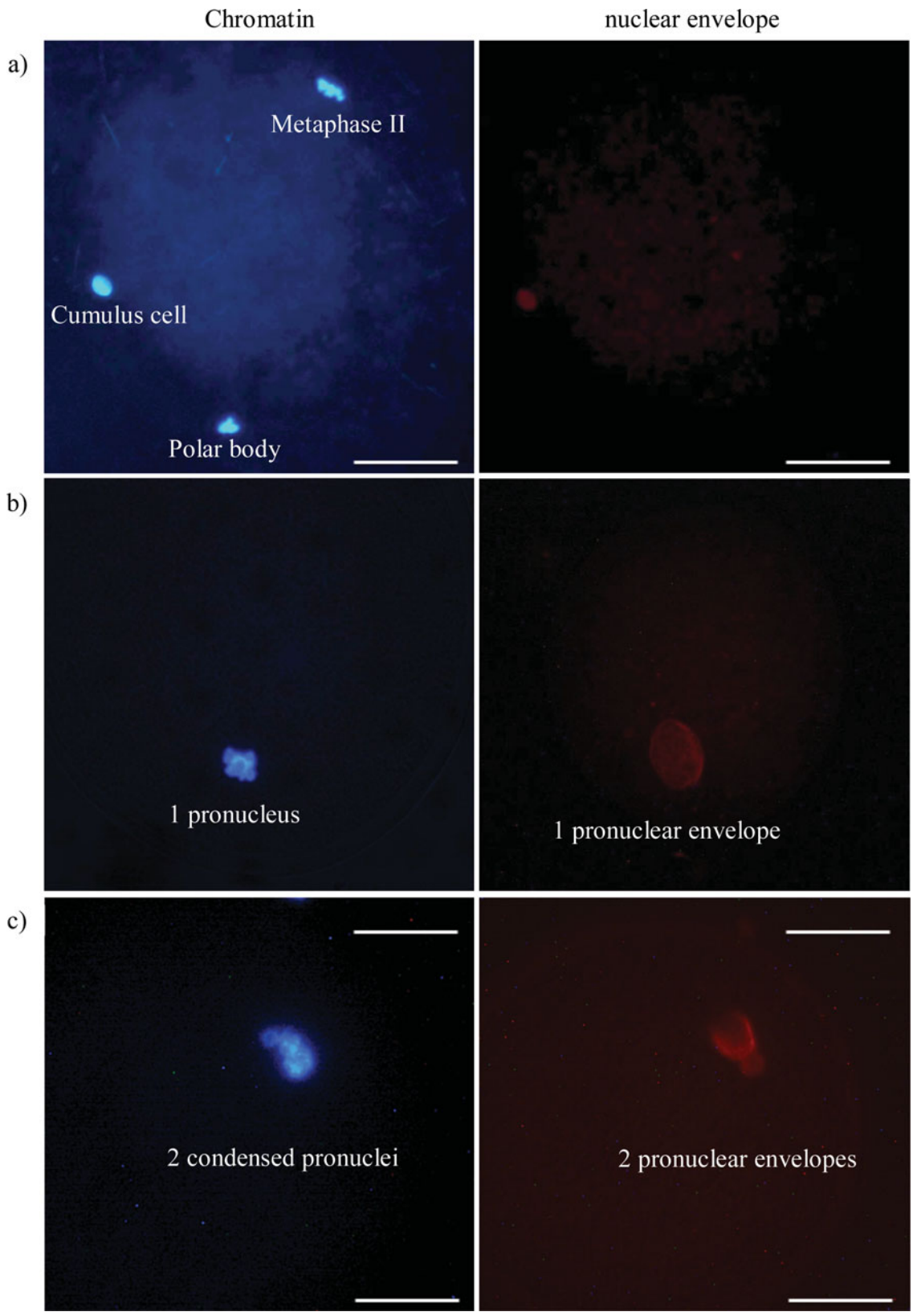

Figure 1 Nuclear status assessed by staining of chromatin with Hoechst stain and nuclear envelope by lamin A/C antibody. (*a*) Mature oocyte with metaphase II and one polar body with no detectable nuclear envelope. (*b*) Oocyte with one pronucleus showing chromatin and a nuclear envelope without sperm cell. (*c*) Oocyte with two condensed pronuclei with a compact mass of chromatin within the nuclear envelope. Scale bar represent 60 μm.

For the oocytes collected in France, three repetitions were performed. After 27 h IVM in MM and pFF, 16 oocytes out of 21 (76%) and 14 oocytes out of 21 (67%) were mature. For the oocytes collected in Italy, seven repetitions were performed and in total, 219 oocytes were analysed after IVM. After 27 h IVM in MM and pFF, 57 oocytes out of 108 (53%) and 60 oocytes out of 111 (54%) were mature. In both experiments, the maturation rates in MM and pFF were not statistically different ($P > 0.05$).

Fertilization and development rates after IVM, IVF and *in vitro* culture

Influence of IVM medium on fertilization rates after IVF

After oocytes collection from slaughterhouse or OPU, IVM in MM or pFF, post-maturation with OF and gametes co-incubation, nuclear status was assessed. We observed oocytes with metaphase II and one polar body with no detectable nuclear envelope (Fig. 1*a*), oocytes with one pronucleus (PN) showing DNA stained with Hoechst stain and a nuclear envelope stained positive with lamin A/C antibody without sperm cell (Fig. 1*b*) and oocytes with two PN in the cytoplasm, each PN showing the presence of DNA and a nuclear envelope (Fig. 1*c*). All the 2PN oocytes showed condensed PN with a compact mass of chromatin within the nuclear envelope and very few 2PN oocytes showed one or two polar bodies. Degenerated oocytes, having shrunken, dense or fragmented cytoplasm, were discarded.

For the oocytes collected in a slaughterhouse, three repetitions were performed (Fig. 2*a*). The percentage of oocytes containing 2PN 18 h post IVF was not statistically different between the two maturation conditions (IVM in MM: 22/33, 67%; IVM in pFF: 24/42, 57%; $P > 0.05$). For the oocytes collected by OPU, two repetitions were performed (Fig. 2*a*). The percentage of oocytes containing 2PN 18 h post IVF was not statistically different (IVM in MM: 4/10, 40%; IVM in pFF: 4/9, 44%; $P > 0.05$). When oocytes collected in a slaughterhouse and by OPU were pooled, the 2PN rates from MM (26/43, 60%) and pFF (28/51, 55%) were not statistically different ($P > 0.05$).

Influence of post-maturation with OF on fertilization rates after IVF

After oocytes collection from slaughtered mares, IVM in MM, pre-incubation or not with OF and gametes co-incubation, nuclear status was assessed. Two repetitions were performed (Fig. 2*b*). The percentage of oocytes containing 2PN 18 h post IVF was not statistically different between post-maturation with OF (19/31, 61%) and without OF (17/34, 50%) ($P > 0.05$).

Control of parthenogenetic activation

For the control groups for parthenogenetic activation, three repetitions were performed during the time of the experiments. Equine immature oocytes collected in France from slaughtered mares were *in vitro* matured in MM or pFF, incubated with OF and co-incubated with or without spermatozoa. Three oocytes containing 2 PN were observed in the control group incubated without spermatozoa (3/26, 11% and 0/7 after IVM in MM and pFF respectively; Fig. 2*c*). In the other oocytes incubated without spermatozoa we observed either a metaphase II (62%, 16/26 and 57%, 4/7) or 1 PN (27%, 7/26 and 43%, 3/7) after IVM in MM and pFF respectively. The percentage of 2PN oocytes was significantly different for incubation with vs without spermatozoa after IVM in MM and pFF ($P < 0.05$).

Development rates after IVM, IVF and in vitro *culture*

After oocytes collection from slaughtered mares or by OPU, IVM in MM or pFF, post-maturation with OF, IVF and *in vitro* culture in one of the four tested media, nuclear status was assessed after 30 h *in vitro* culture, i.e. 48 h post IVF, or after 54 h *in vitro* culture, i.e. 72 h post IVF. For each medium, three repetitions were performed.

In total, 168 oocytes/embryos were analysed 48 h post IVF (39 from SOF, 45 from DMEM-F12–51445C, 42 from DMEM-F12-D8437 and 42 from DMEM-F12-D8900). In 60 of them (36%), we observed a metaphase II and a polar body or one pronucleus. In 89 of them (53%), we observed two fully decondensed pronuclei: the chromatin was diffuse, some nucleoli were observed and the filamentous chromatin filled the whole nuclear envelope (Fig. 3*a*, *c*–*e*). Very few 2PN oocytes showed polar bodies. Moreover, pronuclear apposition was the dominant feature of these 89 oocytes (Fig. 3*a*, *c*–*e*). In 19 of the oocytes/embryos (11%), we observed several nuclei (from three to 16) but the quality of these embryonic structures was poor as the cleavage was abnormal: the number of cells and nuclei were different and some cells had no or several nuclei (Fig. 3*b*, *f*). The percentage of 2PN oocytes and abnormally cleaved embryos with respect to the number of non-degenerated oocytes for each culture medium is presented in Fig. 4.

After culture 48 h post IVF in SOF medium (Figs 3*a* and 4*a*), the percentage of 2PN oocytes and abnormal embryos from slaughtered mares was not statistically different between the two maturation media (73% for MM and 67% for pFF; $P > 0.05$). One embryo (from pFF) contained eight nuclei but no cleavage of the cytoplasm was observed. The percentage of 2PN oocytes and abnormal embryos from OPU was not statistically different between the two maturation

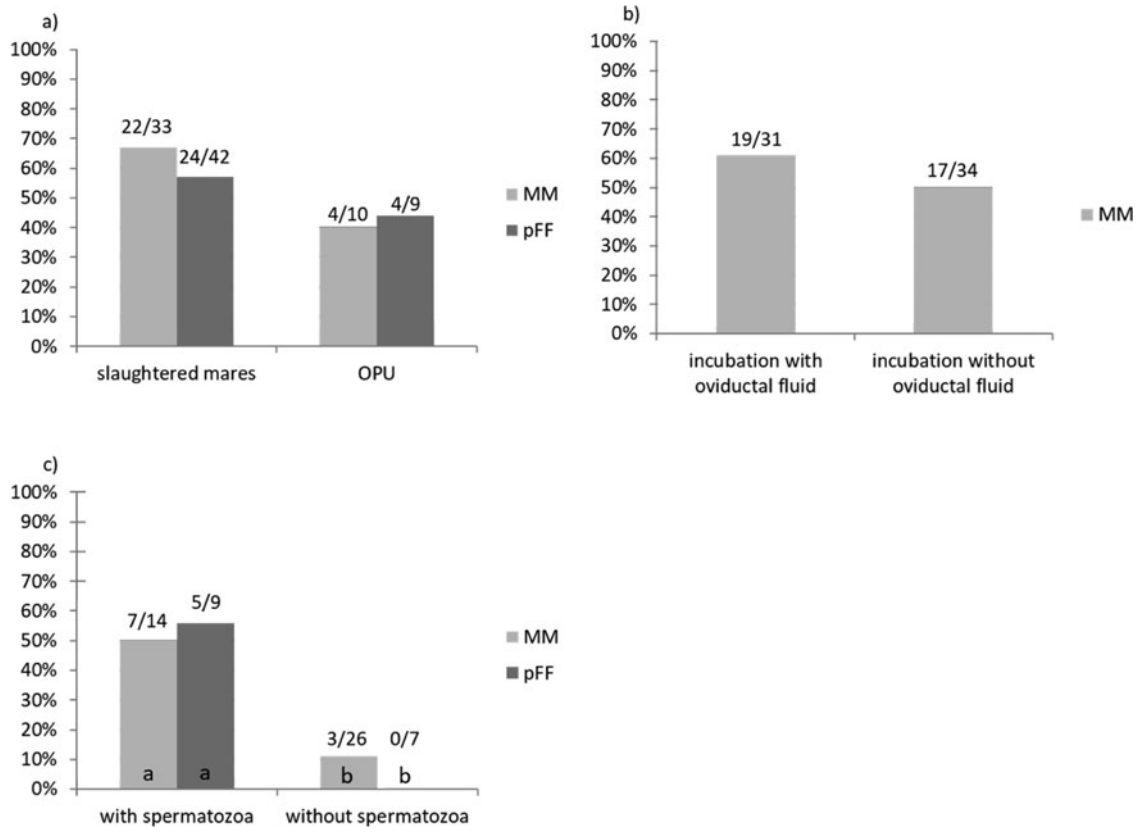

Figure 2 (*a*) Percentage of oocytes containing two pronuclei with respect to the number of non-degenerated oocytes after collection from slaughtered mares or by ovum pick up (OPU), *in vitro* maturation in maturation medium (MM) or preovulatory follicular fluid (pFF), incubation in oviductal fluid and *in vitro* fertilization. The percentages were not statistically different between the two maturation media ($P > 0.05$). (*b*) Percentage of oocytes containing two pronuclei with respect to the number of non-degenerated oocytes for the oocytes collected from slaughtered mares and incubated with oviductal fluid or not. The percentages were not statistically different between the two conditions ($P > 0.05$). (*c*) Percentage of oocytes containing two pronuclei with respect to the number of non-degenerated oocytes for the oocytes collected from slaughtered mares and incubated with or without spermatozoa (parthenogenetic controls). The percentages were statistically different between the two conditions: with and without spermatozoa, within a maturation medium. $^{a,b}P < 0.05$).

media (60% for MM and 75% for pFF, $P > 0.05$). Six embryos (three from MM and three from pFF) contained from five to 16 nuclei, but the cleavage stopped at two to three cells (Fig. 3*b*). When oocytes collected in a slaughterhouse and by OPU were pooled, the percentages of 2PN oocytes and embryos from MM (69%) and pFF (70%) were not statistically different ($P > 0.05$).

After culture in DMEM-F12–51445C medium (Figs 3*c* and 4*b*), the percentage of 2PN oocytes and abnormal embryos from slaughtered mares was not statistically different between the two maturation media (50% for MM and 71% for pFF, $P > 0.05$). Three embryos (one from MM and two from pFF) contained from seven to 16 nuclei, however they did not cleave. The percentage of 2PN oocytes and abnormal embryos from OPU was not statistically different between the two maturation media (50% for MM and 71% for pFF, $P > 0.05$). No cleavage was observed. When oocytes from slaughterhouse and OPU were pooled, the percentages of 2PN oocytes and abnormal embryos from MM (50%) and pFF (71%) were not statistically different ($P > 0.05$).

After culture in DMEM-F12-D8437 medium (Figs 3*d* and 4*c*), the percentage of 2PN oocytes and abnormal embryos from slaughtered mares was not statistically different between the two maturation media (75% for MM and 71% for pFF, $P > 0.05$). One embryo (from MM) contained 12 PN, but no cleavage of the cytoplasm was observed. The percentage of 2PN oocytes and abnormal embryos from OPU was not statistically different between the two maturation media (80% for MM and 75% for pFF, $P > 0.05$, Fig. 4c). No cleavage was observed. When oocytes collected in a slaughterhouse and by OPU were pooled, the percentages of 2PN oocytes and abnormal embryos from MM (76%) and pFF (71%) were not statistically different ($P > 0.05$).

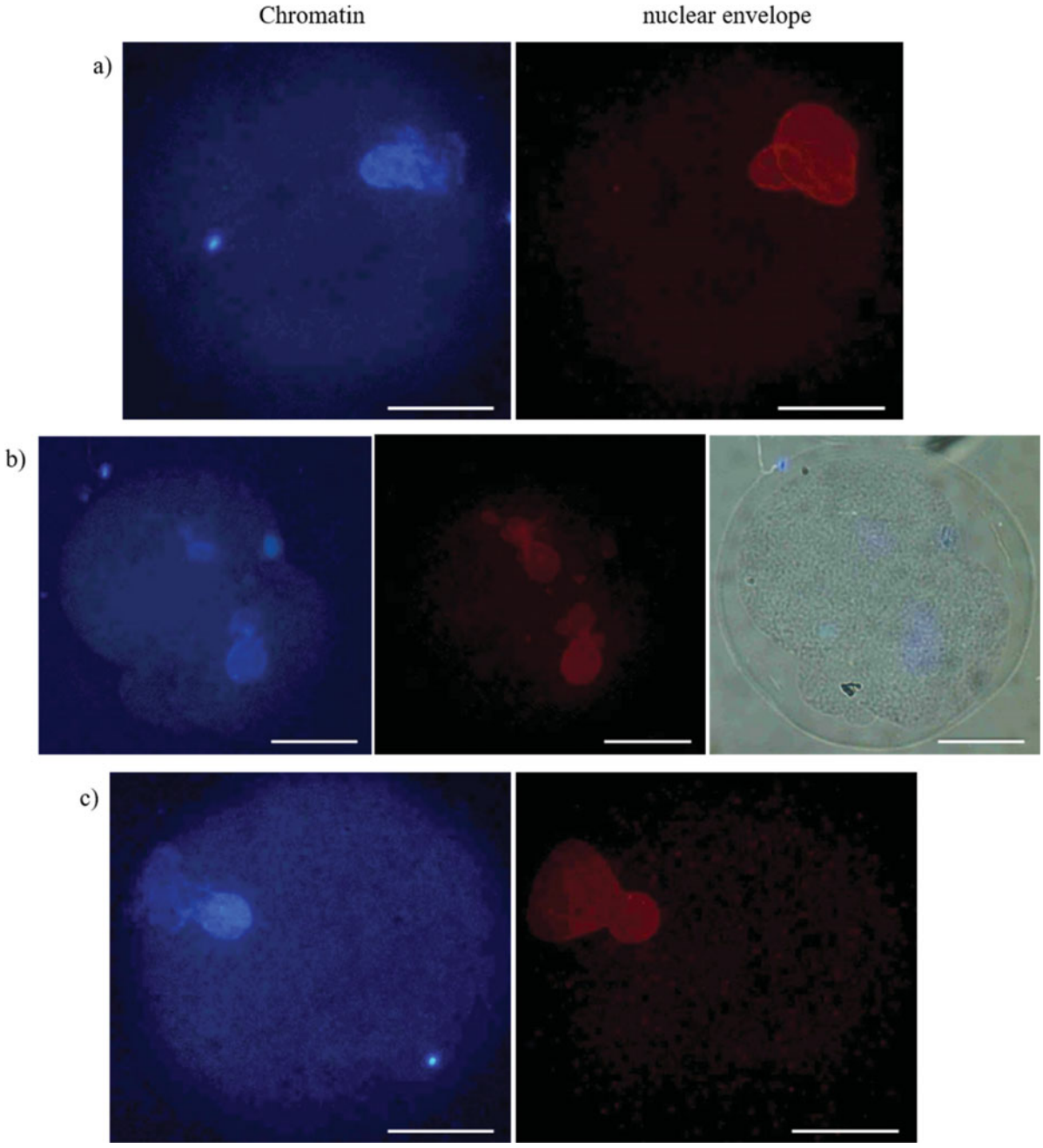

Figure 3 Nuclear status assessed by staining of chromatin with Hoechst and nuclear envelope by lamin A/C antibody. (*a*) An oocyte with two pronuclei fully decondensed after *in vitro* culture in SOF medium. (*b*) An abnormally cleaved embryo with two cells and four nuclei after *in vitro* culture in SOF medium. (*c*) An oocyte with two pronuclei fully decondensed after *in vitro* culture in DMEM-F12–51445C. (*d*) An oocyte with two pronuclei fully decondensed after *in vitro* culture in DMEM-F12-D8437. (*e*) An oocyte with two pronuclei fully decondensed after *in vitro* culture in DMEM-F12-D8900. (*f*) An abnormally cleaved embryo with six cells and five nuclei after *in vitro* culture in DMEM-F12-D8900. Scale bar represent 60 µm.

After culture in DMEM-F12-D8900 medium (Figs 3*e* and 4*d*), the percentage of 2PN oocytes and abnormal embryos from slaughtered mares was not statistically different between the two maturation media (40% for MM and 57% for pFF, $P > 0.05$). Six embryos (two from MM and four from pFF) contained from 3 to 10 PN but the cleavage stopped at three to six cells (Fig. 3f). The percentage of 2PN oocytes and abnormal embryos from OPU was not statistically different between the two maturation media (56% for MM and 67% for pFF, $P > 0.05$). Two embryos (from MM) contained three and nine nuclei, but the cleavage stopped at three to four cells with fragmentations. When oocytes from slaughterhouse and OPU were pooled, the percentages

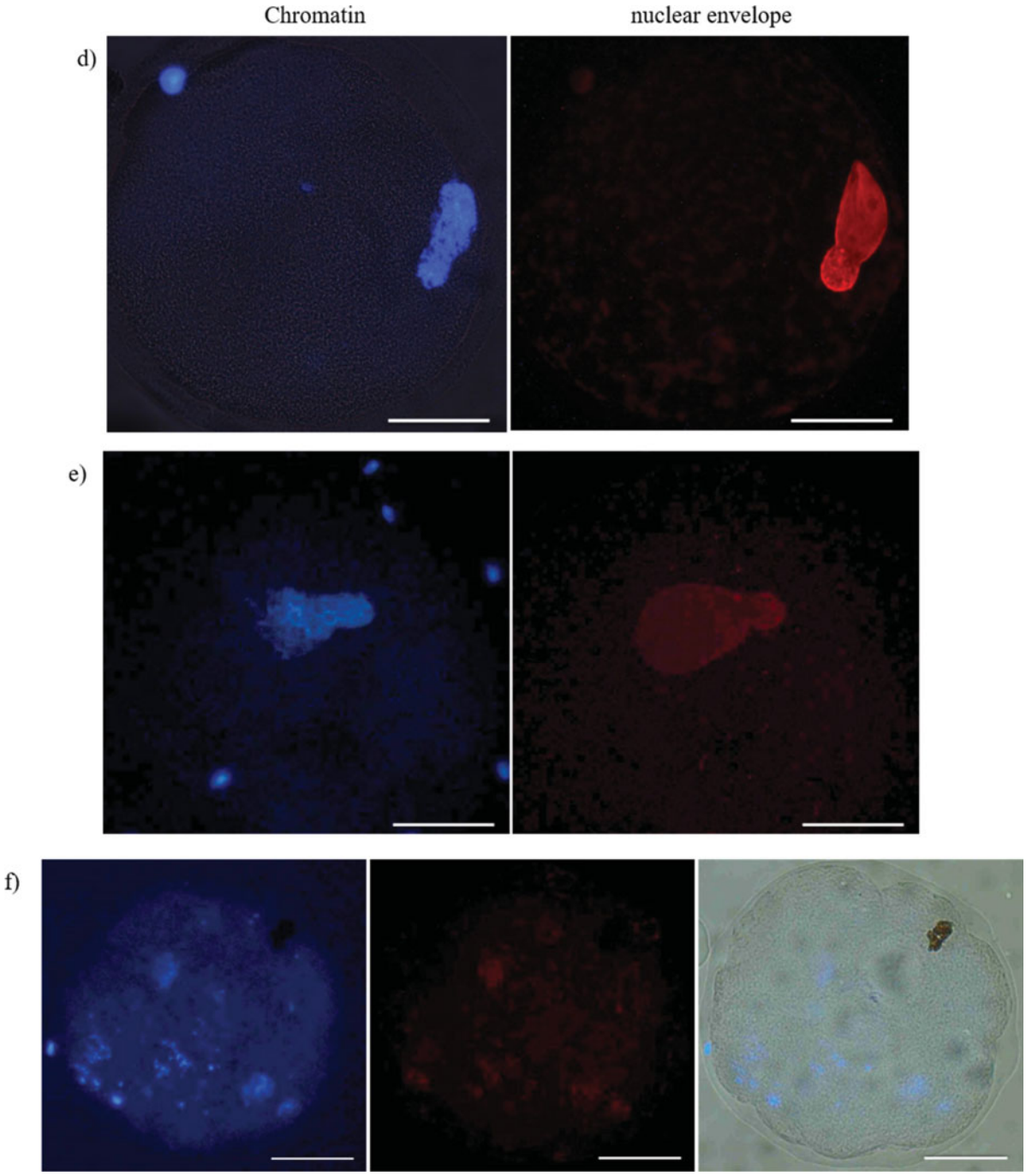

Figure 3 Continued.

of 2PN oocytes and abnormal embryos from MM (47%) and pFF (61%) were not statistically different ($P > 0.05$).

Finally, when data from the four embryo culture media were pooled, the percentages of 2PN oocytes and abnormal embryos from MM (48/80, 60%) and pFF (60/88, 68%) were not statistically different ($P > 0.05$). Moreover, the percentages of abnormally cleaved embryos from MM (9/48, 19%) and pFF (10/60, 17%) were not statistically different ($P > 0.05$). As the four embryo culture media were not tested at the same time, no statistical comparison of these media was performed.

In total, 121 oocytes/embryos were analysed 72 h post IVF (40 from SOF, 26 from DMEM-F12–51445C, 37 from DMEM-F12-D8437 and 18 from DMEM-F12-D8900). None of them went further in its development during these additional 24 h of culture, and most of

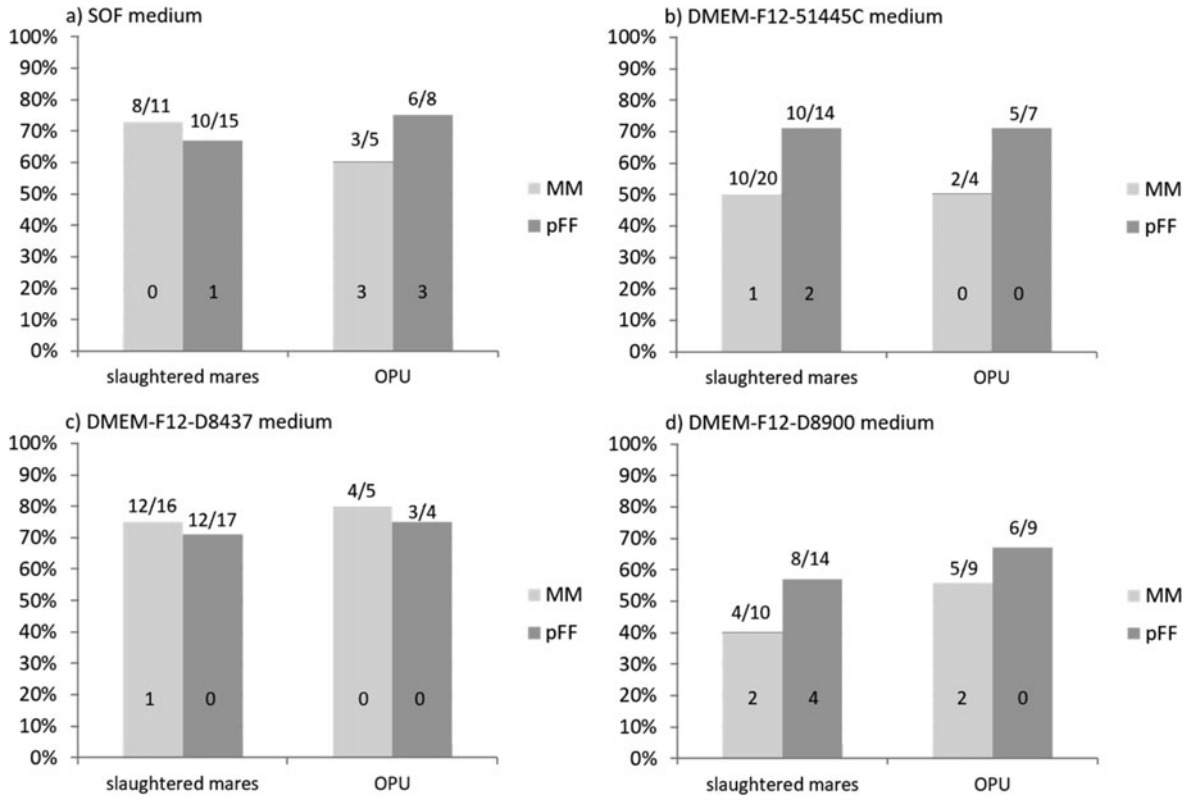

Figure 4 Percentage of 2PN oocytes and abnormally cleaved embryos after collection from slaughtered mares or by ovum pick up (OPU), *in vitro* maturation in maturation medium (MM) or preovulatory follicular fluid (pFF), incubation in oviductal fluid, *in vitro* fertilization and *in vitro* culture 48 h post IVF in (*a*) SOF medium; (*b*) DMEM-F12–51445C medium; (*c*) DMEM-F12-D8437 medium; and (*d*) DMEM-F12-D8900 medium. The fractions at the top represent the number of 2PN oocytes and embryos out of the number of non-degenerated oocytes. The numbers at the bottom represent the number of abnormally cleaved embryos. The percentages were not statistically different between MM and pFF ($P > 0.05$).

them degenerated (88% from SOF, 88% from DMEM-F12–51445C, 41% from DMEM-F12-D8437 and 100% from DMEM-F12-D8900).

Fertilization and development rates after IVM, ICSI and *in vitro* culture

Influence of IVM medium on fertilization and development rates 72 h after ICSI

After oocytes collection from slaughtered mares, IVM in MM or pFF, ICSI and *in vitro* culture for 72 h, nuclear status was assessed. In total, 117 oocytes/embryos were analysed. Among these, 58 (50%) were matured but not fertilized and 59 (50%) were fertilized. Twenty-five (21%) oocytes remained uncleaved after ICSI and 72 h *in vitro* culture. They showed two polar bodies (PB) and two pronuclei (PN) and were called zygotes.

In samples cultured in DMEM-F12–51445C, the total fertilization rate (including zygotes and cleaved embryos) did not differ significantly between the two IVM media (18/36, 50% versus 18/38, 47%, for MM and pFF, respectively; $P > 0.05$, Fig. 5*a*). In samples cultured in DMEM-F12-D8900, the total fertilization rate was higher in oocytes matured in pFF than in MM (15/22, 68% versus 8/21, 38%; $P < 0.05$, Fig. 5*b*). Independent of culture conditions, all zygotes either derived from *in vitro* culture in DMEM-F12–51445C (Fig. 6*a*, *b*) or in DMEM-F12-D8900 (data not shown) showed decondensed pronuclei, their chromatin was diffuse, some nucleoli were observed and the filamentous chromatin filled the whole nuclear area. Pronuclear apposition was found in 37% (7/19) of samples derived from *in vitro* culture in DMEM-F12–51445C (2/9 after IVM in MM and 5/10 after IVM in pFF) whereas it was never found in zygotes derived from *in vitro* culture in DMEM-F12-D8900.

The total cleavage rates with respect to the number of metaphase II (MII) injected oocytes for each IVM and culture medium are presented in Fig. 5. In both embryo culture media, the cleavage rates did not differ between maturation conditions: in DMEM-F12–51445C, the total cleavage rates were 9/36 (25%) versus 8/38 (21%) for MM and pFF respectively ($P > 0.05$); in DMEM-F12-D8900, the total cleavage rates were 7/21 (33%) versus 10/22 (45%) for MM and pFF respectively ($P > 0.05$).

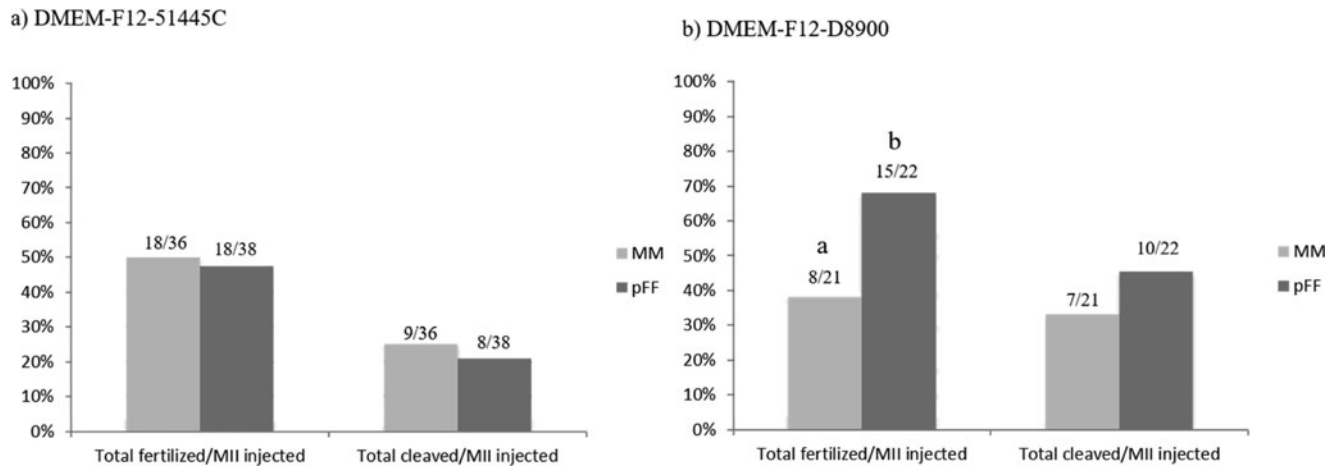

Figure 5 Percentage of fertilized oocytes (with respect to the number of mature oocytes) and cleaved embryos (with respect to the number of mature oocytes) after collection from slaughtered mares, *in vitro* maturation in maturation medium (MM) or preovulatory follicular fluid (pFF), *in vitro* fertilization by intracytoplasmic sperm injection (ICSI) and *in vitro* culture 72 h post-ICSI in (*a*) DMEM-F12–51445C medium; or (*b*) DMEM-F12-D8900 medium. The fractions at the top represent the number of fertilized oocytes out of the number of mature oocytes and the number of cleaved embryos out of the number of mature oocytes. Chi-squared test between maturation media: $^{a,b}P < 0.05$.

Representative micrographs of equine early embryos obtained after IVM in MM or in pFF, ICSI and 72 h *in vitro* embryo culture are presented in Fig. 6. Independent of culture conditions, most of the embryos, either derived from *in vitro* culture in DMEM-F12–51445C (Fig. 6e–h) or in DMEM-F12-D8900, showed nuclei of regular morphology.

The percentages of zygotes and embryos at different developmental stages observed after IVM in MM or pFF, IVF by ICSI and *in vitro* culture in DMEM-F12–51445C or in DMEM-F12-D8900 are reported in Table 1. Within each developmental stage (2PB2PN, 2-cell, 4-cell and 8/16-cell stage) no significant differences were found between the two IVM media. As the two embryo culture media were not tested at the same time, no statistical comparison of these two embryo culture media was performed.

By pooling data of embryo culture media (DMEM-F12–51445C + D8900 in Table 1), no significant differences were found between oocytes matured in MM or pFF.

Influence of post-maturation with OF on fertilization and development rates 10 days after ICSI

After oocytes collection from slaughtered mares, IVM in MM, post-maturation or not with OF, ICSI and *in vitro* culture, cleavage rates were assessed at day 3 and nuclear status was assessed at day 10 post-ICSI. Data are presented in Table 2. The percentages of cleaved embryos at day 3 were not significantly different between oocytes incubated with OF (62%, 29 cleaved embryos/47 injected oocytes) or not (65%, 28/43) ($P > 0.05$). The percentages of morula/MII injected oocytes at day 10 were not significantly different for oocytes incubated with OF (4%, 2/47) compared with oocytes incubated without (0%, 0/43) ($P > 0.05$). The percentages of blastocyst/MII injected oocytes at day 10 were not significantly different between oocytes incubated with OF (6%, 3/47) or without (7%, 3/43) ($P > 0.05$). Representative micrographs of equine blastocysts obtained after IVM in MM, incubation or not in OF, ICSI and 10 days *in vitro* embryo culture in DMEM-F12-D8900 are presented in Fig. 7. They were observed either in culture (Fig. 7*a*, *b*) or after fixation and Hoechst staining (Fig. 7*c*, *d*). In this experiment, all blastocysts reached a good expansion level with a consistent number of nuclei of regular morphology.

Discussion

The aim of our work was to analyse the effect of IVM in follicular fluid and pre-incubation in OF on *IVM*, fertilization using conventional IVF or ICSI, and development of equine oocytes. We have been faced with the difficulty with equine oocyte collection. As the number of slaughtered mares is very low and the collection rate of equine oocytes is low compared with bovine oocytes for example (Hawley *et al.*, 1995), the number of oocytes from slaughterhouses available for our experiments was low. Moreover, the collection of equine oocytes using OPU is expensive and time-consuming, thus a maximum of 4 to 5 OPU sessions per morning were performed, and the number of oocytes collected by OPU was low. The authors are aware of the small sample size in their experiments

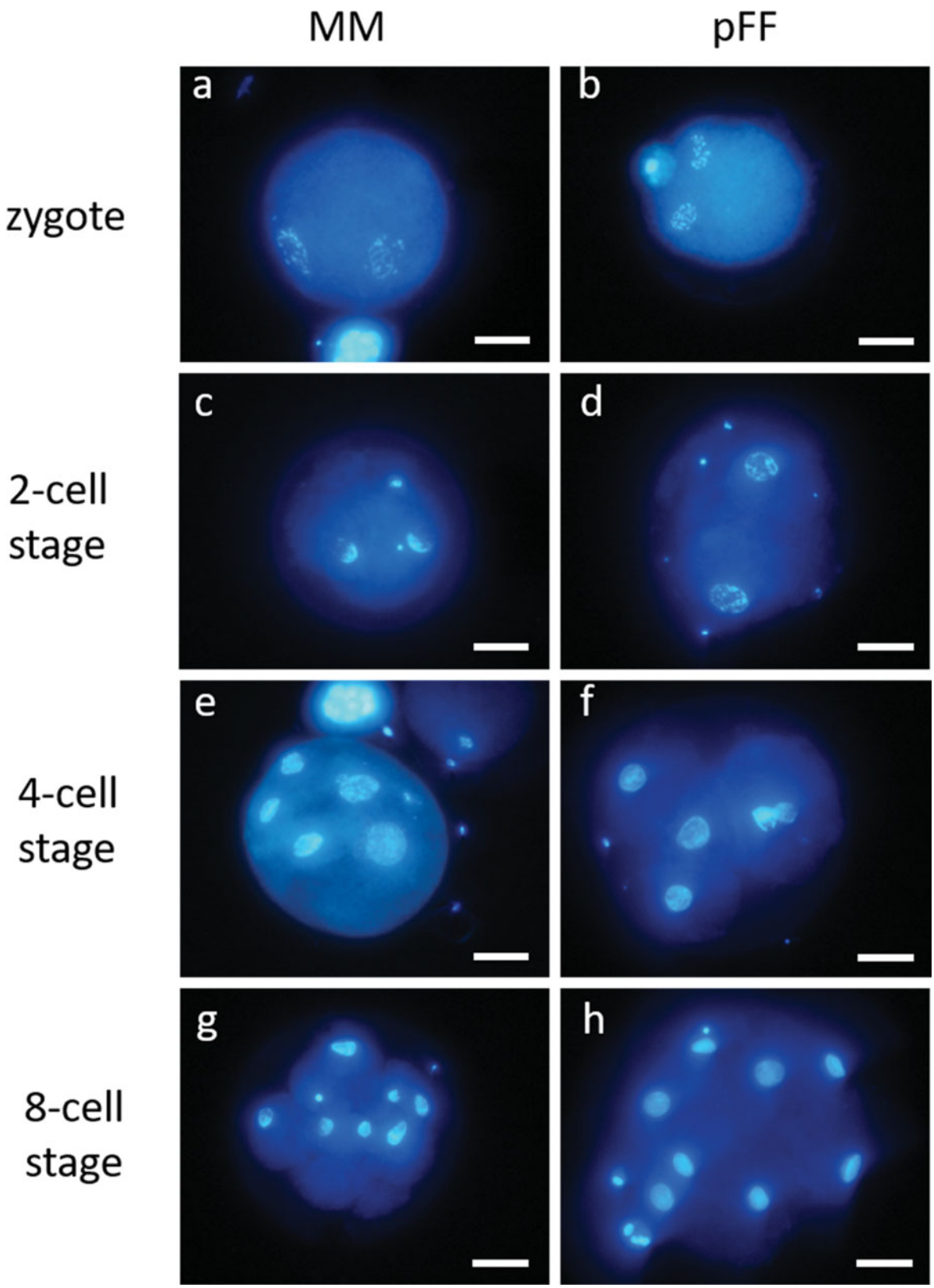

Figure 6 Representative photomicrographs of equine early embryos obtained after IVM in maturation medium (MM) or preovulatory follicular fluid (pFF), fertilized by ICSI and *in vitro* cultured in DMEM-F12–51445C for 72 h. Nuclear status assessed by chromatin staining with Hoechst and examined under ultraviolet (UV) light. (*a*, *b*) Zygotes showing two decondensed pronuclei. (*c*, *d*) Two-cell stage embryo. (*e*, *f*) Four-cell stage embryo. (*g*, *h*) Eight-cell stage embryo. Scale bar represent 60 μm.

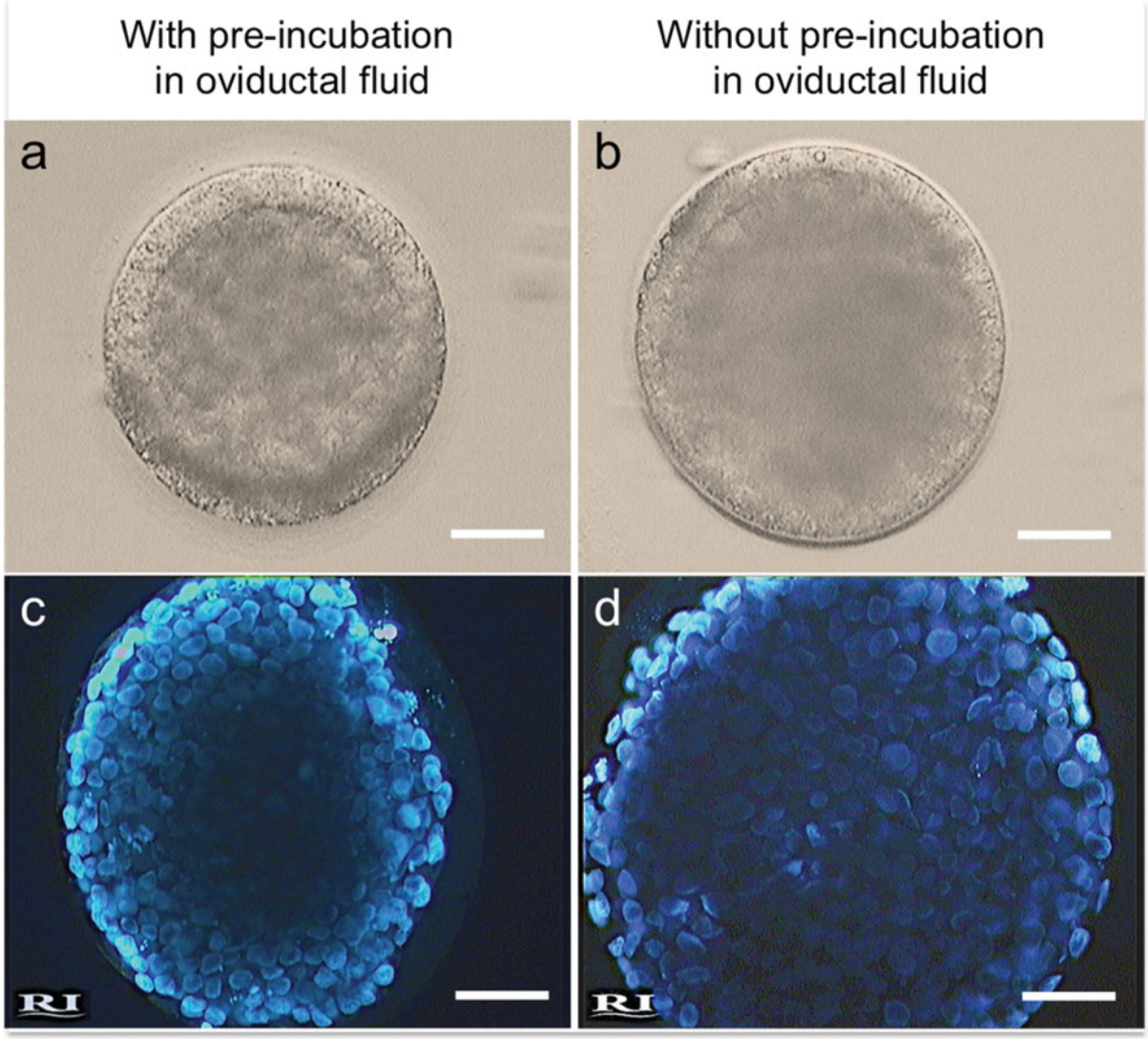

Figure 7 Representative photomicrographs of equine blastocysts obtained after IVM in maturation medium (MM), incubation or not in oviductal fluid and *in vitro* culture in DMEM-F12-D8900 for 10 days and observed under phase contrast (*a*, *b*) or UV light (*c*, *d*) after Hoechst staining. Two expanded blastocysts with the outer layer of trophoblastic cells and a high number of nuclei are shown. Scale bar represent 60 μm.

due to the difficulty in collecting equine oocytes, but the statistical tests have been adapted to small sample size and our conclusions are relevant.

Our first objective was to compare IVM in MM or preovulatory follicular fluid. Our hypothesis was that the low IVF and development rates observed in the equine may be due, at least partly, to a poor cytoplasmic maturation related to unsuited IVM conditions. Preovulatory follicular fluid, in which maturation naturally occurs, may sustain a better cytoplasmic maturation and thus a better competence for IVF and development. In our study, the nuclear maturation rates in MM and pFF were not statistically different, neither for the oocytes collected in France (76% of the oocytes are in metaphase II in MM versus 67% in pFF) nor for the oocytes collected in Italy (53% in MM versus 54% in pFF). Thus, the semi-synthetic MM provides nuclear maturation rates similar to follicular fluid. In previous studies, the maturation rates of equine oocytes were similar: 68% in pFF (Caillaud *et al.*, 2008), 54–58% in pFF (Conforti *et al.*, 2005), 61% in MM (Ambruosi *et al.*, 2013), 71% in MM (Deleuze *et al.*, 2009). In our study, the fertilization rates after ICSI were not different between maturation in MM (46%) vs pFF (55%). This result shows that the semi-synthetic MM supports cytoplasmic maturation to a similar extent to preovulatory follicular fluid. Moreover, the percentage of oocytes containing two pronuclei after IVF was not statistically different between maturation in MM

Table 1 Percentage of equine zygotes (oocytes showing two polar bodies and two pronuclei, 2PB2PN) and embryos at different developmental stages after oocyte collection from slaughtered mares, IVM in maturation medium (MM) or preovulatory follicular fluid (pFF), intracytoplasmic sperm injection (ICSI) and *in vitro* culture in DMEM-F12–51445C medium or DMEM-F12-D8900 medium for 72 h post-ICSI

IVM medium	Embryo culture medium	N° of cultured oocytes	N° (%) of MII and injected oocytes	N° (%) of total fertilized oocytes[a]	N° (%) of zygotes and cleaved embryos found 72 h post-ICSI[b]				
					2PB2PN	2-cell stage	4-cell stage	8/16-cell stage	Total cleaved
MM	DMEM-F12–51445C	69	36 (52)	18 (50)	9 (25)	1 (3)	6 (17)	2 (5)	9 (25)
pFF		71	38 (53)	18 (47)	10 (26)	3 (8)	4 (10)	1 (3)	8 (21)
MM + pFF	**DMEM-F12–51445C**	**140**	**74 (53)**	**36 (49)**	**19 (26)**	**4 (5)**	**10 (14)**	**3 (4)**	**17 (23)**
MM	DMEM-F12 D8900	39	21 (54)	8 (38)*	1 (5)	3 (14)	3 (14)	1 (5)	7 (33)
pFF		40	22 (55)	15 (68)*	5 (23)	5 (23)	2 (9)	3 (14)	10 (46)
MM + pFF	**DMEM-F12-D8900**	**79**	**43 (54)**	**23 (53)**	**6 (14)**	**8 (19)**	**5 (12)**	**4 (9)**	**17 (40)**
MM	Total DMEM-F12–51445C + D8900	108	57 (53)	26 (46)	10 (17)	4 (7)	9 (16)	3 (5)	16 (28)
pFF		111	60 (54)	33 (55)	15 (25)	8 (13)	6 (10)	4 (7)	18 (30)

[a]Numbers and percentages of fertilized oocytes out of the number of MII injected oocytes.
[b]Numbers and percentages of zygotes and cleaved embryos out of the number of MII injected oocytes.
Chi-squared test between media, for fertilization and cleavage rates: $*P < 0.05$.

Table 2 Percentage of equine embryos at morula or blastocyst stages after oocyte collection from slaughtered mares, IVM in maturation medium (MM), incubation or not with OF, intracytoplasmic sperm injection (ICSI) and *in vitro* culture for 10 days post-ICSI in DMEM-F12-D8900

IVM medium	Pre-incubation with oviductal fluid	No. of cultured oocytes	No. (%) of MII and injected oocytes	No. (%) of morula (day 10)[a]	No. (%) of blastocyst (day 10)[a]	No. (%) of morula + blastocyst (day 10)[a]
MM	Pre-incubation	103	47 (46)	2 (4)	3 (6)	5 (11)
MM	No pre-incubation	109	43 (39)	0 (0)	3 (7)	3 (7)
Total	Total	212	90 (42)	2 (2)	6 (7)	8 (9)

[a]Numbers and percentages of embryos out of the number of MII injected oocytes.

versus pFF, neither for the oocytes collected in a slaughterhouse (67% in MM versus 57% in pFF) nor for the oocytes collected by OPU (40% in MM versus 44% in pFF). As very few oocytes containing two pronuclei extruded PB, the significance of this nuclear stage may be questionable. One could hypothesize that either the PB were extruded and degenerated quickly or the expulsion of the PB did not occur as a consequence of an abnormal fertilization. On the one hand, using equine zygotes flushed from oviducts at different time intervals from ovulation and *in vitro* fertilized oocytes, Bézard and collaborators showed that PB degeneration and undetectability may occur quickly after fertilization both *in vitro* and in vivo (Bézard *et al*., 1989). On the other hand, Leemans and collaborators showed that the absence of the second polar body extrusion is due to oocyte cytokinesis induced by procaine instead of sperm penetration of equine oocytes (Leemans *et al*., 2015). In our study, the presence of some equine oocytes containing two pronuclei in the parthenogenetic control group incubated without spermatozoa supports this second hypothesis. Previous studies have shown that spontaneous parthenogenetic division of unfertilized equine oocytes is rare either *in vitro* or *in vivo* (Zhang *et al*., 1989; Zhang *et al*., 1990). Finally, after culture in embryo culture media, we observed no difference between maturation in MM vs pFF for the percentage of zygotes after ICSI (17% vs. 25% respectively) and the percentage of cleaved embryos after ICSI (28% versus 30%). Thus, our MM and the preovulatory follicular fluid support the acquisition of competence for maturation, fertilization and development to a similar extent. However, in our conditions, IVM occurs in a static medium, whereas in vivo maturation naturally takes place in a dynamic medium in which subtle changes occur during final maturation of the preovulatory follicle. IVM in a culture system in which hormones would be added sequentially to mimic changes observed in vivo might provide better conditions for equine oocyte maturation. For example, porcine oocytes matured in a medium in which hormones were added sequentially exhibited greater developmental competence to blastocyst stage (Kawashima *et al*., 2008).

The influence of oviductal secretions on oocytes has been widely studied in mammals (Aviles *et al*., 2010; Coy *et al*., 2012; Lopera-Vasquez *et al*., 2015). A beneficial effect on equine oocytes has been observed with incubation of equine oocytes with OF collected 6 h after ovulation (Ambruosi *et al*., 2013) or co-culture of equine oocytes with equine or porcine oviduct epithelial cells (Mugnier *et al*., 2009). In our study, incubation of equine oocytes with OF collected before ovulation, at the end of follicular growth, did not influence fertilization or development rates. This result suggests that oviductal factors with a beneficial effect on oocyte may be present in the oviduct during a specific time interval after ovulation. Several oviductal factors, which influence fertilization rates, have been studied in the equine oviduct: Deleted in malignant brain tumour one is secreted in the oviduct from early follicular phase to post-ovulatory stage (Ambruosi *et al*., 2013) and osteopontin and atrial natriuretic peptide A are present in the equine oviduct from emergence of the dominant follicle to preovulatory stage (Mugnier *et al*., 2009). Further studies are in progress to clarify the role and secretion pattern of oviductal factors with a beneficial effect on equine oocytes.

Several culture media have been used for *in vitro* development of equine fertilized oocytes. SOF medium has been used previously for *in vitro* culture of equine zygotes after ICSI with a cleavage rate of 61% (Tremoleda *et al*., 2003), 69% (Galli *et al*., 2002) and 64% (Lazzari *et al*., 2002) 48 h post-ICSI. In our conditions, 26% of the oocytes after IVF were abnormally cleaved and 74% contained two fully decondensed and apposed pronuclei. The pronuclei decondensation and apposition evidence the first step of embryo development. However, the lack of normal embryo cleavage throws doubt on the fertilization technique. The use of procaine during the IVF procedure in order to induce sperm hyperactivation may have a detrimental effect on oocytes and zygotes. Leemans and collaborators have shown that procaine induces oocyte cytokinesis and that cleaved oocytes did not develop beyond 8–16 cells with daughter cells containing aberrant DNA fragments (Leemans *et al*., 2015). DMEM-F12 medium has been used previously for *in vitro* culture of equine zygotes after ICSI with a cleavage rate of 68% (Smits *et al*., 2012a), 69% (Choi *et al*., 2011) and 75% (Choi *et al*., 2006) 3 days post-ICSI. Moreover, DMEM-F12 medium has been used previously for *in vitro* culture of equine zygotes after IVF with a cleavage rate of 66% (McPartlin *et al*., 2009). In our conditions, three references of DMEM-F12 were tested, with identical composition but different forms (liquid or powder, sodium bicarbonate included or added subsequently). Following ICSI and 72 h culture in DMEM-F12, the percentages of zygotes (26% in DMEM-F12–51445C and 14% in DMEM-F12-D8900) and the percentages of cleaved embryos (23% in DMEM-F12–51445C and 40% in DMEM-F12-D8900) were consistent, and the morphology of the ICSI embryos was normal. Following ICSI and 10 days culture in DMEM-F12-D8900, the blastocysts formation rate was correct and repeatable and blastocysts quality was good, as blastocyst expansion and hatching throughout the zona pellucida were observed. Following IVF and culture in DMEM-F12, no normal embryo cleavage and

development were observed. As the IVM and embryo culture media were the same in the IVF and ICSI experiments, it can be speculated that the IVM and embryo culture conditions are appropriate and that the IVF conditions have to be improved to increase the quality of the embryos and their developmental competence.

Thus, in our study, exposure to physiological fluids for oocyte maturation and post-maturation does not improve *in vitro* embryo production in the horse. *In vitro* culture of ICSI fertilized oocytes provides morphologically normal embryos. However, when procaine is used during IVF, no embryo able to develop properly is observed. Improving the IVF technique will improve the developmental competence of IVF embryos and bring them to a state in which they will be transferred into surrogate mares.

Author's contributions

MED and GG conceived of the study and participated in its design. CD, OP, FR, SD and GG performed the experiments and analysis in France, NAM, MN, GML and MED performed the experiments and analysis in Italy. MED and GG wrote the manuscript and all authors read and approved the final manuscript.

Acknowledgements

We would like to thank the staff of the experimental stud at the 'Unité Expérimentale de Physiologie Animale de l'Orfrasière (UEPAO)' for providing equine oocytes and sperm. We also thank Albert Arnould, Jean-Noël Couet, Thierry Delpuech, Pascal Papillier and Gaël Ramé at the 'Unité Mixte de Recherche de Physiologie de la Reproduction et des Comportements (UMR PRC)' and the staff of the slaughterhouses for technical help. This work has benefited from the facilities and expertise of the 'Plateforme d'Imagerie Cellulaire' (PIC) of the UMR 85 'Physiologie de la Reproduction et des Comportements'.

Financial support

This work was supported by the 'Institut Français du Cheval et de l'Equitation' (IFCE). NAM was supported by the Italian Ministry of Health, Project GR-2011–02351396.

Conflict of interest

The authors declare no conflict of interest.

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