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# 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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## 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 (Bézar *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'Éthique 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.

## 152 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 as-

pirated 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<sup>®</sup>, 0.25 ml/animal i.v., 10 mg/ml detomidine, Centravet, Plancoet, France) and butorphanol (Dolorex<sup>®</sup>, 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<sup>®</sup>, 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, Beaucauze, 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<sub>3</sub> 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<sub>2</sub> 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.

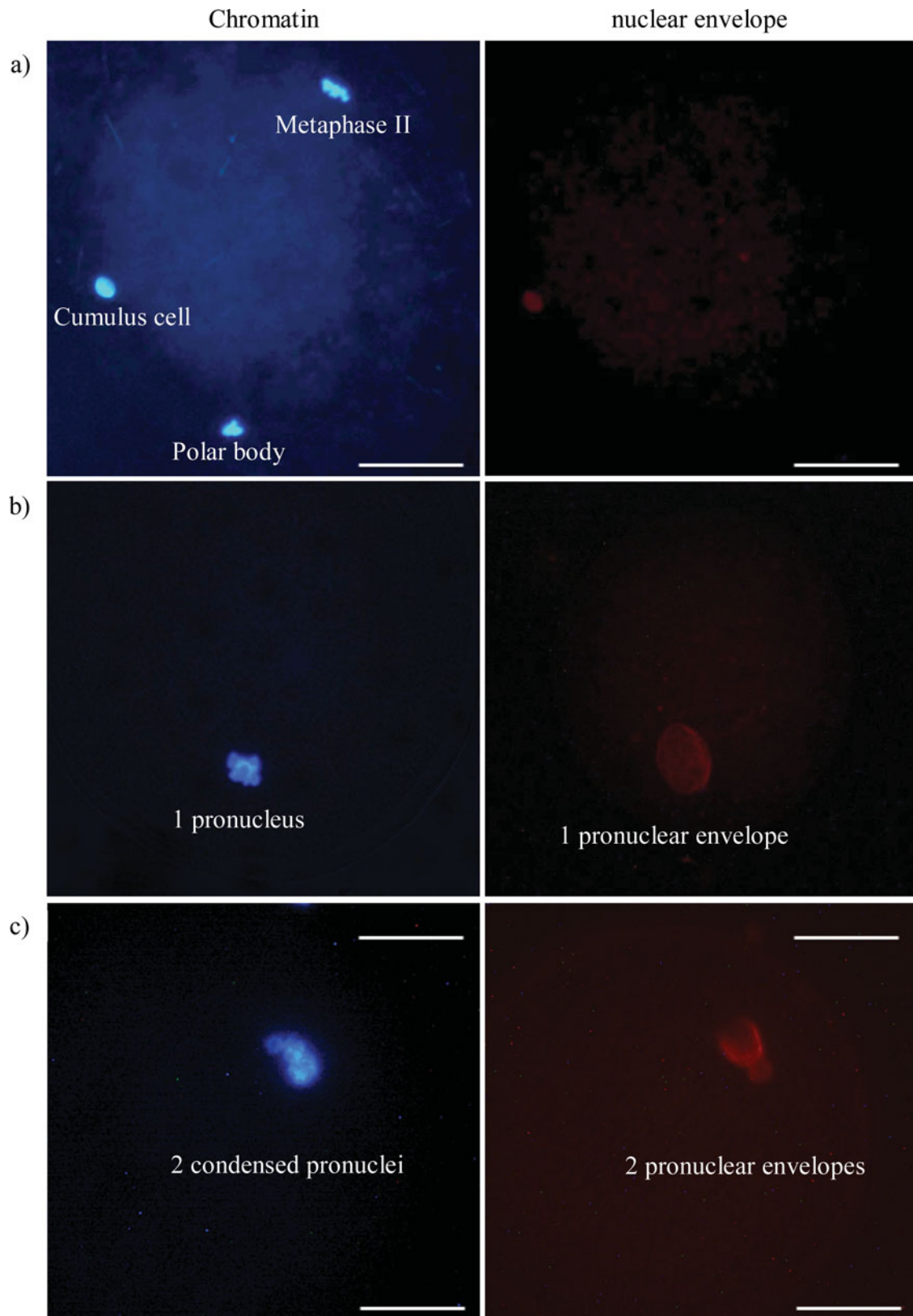
## Post-maturation of equine oocytes in OF

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<sub>2</sub> 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.

251	Genital tracts with both ovaries containing several	Equine oocytes were washed in capacitating MW	304
252	follicles larger than 5 mm were used. The oviducts	and groups of 10 were transferred to droplets of	305
253	were dissected free from surrounding tissues. The	100 µl of spermatozoa suspension and co-incubated for	306
254	oviductal content from the ampulla was expelled by	18 h in an atmosphere of 5% CO <sub>2</sub> in air at 38.5°C in	307
255	gentle squeezing using a sterile microscope slide and	100% humidity.	308
256	collected by introducing the tip of a pipette into		
257	the ampulla and aspirating while making a manual	<i>Control of parthenogenetic activation</i>	309
258	ascendant pressure from the isthmus to the ampulla	Equine oocytes were washed in capacitating MW	310
259	(Carrasco <i>et al.</i> , 2008). After centrifugation at 10,000 g	and transferred to droplets of 100 µl of capacitating	311
260	for 15 min, the supernatant containing secreted and	MW supplemented with 5 mM procaine without	312
261	intracellular components was immediately stored at	spermatozoa and incubated for 18 h in an atmosphere	313
262	−20°C until use as 'OF'.	of 5% CO <sub>2</sub> in air at 38.5°C in 100% humidity.	314
263	<b><i>In vitro</i> fertilization (IVF) procedure</b>		
264	The IVF procedure was performed in France, using	<i>In vitro culture of equine zygotes</i>	315
265	oocytes collected from slaughtered mares or by OPU.	After 18 h co-incubation with spermatozoa, equine zy-	316
		gotes were washed in the culture medium and flushed	317
266	<i>Preparation of equine sperm and IVF</i>	to remove attached spermatozoa. Groups of 10 were	318
267	Fresh equine semen was collected with a closed	transferred to droplets of 30 µl of culture medium for	319
268	artificial vagina from three adult Welsh pony stallions	30 h or 54 h (48 h or 72 h post IVF) in an atmosphere of	320
269	of proven fertility from our experimental stud. Semen	5% CO <sub>2</sub> , 5% O <sub>2</sub> and 90% N <sub>2</sub> at 38.5°C in 100% humidity.	321
270	was filtered through gauze, sperm motility was	Four culture media were tested. Synthetic OF	322
271	visually evaluated under light microscopy on a heated	(SOF) medium was SOF (Minitub) supplemented	323
272	stage and sperm concentration was assessed using a	with 2% Basal Medium Eagle amino acids solution,	324
273	spectrophotometer (Ciba-Geigy). Then, 1 ml of semen	1% Minimum Essential Medium non-essential amino	325
274	was diluted in 2 ml of pre-warmed modified Whitten's	acids solution, 0.33 mg/ml sodium pyruvate, 6 mg/ml	326
275	Medium (MW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM	BSA fatty acid free, 5% FCS and 25 µg/ml genta-	327
276	MgCl <sub>2</sub> , 22 mM HEPES, 4.8 mM lactic acid hemicalcium	mycin. DMEM-F12-51445C medium was Dulbecco's	328
277	salt, 1 mM pyruvic acid) supplemented with 5.5 mM	Modified Eagle's Medium Ham's Nutrient Mixture	329
278	glucose (anhydrous), pH 7.25 (McPartlin <i>et al.</i> , 2009).	F12 with 3151 mg/l dextrose, 2.5 mM L-glutamine, 15	330
279	Diluted sperm was transported to the laboratory	mM HEPES, 55 mg/l sodium pyruvate (ref. 51445C)	331
280	within a few minutes at 37°C and centrifuged in	supplemented with 10% FCS and 25 µg/ml gentamy-	332
281	15 ml conical tubes at 100 g for 1 min at 37°C	cin. DMEM-F12-D8437 medium was DMEM-F12 with	333
282	to remove particulate matter and dead sperm. The	2.5 mM L-glutamine, 15 mM HEPES, 1.2 g/l sodium	334
283	supernatant was then transferred to a 14 ml round-	bicarbonate (ref D8437) supplemented with 10%	335
284	bottom tube and centrifuged at 600 g for 5 min	FCS and 25 µg/ml gentamycin. DMEM-F12-D8900	336
285	at 37°C. The pellet was re-suspended in 1.5 ml of	medium was DMEM-F12 with 2.5 mM L-glutamine	337
286	pre-warmed MW supplemented with glucose, and	and 15 mM HEPES (ref D8900) supplemented with	338
287	the concentration was determined by counting on a	1.2 g/l NaHCO <sub>3</sub> , 10% FCS and 25 µg/ml gentamycin.	339
288	Thoma chamber under a microscope (Olympus, IMT-	The three references of DMEM-F12 had identical	340
289	2, Paris, France). Spermatozoa were then diluted at	composition but different forms (liquid or powder,	341
290	10 × 10 <sup>6</sup> /ml in pre-warmed MW supplemented with	sodium bicarbonate included or added subsequently).	342
291	5.5 mM glucose, 25 mM NaHCO <sub>3</sub> and 7 mg/ml	<i>Assessment of nuclear status</i>	343
292	BSA, pH 7.25 (capacitating MW) (McPartlin <i>et al.</i> ,	Nuclear status was assessed either after 27 h IVM,	344
293	2009). Spermatozoa were incubated in 500 µl aliquots	after 18 h IVF or after 30 h or 54 h <i>in vitro</i> development.	345
294	in polyvinyl alcohol-coated 5 ml round-bottom tubes	Oocytes and zygotes were washed by aspiration in and	346
295	at 37°C in a humidified atmosphere during 6	out of a pipette in PBS, fixed in 4% paraformaldehyde	347
296	h. The motility was visually evaluated under a	in PBS for 20 min at room temperature, washed in	348
297	microscope (Olympus, IMT-2, Paris, France) at the	PBS and processed for DNA and nuclear membrane	349
298	beginning and at the end of the incubation period.	staining. They were incubated for 30 min at room	350
299	Spermatozoa were then diluted at 1 × 10 <sup>6</sup> /ml in	temperature in 0.2% Triton X-100 in PBS. Non-specific	351
300	capacitating MW supplemented with 5 mM procaine	reactions were blocked by incubation for 1 h at room	352
301	to induce hyperactivated motility. Droplets of 100 µl of	temperature in 10% goat serum in PBS. Oocytes and	353
302	spermatozoa suspension were laid down onto culture	zygotes were incubated overnight at 4°C or 2 h at	354
303	dishes and covered with mineral oil.	room temperature with an anti-lamin A/C antibody	355
		(ThermoScientific) diluted 1:100 in PBS containing	356



357	0.2% BSA and 0.1% Tween. After four washings for	1.2 g/l NaHCO <sub>3</sub> ) plus 10% FCS with 25 µg/ml	409
358	5 min in PBS containing 0.2% BSA and 0.1% Tween,	gentamycin under mineral oil in droplets of 10 µl	410
359	they were incubated for 1 h at room temperature with	and cultured individually for 72 h in a humidified	411
360	an AlexaFluor 594-conjugated-anti-mouse antibody	atmosphere of 5% CO <sub>2</sub> , 5% O <sub>2</sub> and 90% N <sub>2</sub> at 38.5°C.	412
361	(Life Technologies) diluted 1:400 in PBS. They were	At the end of the culture period, early embryos	413
362	then washed five times for 5 min in PBS containing	and uncleaved ova were removed from culture, fixed	414
363	0.1% Tween and two times for 5 min in PBS. They	and evaluated using the procedures as described	415
364	were incubated with 1 µg/ml bis-benzimide (Hoechst	below. In a second embryo culture experiment,	416
365	33342) in PBS for 5 min and mounted on microscope	aimed to test the influence of pre-incubation with	417
366	slides in Mowiol V4-88 (133 mg/ml; Hoechst, Frank-	OF on embryo development, injected oocytes were	418
367	furt, Germany) and <i>n</i> -propyl gallate (5 mg/ml). The	cultured in DMEM-F12 D8900 for up to 10 days.	419
368	slides were kept at 4°C in the dark until observation.	At day 10 of culture, embryos (developed, delayed	420
369	Oocytes were observed under an epifluorescence	and degenerated embryos and uncleaved ova) were	421
370	microscope (Zeiss). Controls were performed using	morphologically evaluated, fixed and stained for	422
371	no primary antibodies to ascertain the absence of	assessing nuclear chromatin, as described below.	423
372	non-specific binding or no secondary antibodies to		
373	ascertain the absence of auto-fluorescence.		
374	<b>Intracytoplasmic sperm injection (ICSI) procedure</b>		
375	The ICSI procedure was performed in Italy, using	<i>Nuclear chromatin evaluation</i>	424
376	oocytes collected from slaughtered mares and matured	Nuclear chromatin configuration of embryos and un-	425
377	<i>in vitro</i> with the procedure described above.	cleaved ova was evaluated under an epifluorescence	426
		microscope (Nikon Eclipse 600 equipped with B-2	427
378	<i>Preparation of equine sperm and ICSI procedure</i>	A, 346 nm excitation/460 nm emission filter) after	428
379	Fresh semen samples from three mature stallions with	staining with Hoechst 33258, as previously described	429
380	a reproductive history of normal fertility were used.	(Dell'Aquila <i>et al.</i> , 2001; Hinrichs <i>et al.</i> , 2005; Lange	430
381	The stallions were located in the reproductive centre	Consiglio <i>et al.</i> , 2009). Normally cleaved embryos	431
382	Pegasus (Veterinary Clinics and Animal Productions	were defined by the presence of nuclei of regular	432
383	Unit – DETO, Polo di Valenzano, University of	morphology within each blastomere. The number of	433
384	Bari Aldo Moro, Valenzano, Bari, Italy) and were	morphologically normal nuclei was counted for each	434
385	routinely used in artificial insemination programs.	embryo. Embryos were classified as morulae when	435
386	Semen was collected with a Missouri artificial vagina	they had more than 32 nuclei. Embryos with more	436
387	with an in-line gel filter, and was extended with	than 64 nuclei and having an outer layer of apparent	437
388	INRA 96 (IMV Technologies, Piacenza, Italy) at a	differentiating trophoblast cells were considered to be	438
389	concentration of 20 to 25 × 10 <sup>6</sup> sperm cells/ml and	blastocysts (Choi <i>et al.</i> , 2006). In the group of uncleaved	439
390	used immediately. Sperm cells for ICSI were prepared	ova, normal fertilization was defined by the presence	440
391	by the swim-up procedure in Earle's balanced salt	of two polar bodies with two pronuclei (PN). Oocytes	441
392	solution supplemented with 0.4% BSA and 50 µg/ml	showing one PN with intact sperm cell were classified	442
393	gentamicin as previously described (Dell'Aquila <i>et al.</i> ,	as activated oocytes. Oocytes showing a metaphase	443
394	2001, 2003; Ambruosi <i>et al.</i> , 2009).	plate and one polar body with an intact sperm	444
395	Intracytoplasmic sperm injection was carried out as	cell were classified as unfertilized; oocytes having	445
396	previously reported (Ambruosi <i>et al.</i> , 2009; Dell'Aquila	degenerated, irregularly clustered or faint chromatin	446
397	<i>et al.</i> , 2001, 2003). All procedures were performed	were classified as degenerating.	447
398	at 38.5°C in Quinn's Advantage Fertilization HTF		
399	Universal medium (Cooper Surgical, Trumbull, CT,	<b>Statistical analysis</b>	448
400	USA) supplemented with 5 mg/ml human serum	The percentages of mature oocytes, fertilized oocytes,	449
401	albumin (HSA).	cleaved embryos, morulae and blastocysts were	450
		compared between groups using chi-square analysis.	451
		Differences were considered statistically significant at	452
		<i>P</i> < 0.05.	453
402	<i>In vitro culture of embryos</i>		
403	In a first embryo culture experiment, aimed to test	<b>Results</b>	454
404	the influence of IVM medium (MM versus pFF)		
405	on fertilization and early embryonic development,	<b>Maturation rates after IVM</b>	455
406	injected oocytes were put immediately after ICSI in	After 27 h IVM in MM or pFF, nuclear status of the	456
407	one of the two embryo culture medium (either DMEM-	oocytes was assessed. Oocytes with metaphase II and	457
408	F12-51445C or DMEM-F12-D8900 supplemented with	one polar body were considered mature (Fig. 1a).	458



**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  $\mu$ m.

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

#### 469 **Fertilization and development rates after IVM, IVF** 470 **and *in vitro* culture**

471 *Influence of IVM medium on fertilization rates after IVF*  
 472 After oocytes collection from slaughterhouse or OPU,  
 473 IVM in MM or pFF, post-maturation with OF and  
 474 gametes co-incubation, nuclear status was assessed.  
 475 We observed oocytes with metaphase II and one polar  
 476 body with no detectable nuclear envelope (Fig. 1a),  
 477 oocytes with one pronucleus (PN) showing DNA  
 478 stained with Hoechst stain and a nuclear envelope  
 479 stained positive with lamin A/C antibody without  
 480 sperm cell (Fig. 1b) and oocytes with two PN in  
 481 the cytoplasm, each PN showing the presence of  
 482 DNA and a nuclear envelope (Fig. 1c). All the 2PN  
 483 oocytes showed condensed PN with a compact mass  
 484 of chromatin within the nuclear envelope and very  
 485 few 2PN oocytes showed one or two polar bodies.  
 486 Degenerated oocytes, having shrunken, dense or  
 487 fragmented cytoplasm, were discarded.

488 For the oocytes collected in a slaughterhouse, three  
 489 repetitions were performed (Fig. 2a). The percentage  
 490 of oocytes containing 2PN 18 h post IVF was not  
 491 statistically different between the two maturation  
 492 conditions (IVM in MM: 22/33, 67%; IVM in pFF:  
 493 24/42, 57%;  $P > 0.05$ ). For the oocytes collected by  
 494 OPU, two repetitions were performed (Fig. 2a). The  
 495 percentage of oocytes containing 2PN 18 h post IVF  
 496 was not statistically different (IVM in MM: 4/10,  
 497 40%; IVM in pFF: 4/9, 44%;  $P > 0.05$ ). When oocytes  
 498 collected in a slaughterhouse and by OPU were  
 499 pooled, the 2PN rates from MM (26/43, 60%) and pFF  
 500 (28/51, 55%) were not statistically different ( $P > 0.05$ ).

#### 501 *Influence of post-maturation with OF on fertilization rates* 502 *after IVF*

503 After oocytes collection from slaughtered mares, IVM  
 504 in MM, pre-incubation or not with OF and gametes  
 505 co-incubation, nuclear status was assessed. Two  
 506 repetitions were performed (Fig. 2b). The percentage  
 507 of oocytes containing 2PN 18 h post IVF was not  
 508 statistically different between post-maturation with  
 509 OF (19/31, 61%) and without OF (17/34, 50%) ( $P >$   
 510 0.05).

#### *Control of parthenogenetic activation*

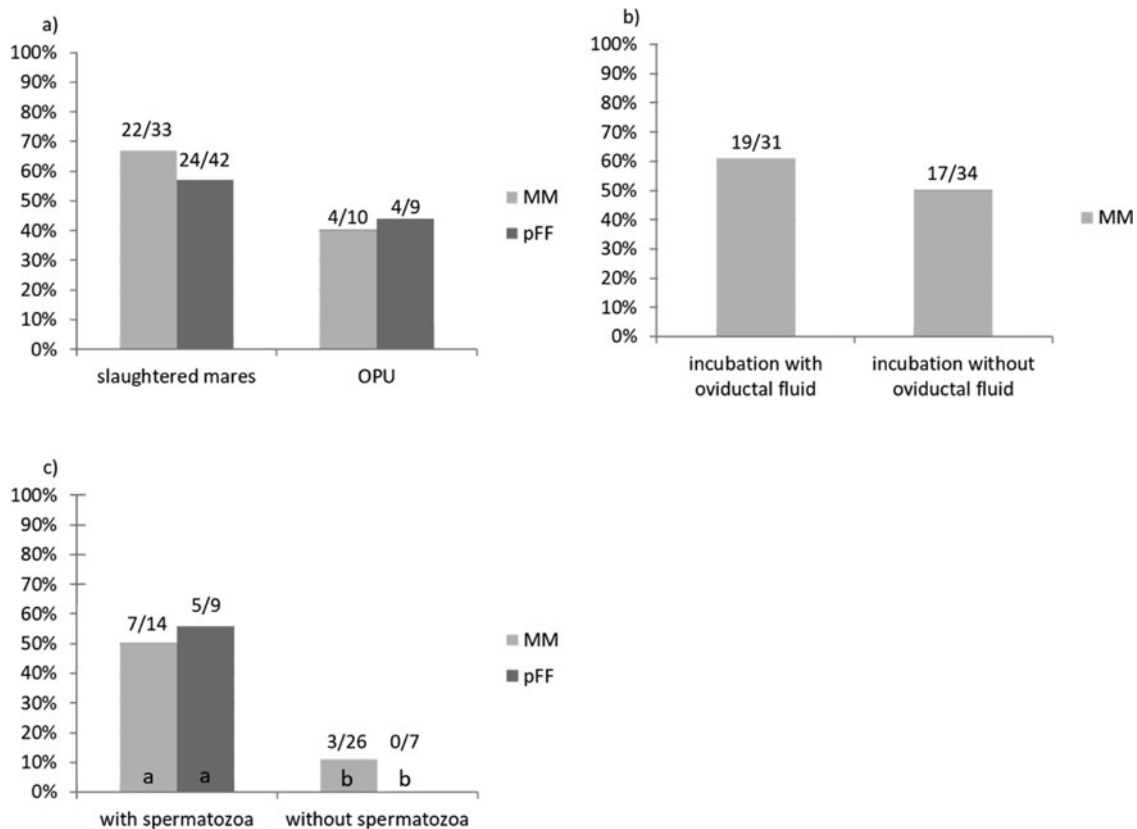
511 For the control groups for parthenogenetic activation,  
 512 three repetitions were performed during the time of  
 513 the experiments. Equine immature oocytes collected  
 514 in France from slaughtered mares were *in vitro*  
 515 matured in MM or pFF, incubated with OF and co-  
 516 incubated with or without spermatozoa. Three oocytes  
 517 containing 2 PN were observed in the control group  
 518 incubated without spermatozoa (3/26, 11% and 0/7  
 519 after IVM in MM and pFF respectively; Fig. 2c). In  
 520 the other oocytes incubated without spermatozoa we  
 521 observed either a metaphase II (62%, 16/26 and 57%,  
 522 4/7) or 1 PN (27%, 7/26 and 43%, 3/7) after IVM  
 523 in MM and pFF respectively. The percentage of 2PN  
 524 oocytes was significantly different for incubation with  
 525 vs without spermatozoa after IVM in MM and pFF ( $P$   
 526  $< 0.05$ ).  
 527

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

528 After oocytes collection from slaughtered mares or by  
 529 OPU, IVM in MM or pFF, post-maturation with OF,  
 530 IVF and *in vitro* culture in one of the four tested media,  
 531 nuclear status was assessed after 30 h *in vitro* culture,  
 532 i.e. 48 h post IVF, or after 54 h *in vitro* culture, i.e. 72  
 533 h post IVF. For each medium, three repetitions were  
 534 performed.  
 535

536 In total, 168 oocytes/embryos were analysed 48 h  
 537 post IVF (39 from SOF, 45 from DMEM-F12-51445C,  
 538 42 from DMEM-F12-D8437 and 42 from DMEM-  
 539 F12-D8900). In 60 of them (36%), we observed a  
 540 metaphase II and a polar body or one pronucleus. In  
 541 89 of them (53%), we observed two fully decondensed  
 542 pronuclei: the chromatin was diffuse, some nucleoli  
 543 were observed and the filamentous chromatin filled  
 544 the whole nuclear envelope (Fig. 3a, c-e). Very few 2PN  
 545 oocytes showed polar bodies. Moreover, pronuclear  
 546 apposition was the dominant feature of these 89  
 547 oocytes (Fig. 3a, c-e). In 19 of the oocytes/embryos  
 548 (11%), we observed several nuclei (from three to 16)  
 549 but the quality of these embryonic structures was  
 550 poor as the cleavage was abnormal: the number  
 551 of cells and nuclei were different and some cells  
 552 had no or several nuclei (Fig. 3b, f). The percentage  
 553 of 2PN oocytes and abnormally cleaved embryos  
 554 with respect to the number of non-degenerated  
 555 oocytes for each culture medium is presented  
 556 in Fig. 4.

557 After culture 48 h post IVF in SOF medium (Figs 3a  
 558 and 4a), the percentage of 2PN oocytes and abnormal  
 559 embryos from slaughtered mares was not statistically  
 560 different between the two maturation media (73%  
 561 for MM and 67% for pFF;  $P > 0.05$ ). One embryo  
 562 (from pFF) contained eight nuclei but no cleavage  
 563 of the cytoplasm was observed. The percentage of  
 564 2PN oocytes and abnormal embryos from OPU was  
 565 not statistically different between the two maturation  
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**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. <sup>a,b</sup> $P < 0.05$ ).

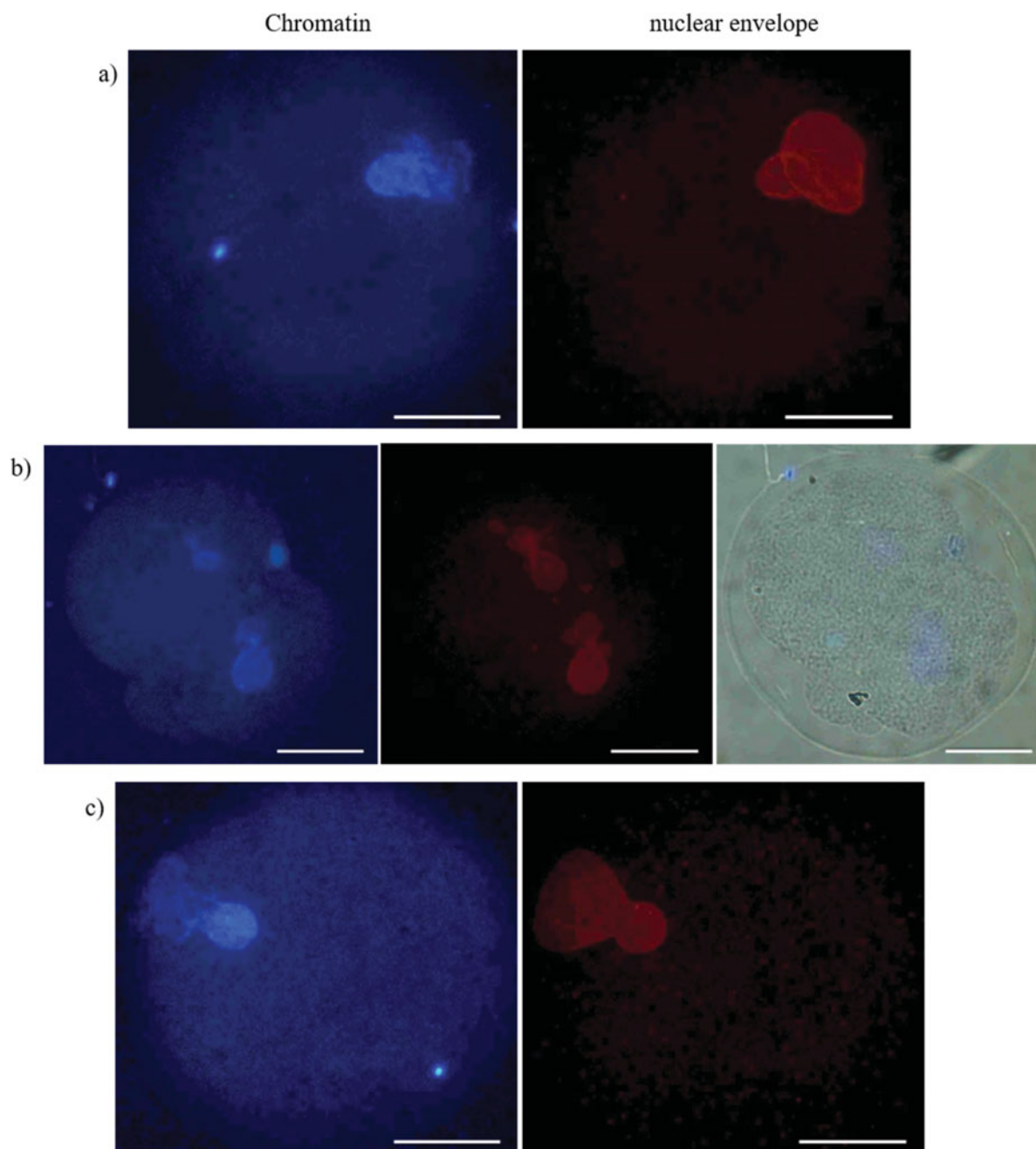
566 media (60% for MM and 75% for pFF,  $P > 0.05$ ).  
 567 Six embryos (three from MM and three from pFF)  
 568 contained from five to 16 nuclei, but the cleavage  
 569 stopped at two to three cells (Fig. 3b). When oocytes  
 570 collected in a slaughterhouse and by OPU were  
 571 pooled, the percentages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

574 After culture in DMEM-F12-51445C medium  
 575 (Figs 3c and 4b), the percentage of 2PN oocytes and  
 576 abnormal embryos from slaughtered mares was not  
 577 statistically different between the two maturation  
 578 media (50% for MM and 71% for pFF,  $P > 0.05$ ). Three  
 579 embryos (one from MM and two from pFF) contained  
 580 from seven to 16 nuclei, however they did not cleave.  
 581 The percentage of 2PN oocytes and abnormal embryos  
 582 from OPU was not statistically different between the  
 583 two maturation media (50% for MM and 71% for pFF,  
 584  $P > 0.05$ ). No cleavage was observed. When oocytes

585 from slaughterhouse and OPU were pooled, the  
 586 percentages of 2PN oocytes and abnormal embryos  
 587 from MM (50%) and pFF (71%) were not statistically  
 588 different ( $P > 0.05$ ).

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

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**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  $\mu\text{m}$ .

604 After culture in DMEM-F12-D8900 medium (Figs 3e  
 605 and 4d), the percentage of 2PN oocytes and abnormal  
 606 embryos from slaughtered mares was not statistically  
 607 different between the two maturation media (40% for  
 608 MM and 57% for pFF,  $P > 0.05$ ). Six embryos (two from  
 609 MM and four from pFF) contained from 3 to 10 PN  
 610 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

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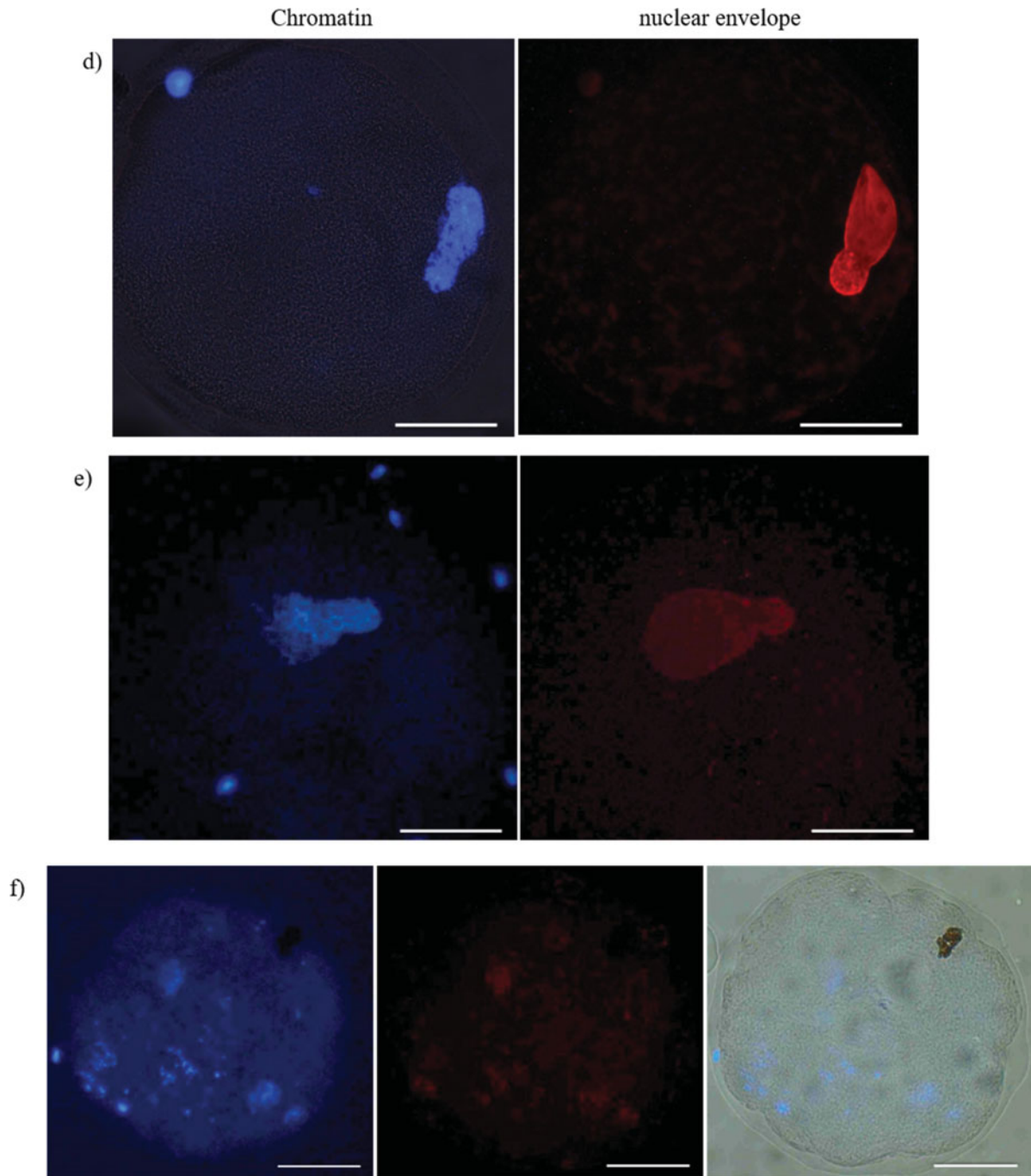


Figure 3 Continued.

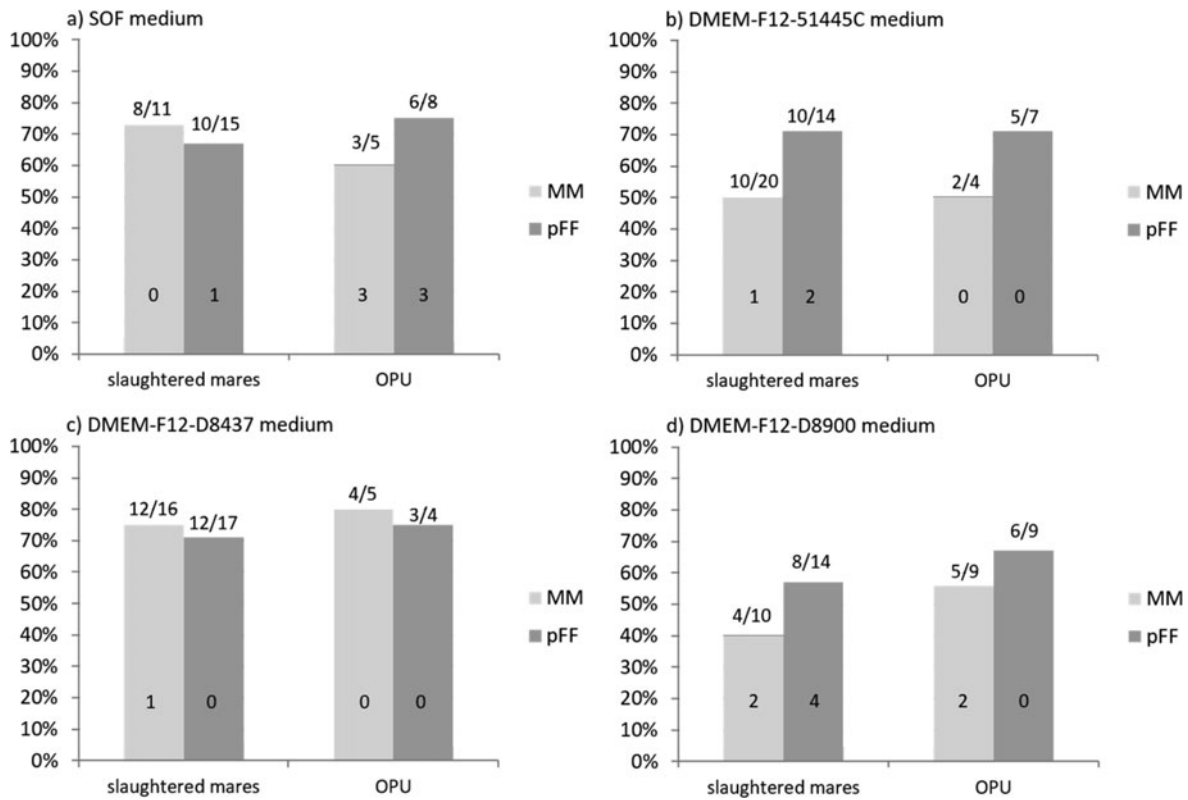
618 of 2PN oocytes and abnormal embryos from MM (47%)  
 619 and pFF (61%) were not statistically different ( $P >$   
 620 0.05).

621 Finally, when data from the four embryo culture  
 622 media were pooled, the percentages of 2PN oocytes  
 623 and abnormal embryos from MM (48/80, 60%) and  
 624 pFF (60/88, 68%) were not statistically different ( $P >$   
 625 0.05). Moreover, the percentages of abnormally cleaved  
 626 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

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**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 ( $P > 0.05$ ).

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

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

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

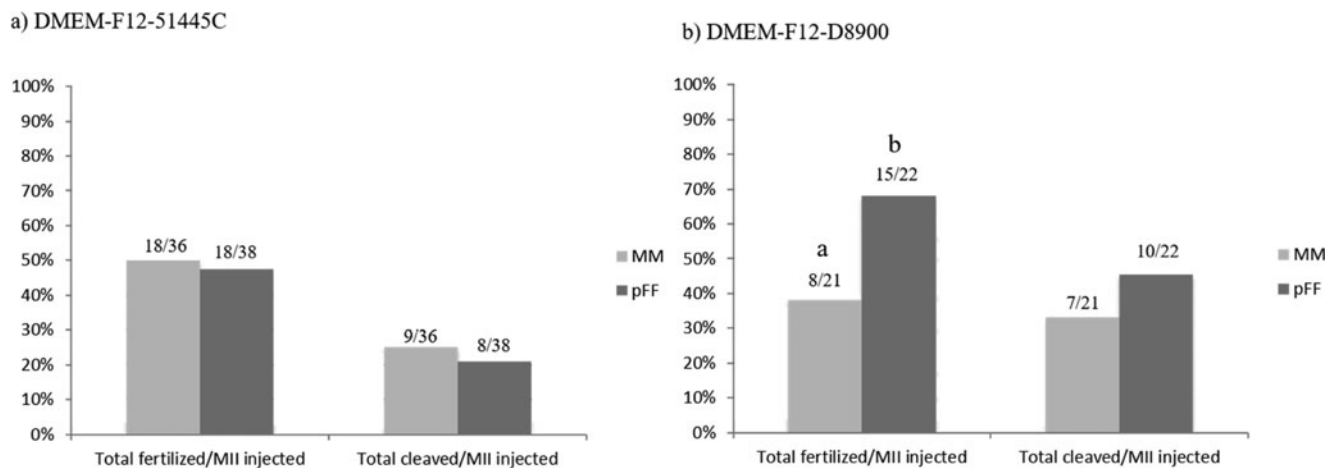
643 After oocytes collection from slaughtered mares, IVM  
 644 in MM or pFF, ICSI and *in vitro* culture for 72 h, nuclear  
 645 status was assessed. In total, 117 oocytes/embryos  
 646 were analysed. Among these, 58 (50%) were matured  
 647 but not fertilized and 59 (50%) were fertilized. Twenty-  
 648 five (21%) oocytes remained uncleaved after ICSI and  
 649 72 h *in vitro* culture. They showed two polar bodies  
 650 (PB) and two pronuclei (PN) and were called zygotes.

651 In samples cultured in DMEM-F12-51445C, the  
 652 total fertilization rate (including zygotes and cleaved  
 653 embryos) did not differ significantly between the two  
 654 IVM media (18/36, 50% versus 18/38, 47%,  
 655 for MM and pFF, respectively;  $P > 0.05$ , Fig. 5a).  
 656 In samples cultured in DMEM-F12-D8900, the total

657 fertilization rate was higher in oocytes matured in  
 658 pFF than in MM (15/22, 68% versus 8/21, 38%;  $P <$   
 659  $0.05$ , Fig. 5b). Independent of culture conditions, all  
 660 zygotes either derived from *in vitro* culture in DMEM-  
 661 F12-51445C (Fig. 6a, b) or in DMEM-F12-D8900 (data  
 662 not shown) showed decondensed pronuclei, their  
 663 chromatin was diffuse, some nucleoli were observed  
 664 and the filamentous chromatin filled the whole nuclear  
 665 area. Pronuclear apposition was found in 37% (7/19) of  
 666 samples derived from *in vitro* culture in DMEM-F12-  
 667 51445C (2/9 after IVM in MM and 5/10 after IVM in  
 668 pFF) whereas it was never found in zygotes derived  
 669 from *in vitro* culture in DMEM-F12-D8900.

670 The total cleavage rates with respect to the number  
 671 of metaphase II (MII) injected oocytes for each IVM  
 672 and culture medium are presented in Fig. 5. In both  
 673 embryo culture media, the cleavage rates did not  
 674 differ between maturation conditions: in DMEM-F12-  
 675 51445C, the total cleavage rates were 9/36 (25%) versus  
 676 8/38 (21%) for MM and pFF respectively ( $P > 0.05$ ); in  
 677 DMEM-F12-D8900, the total cleavage rates were 7/21  
 678 (33%) versus 10/22 (45%) for MM and pFF respectively  
 679 ( $P > 0.05$ ).

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**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: <sup>a,b</sup> $P < 0.05$ .

680 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 681 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. 682 683 684 685 686

687 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. 688 689 690 691 692 693 694 695 696

697 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. 698 699 700

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

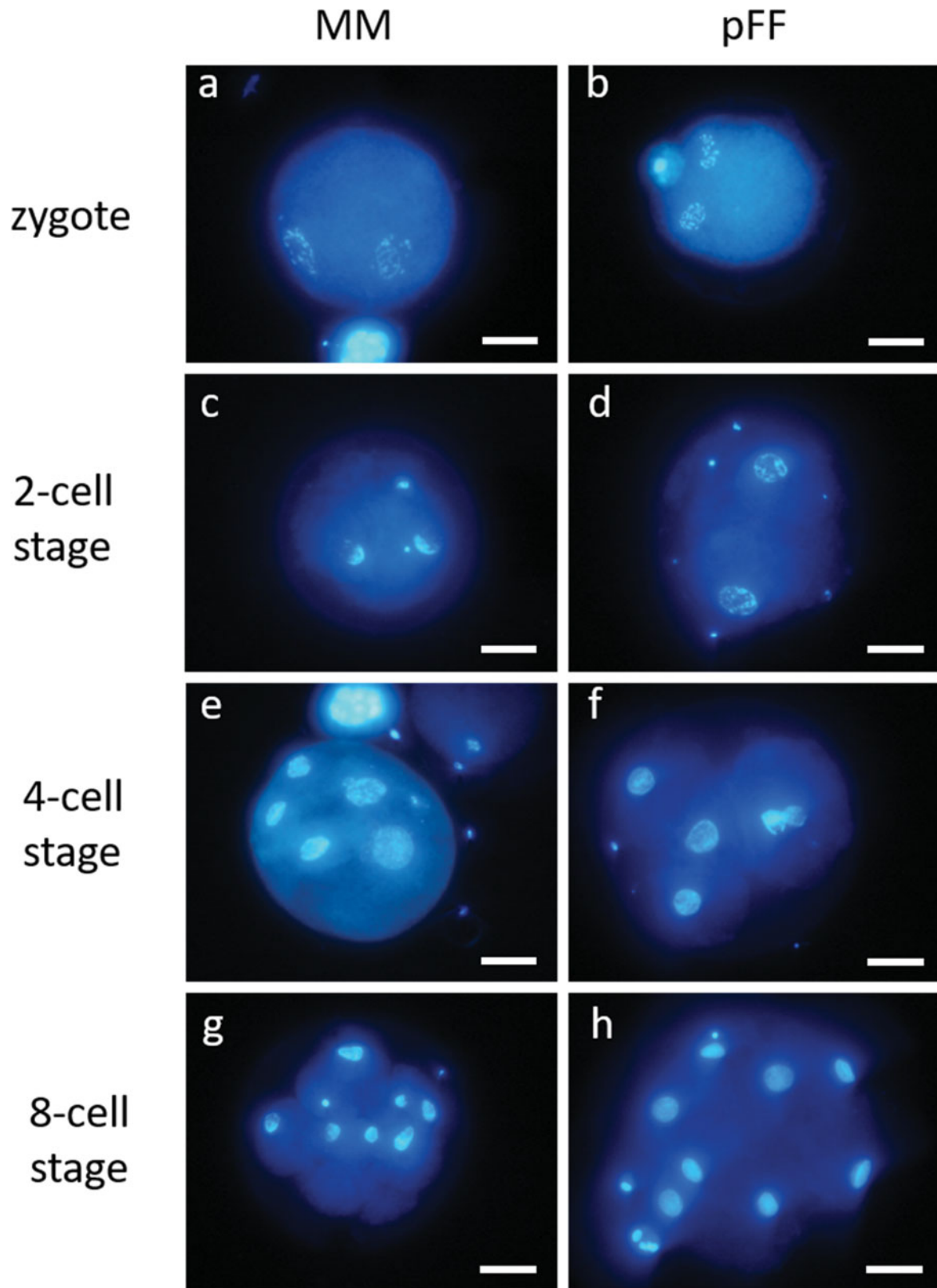
702 After oocytes collection from slaughtered mares, IVM in MM, post-maturation or not with OF, ICSI and *in vitro* culture, cleavage rates were assessed at day 3 and nuclear status was assessed at day 10 post-ICSI. Data are presented in Table 2. The percentages of cleaved embryos at day 3 were not significantly 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 703 704 705 706 707 708 709 710 711

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. 7a, b) or after fixation and Hoechst staining (Fig. 7c, d). In this experiment, all blastocysts reached a good expansion level with a consistent number of nuclei of regular morphology. 712 713 714 715 716 717 718 719 720 721 722 723 724 725

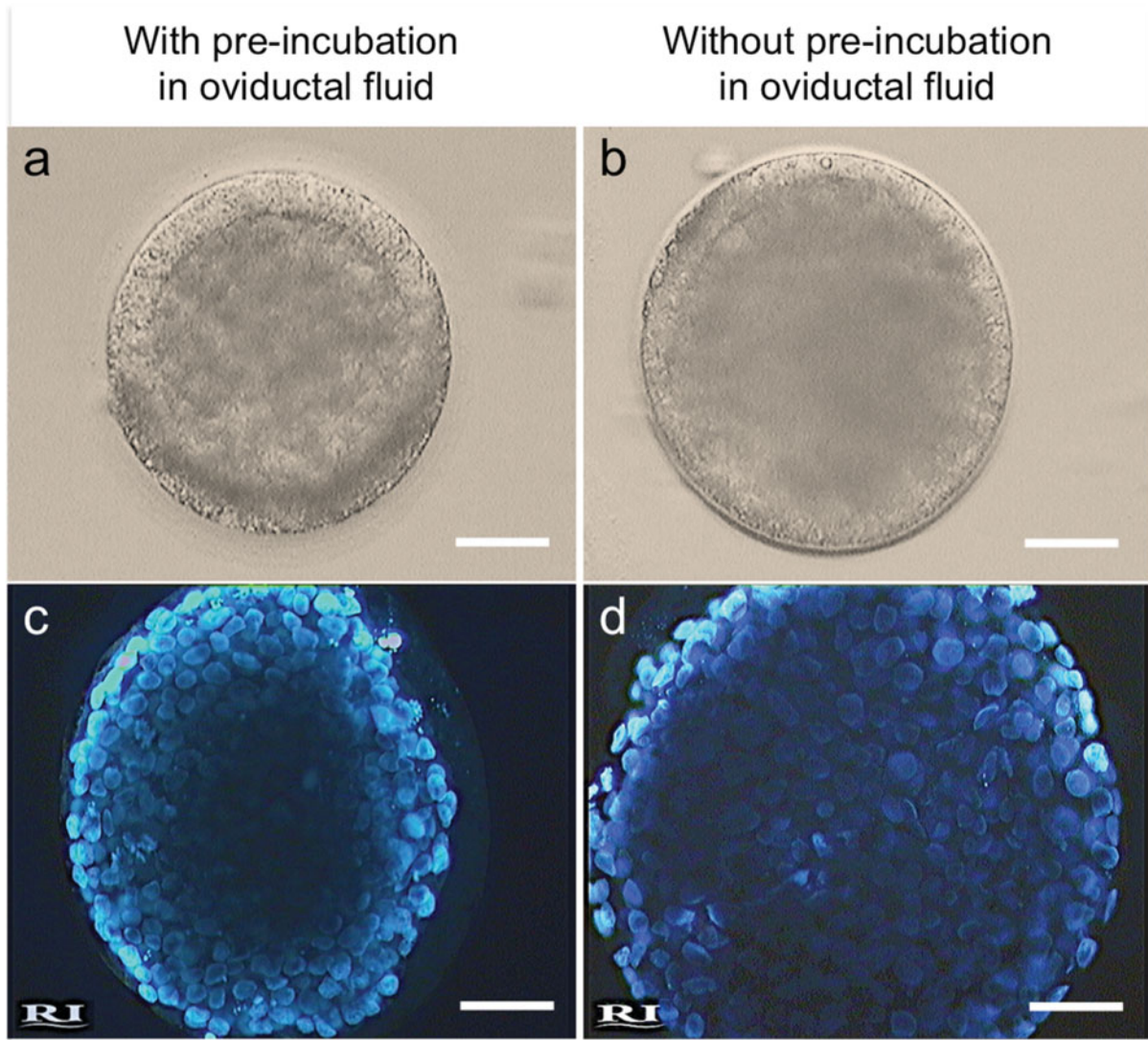
## 726 Discussion

727 The aim of our work was to analyse the effect of IVM in follicular fluid and pre-incubation in OF on 728 IVM, fertilization using conventional IVF or ICSI, and development of equine oocytes. We have been faced 729 with the difficulty with equine oocyte collection. As the number of slaughtered mares is very low and 730 the collection rate of equine oocytes is low compared with bovine oocytes for example (Hawley *et al.*, 1995), 731 the number of oocytes from slaughterhouses available for our experiments was low. Moreover, the collection 732 of equine oocytes using OPU is expensive and time-consuming, thus a maximum of 4 to 5 OPU sessions 733 per morning were performed, and the number of oocytes collected by OPU was low. The authors are 734 aware of the small sample size in their experiments 735 736 737 738 739 740 741





**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  $\mu\text{m}$ .



**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  $\mu$ m.

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

745 Our first objective was to compare IVM in MM  
 746 or preovulatory follicular fluid. Our hypothesis was  
 747 that the low IVF and development rates observed  
 748 in the equine may be due, at least partly, to a  
 749 poor cytoplasmic maturation related to unsuited IVM  
 750 conditions. Preovulatory follicular fluid, in which  
 751 maturation naturally occurs, may sustain a better  
 752 cytoplasmic maturation and thus a better competence  
 753 for IVF and development. In our study, the nuclear  
 754 maturation rates in MM and pFF were not statistically  
 755 different, neither for the oocytes collected in France  
 756 (76% of the oocytes are in metaphase II in MM

757 versus 67% in pFF) nor for the oocytes collected  
 758 in Italy (53% in MM versus 54% in pFF). Thus,  
 759 the semi-synthetic MM provides nuclear maturation  
 760 rates similar to follicular fluid. In previous studies,  
 761 the maturation rates of equine oocytes were similar:  
 762 68% in pFF (Caillaud *et al.*, 2008), 54–58% in pFF  
 763 (Conforti *et al.*, 2005), 61% in MM (Ambruosi *et al.*,  
 764 2013), 71% in MM (Deleuze *et al.*, 2009). In our  
 765 study, the fertilization rates after ICSI were not  
 766 different between maturation in MM (46%) vs pFF  
 767 (55%). This result shows that the semi-synthetic MM  
 768 supports cytoplasmic maturation to a similar extent to  
 769 preovulatory follicular fluid. Moreover, the percentage  
 770 of oocytes containing two pronuclei after IVF was  
 771 not statistically different between maturation in MM

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**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 <sup>a</sup>	N° (%) of zygotes and cleaved embryos found 72 h post-ICSI <sup>b</sup>				
					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)
<b>MM + pFF</b>	<b>DMEM-F12-51445C</b>	<b>140</b>	<b>74 (53)</b>	<b>36 (49)</b>	<b>19 (26)</b>	<b>4 (5)</b>	<b>10 (14)</b>	<b>3 (4)</b>	<b>17 (23)</b>
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)
<b>MM + pFF</b>	<b>DMEM-F12-D8900</b>	<b>79</b>	<b>43 (54)</b>	<b>23 (53)</b>	<b>6 (14)</b>	<b>8 (19)</b>	<b>5 (12)</b>	<b>4 (9)</b>	<b>17 (40)</b>
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)

<sup>a</sup>Numbers and percentages of fertilized oocytes out of the number of MII injected oocytes.

<sup>b</sup>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) <sup>a</sup>	No. (%) of blastocyst (day 10) <sup>a</sup>	No. (%) of morula + blastocyst (day 10) <sup>a</sup>
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)

<sup>a</sup>Numbers and percentages of embryos out of the number of MII injected oocytes.

772 versus pFF, neither for the oocytes collected in a  
773 slaughterhouse (67% in MM versus 57% in pFF) nor  
774 for the oocytes collected by OPU (40% in MM versus  
775 44% in pFF). As very few oocytes containing two  
776 pronuclei extruded PB, the significance of this nuclear  
777 stage may be questionable. One could hypothesize  
778 that either the PB were extruded and degenerated  
779 quickly or the expulsion of the PB did not occur  
780 as a consequence of an abnormal fertilization. On  
781 the one hand, using equine zygotes flushed from  
782 oviducts at different time intervals from ovulation and  
783 *in vitro* fertilized oocytes, Bézard and collaborators  
784 showed that PB degeneration and undetectability may  
785 occur quickly after fertilization both *in vitro* and  
786 *in vivo* (Bézard *et al.*, 1989). On the other hand,  
787 Leemans and collaborators showed that the absence  
788 of the second polar body extrusion is due to oocyte  
789 cytokinesis induced by procaine instead of sperm  
790 penetration of equine oocytes (Leemans *et al.*, 2015). In  
791 our study, the presence of some equine oocytes con-  
792 taining two pronuclei in the parthenogenetic control  
793 group incubated without spermatozoa supports this  
794 second hypothesis. Previous studies have shown that  
795 spontaneous parthenogenetic division of unfertilized  
796 equine oocytes is rare either *in vitro* or *in vivo* (Zhang  
797 *et al.*, 1989; Zhang *et al.*, 1990). Finally, after culture  
798 in embryo culture media, we observed no difference  
799 between maturation in MM vs pFF for the percentage  
800 of zygotes after ICSI (17% vs. 25% respectively) and  
801 the percentage of cleaved embryos after ICSI (28%  
802 versus 30%). Thus, our MM and the preovulatory  
803 follicular fluid support the acquisition of competence  
804 for maturation, fertilization and development to a  
805 similar extent. However, in our conditions, IVM occurs  
806 in a static medium, whereas *in vivo* maturation  
807 naturally takes place in a dynamic medium in which  
808 subtle changes occur during final maturation of  
809 the preovulatory follicle. IVM in a culture system  
810 in which hormones would be added sequentially  
811 to mimic changes observed *in vivo* might provide  
812 better conditions for equine oocyte maturation. For  
813 example, porcine oocytes matured in a medium in  
814 which hormones were added sequentially exhibited  
815 greater developmental competence to blastocyst stage  
816 (Kawashima *et al.*, 2008).

817 The influence of oviductal secretions on oocytes  
818 has been widely studied in mammals (Aviles *et al.*,  
819 2010; Coy *et al.*, 2012; Lopera-Vasquez *et al.*, 2015). A  
820 beneficial effect on equine oocytes has been observed  
821 with incubation of equine oocytes with OF collected 6  
822 h after ovulation (Ambruosi *et al.*, 2013) or co-culture  
823 of equine oocytes with equine or porcine oviduct  
824 epithelial cells (Mugnier *et al.*, 2009). In our study,  
825 incubation of equine oocytes with OF collected before  
826 ovulation, at the end of follicular growth, did not  
827 influence fertilization or development rates. This result

828 suggests that oviductal factors with a beneficial effect  
829 on oocyte may be present in the oviduct during a  
830 specific time interval after ovulation. Several oviductal  
831 factors, which influence fertilization rates, have been  
832 studied in the equine oviduct: Deleted in malignant  
833 brain tumour one is secreted in the oviduct from early  
834 follicular phase to post-ovulatory stage (Ambruosi  
835 *et al.*, 2013) and osteopontin and atrial natriuretic  
836 peptide A are present in the equine oviduct from  
837 emergence of the dominant follicle to preovulatory  
838 stage (Mugnier *et al.*, 2009). Further studies are in  
839 progress to clarify the role and secretion pattern of  
840 oviductal factors with a beneficial effect on equine  
841 oocytes.

842 Several culture media have been used for *in*  
843 *vitro* development of equine fertilized oocytes. SOF  
844 medium has been used previously for *in vitro* culture  
845 of equine zygotes after ICSI with a cleavage rate  
846 of 61% (Tremoleda *et al.*, 2003), 69% (Galli *et al.*,  
847 2002) and 64% (Lazzari *et al.*, 2002) 48 h post-  
848 ICSI. In our conditions, 26% of the oocytes after  
849 IVF were abnormally cleaved and 74% contained  
850 two fully decondensed and apposed pronuclei. The  
851 pronuclei decondensation and apposition evidence  
852 the first step of embryo development. However,  
853 the lack of normal embryo cleavage throws doubt  
854 on the fertilization technique. The use of procaine  
855 during the IVF procedure in order to induce sperm  
856 hyperactivation may have a detrimental effect on  
857 oocytes and zygotes. Leemans and collaborators have  
858 shown that procaine induces oocyte cytokinesis and  
859 that cleaved oocytes did not develop beyond 8–16  
860 cells with daughter cells containing aberrant DNA  
861 fragments (Leemans *et al.*, 2015). DMEM-F12 medium  
862 has been used previously for *in vitro* culture of equine  
863 zygotes after ICSI with a cleavage rate of 68% (Smits  
864 *et al.*, 2012a), 69% (Choi *et al.*, 2011) and 75% (Choi  
865 *et al.*, 2006) 3 days post-ICSI. Moreover, DMEM-F12  
866 medium has been used previously for *in vitro* culture  
867 of equine zygotes after IVF with a cleavage rate of  
868 66% (McPartlin *et al.*, 2009). In our conditions, three  
869 references of DMEM-F12 were tested, with identical  
870 composition but different forms (liquid or powder,  
871 sodium bicarbonate included or added subsequently).  
872 Following ICSI and 72 h culture in DMEM-F12, the  
873 percentages of zygotes (26% in DMEM-F12-51445C  
874 and 14% in DMEM-F12-D8900) and the percentages  
875 of cleaved embryos (23% in DMEM-F12-51445C  
876 and 40% in DMEM-F12-D8900) were consistent, and  
877 the morphology of the ICSI embryos was normal.  
878 Following ICSI and 10 days culture in DMEM-F12-  
879 D8900, the blastocysts formation rate was correct  
880 and repeatable and blastocysts quality was good, as  
881 blastocyst expansion and hatching throughout the  
882 zona pellucida were observed. Following IVF and  
883 culture in DMEM-F12, no normal embryo cleavage and

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

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

## 901 Author's contributions

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

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## 926 Conflict of interest

927 The authors declare no conflict of interest.

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