Effects of follicular fluid supplementation of in-vitro maturation medium on the fertilization and development of equine oocytes after in-vitro fertilization or intracytoplasmic sperm injection*

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The aim of this study was to compare the effect of the addition of follicular fluid (FF) collected from preovulatory follicles with that of oestrous mare serum (EMS) (acting as the control) to TCM-199 medium on the in-vitro maturation, fertilization and development of equine cumulus-enclosed oocytes. Oocytes (<30 mm in diameter) were obtained from the ovaries of slaughtered mares. After in-vitro maturation in the presence of the two supplements, their fertilization, cleavage and developmental potential were compared after conventional in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) using frozen–thawed spermatozoa. Follicular fluid did not increase the maturation of oocytes to metaphase II stage compared to control. After IVF, there was no difference in fertilization rates between FF-supplemented oocytes and controls (7/87, 8.4% of oocytes showing two pronuclei with FF versus 7/116, 6% with EMS; not significant). However, after ICSI, FF-supplemented oocytes showed significantly increased normal fertilization (32/85, 37.6% of two-pronuclear oocytes) and developmental potential (15/31, 48% cleavage) compared to the control oocytes (7/47, 14.9%, P <0.01; and 2/48, 4%, P < 0.01, respectively). Overall, ICSI resulted in increased fertilization rates compared to IVF, regardless of the presence or absence of FF (39/132, 29.5% with ICSI versus 14/203, 6.9%). These results suggest that follicular fluid supplementation may improve the maturity of equine cumulus-enclosed oocytes sufficiently for the successful use of ICSI, but not sufficiently for normal sperm–egg interaction occurring during IVF.

Key words: equine oocytes/follicular fluid/intracytoplasmic sperm injection/in-vitro fertilization/in-vitro maturation

Introduction

During meiosis, the female germ cell acquires the factors necessary for fertilization and early development. A large number of nuclear and cytoplasmic events take place which are regulated by intercellular co- operative mechanisms operating between the somatic compartment of the follicle and the oocyte itself (Staigmiller and Moor, 1984; Mattioli et al., 1988a; Moor et al., 1992; Mattioli, 1992).

The development of culture conditions able to mimic the preovulatory stage follicular environment and to support nuclear and cytoplasmic maturation of oocytes is important for in-vitro fertilization (IVF) programmes both in farm animals, where in-vitro maturation (IVM) of primary oocytes is widely used (Brackett, 1992; Brackett and Zuelke, 1993; Trousnon et al., 1994a; Gordon, 1995) and in women who fail to respond to ovarian stimulation or have polycystic ovaries (Trousnon et al., 1994b) and for macaque species used as models for human embryology (Lanzendorf et al., 1996; Schramm and Bavister, 1995, 1996). In humans, pregnancies and live births have resulted following in-vitro maturation of oocytes retrieved from ovariectomy specimens in oocyte donation programmes (Cha et al., 1991), from patients with polycystic ovary syndrome (PCOS) (Trousnon et al., 1994b) and more recently in a study in which IVM was used in association with intracytoplasmic sperm injection (ICSI) and assisted hatching (Barnes et al., 1995). In the horse, the IVM technique has yielded >60% oocytes which reach metaphase II following first polar body extrusion (Brinsko et al., 1995; Del Campo et al., 1995; Bruch et al., 1996; Dell’Aquila et al., 1996). However, the lack of an efficient IVF procedure has limited assessment of the developmental potential of IVM equine oocytes and no foals have as yet been produced from oocytes matured and fertilized in vitro (Grondahl et al., 1995; Li et al., 1995; Dell’Aquila et al., 1996; Marcos et al., 1996; Kato et al., 1997a; Tornet et al., 1997). Stallion sperm cells have been shown to undergo in-vitro capacitation (for review see Dell’Aquila et al., 1996) and it has been suggested that IVM may induce alterations in the zona with deleterious effects on sperm penetration in vitro (Grondahl et al., 1995; Li et al., 1995). Alternatively, equine oocytes obtained from abattoir material may be incapable of normal cytoplasmic maturation in vitro (Kato et al., 1997a). An alternative method involving the intrafollicular transfer of immature oocytes into preovulatory follicles of mares stimulated by administration of human chorionic gonadotrophin (HCG) has been proposed (Hinrichs and Di Giorgio, 1991) and is currently undergoing active research (Goudet, 1997). Intracytoplasmic sperm injection (ICSI) has been demonstrated to be efficient in achieving

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fertilization of IVM equine oocytes (Cho et al., 1995a; Cho et al., 1995b; Dell’Aquila et al., 1995; Guignot et al., 1996; Meintjes et al., 1996; Squires et al., 1996; Kato et al., 1997b). Using ICSI, significantly higher fertilization rates were obtained in oocytes with an expanded cumulus as compared to those with a compact cumulus, despite the fact that all oocytes were at metaphase II stage. This may reflect differences in cytoplasmic maturity (Dell’Aquila et al., 1997). Since the majority of oocytes from the ovaries of untreated slaughtered females have a compact cumulus, attempts to improve their cytoplasmic maturity are of great interest.

Studies on oocyte maturation carried out in many species have involved the addition of growth factors, hormones, substances, sera and follicular fluid to the maturation medium. Supplementation with follicular fluid (FF) from large follicles has been demonstrated to support in-vitro nuclear and cytoplasmic maturation of both bovine (Kim et al., 1993, 1996; Carolan et al., 1996) and porcine (Naft et al., 1988, 1989; Mattioli et al., 1988b, 1992; Yoshida et al., 1992a,b; Funahashi and Day, 1993a) oocytes. The objective of this study was to evaluate the effects of follicular fluid from preovulatory follicles on the cytoplasmic maturation of equine compact cumulus–oocyte complexes (COC) as assessed by their fertilization and developmental capacity following IVF or ICSI.

Materials and methods

Oocyte collection

The study was conducted from the autumn of 1995 to the spring of 1996 in southern Italy. Ovaries from mares of unknown reproductive history were obtained at abattoirs located at a maximum distance of 30 km (half an hour) from the IVF laboratory. They were placed in saline (9 g NaCl/l containing 40 mg/l gentamycin sulphate) within 30 min of slaughter and transported to the laboratory in thermal containers at 25–30°C. Oocytes were aspirated from follicles <3 cm in diameter as previously described (Dell’Aquila et al., 1996). Cumulus-enclosed oocytes were collected in HEPES-buffered tissue culture medium (TCM-199) supplemented with 20% oestrous mare serum (EMS), and those oocytes with a complete and compact cumulus investment were washed four times in medium supplemented with either EMS or FF (20%) in relation to the experimental group. The total time between slaughter and culture ranged between 2 and 4 h.

Oestrous mare serum preparation

Oestrous mares were echographically monitored every 6 h and blood was collected 2–3 h after ovulation. Serum was centrifuged twice at 500 g for 15 min and the supernatant was heat-inactivated at 56°C for 30 min, filtered through a 0.22 µm filter, aliquoted and stored as described above.

Maturation medium

The basic medium was TCM-199 (Sigma M-0148, Milan, Italy) pH 7.18 containing Earle’s salts and supplemented with 4.43 mM HEPES (Sigma H-9136), 33.9 mM sodium bicarbonate (Sigma S-5761), 0.1 g/l L-glutamine (Sigma G-7513), 2 mM sodium pyruvate (Sigma P-2256), 2.92 mM calcium lactate (Serva Feinbiochem GmbH & Co., Heidelberg, Germany, No.29760), and 50 µg/ml gentamycin (Sigma G-1272). The medium was further supplemented with either EMS or FF (20%), gonadotrophins [10 µg/ml ovine follicle stimulating hormone (FSH) and 20 µg/ml ovine luteinizing hormone (LH), NIDDK, NHPP, Baltimore, MD, USA], and 17β-oestradiol (1 µg/ml, Sigma E-2257). Up to 20 oocytes were placed in 400 µl of medium well of a four-well multidish (Nunc Intermed, Roskilde, Denmark, No. 176740), covered with pre-equilibrated lightweight paraffin oil (Sigma M-3516) and cultured for 27–30 h at 38.5°C under 5% CO2 in air.

Oocyte preparation for IVF and ICSI

After 28–30 h culture, oocyte morphology was assessed following partial removal of cumulus cells. Those oocytes showing the perivitelline space, an extruded first polar body and an intact oolemma were selected. The cumulus and corona cells of oocytes undergoing ICSI were totally removed by incubation in TCM-199 with 20% EMS containing 80 IU hyaluronidase/ml (Sigma H-3506) and by aspiration in and out of finely drawn glass pipettes. After transport at 37°C to the microinjection laboratory the oocytes were checked for nuclear maturation and the absence of vacuoles and other cytoplasmic abnormalities under the inverted microscope (×200 magnification).

Semen preparation for IVF and ICSI

Semen samples (0.4 ml/straw) from a single ejaculate frozen at a concentration of 1×10^6 sperm cells/ml were rapidly thawed in a water bath at 37°C. Total motility after thawing was 70%, with 50–60% progressive motility. Sperm cells for IVF were prepared using the swim-up procedure described for bull sperm cells by Parrish et al. (1986) in modified Tyrode–lactate medium containing heparin (1 µg/ml; Sigma H3393) (Sp-TALP, Parrish et al., 1988) as applied to stallion spermatozoa by Dell’Aquila et al. (1996). A final sperm concentration of 1×10^6 sperm cells/ml in 400 µl of culture medium containing 5–20 oocytes was incubated in four-well multidishes for 24 h at 38.5°C under 5% CO2 in air. Semen samples for ICSI were prepared by the swim-up procedure in Earle’s balanced salt solution (EBSS, B-4065-L; HyClone Laboratories, Cellbio, Milan, Italy) supplemented with 0.4% human serum albumin (HSA; Immuno, Pisa, Italy), 40 µg/ml streptomycin sulphate (Sigma S-9139) and 25 U/ml penicillin G (Sigma P-3032) as described in Dell’Aquila et al. (1997).

ICSI procedure

ICSI was carried out basically according to the procedures described by Palermo et al. (1992) and Van Steirteghem et al. (1993) as applied to equine germ cells (Cho et al., 1995a,b; Dell’Aquila et al., 1997). All procedures were performed at 38.5°C in HEPES-buffered human tubal fluid (HTF–HEPES 9962; Irvine Scientific, Santa Ana, CA, USA) containing 0.5% HSA. The injected oocytes were then transferred to 25 µl Fert-Talp covered by lightweight paraffin oil and incubated at 38.5°C for 18–20 h under 5% CO2 in air.

Assessment of fertilization (experiment 1)

Compacted-cumulus oocytes were matured in vitro randomly in media supplemented either with 20% FF or with 20% EMS as control and
fertilized by IVF or ICSI. On the day after IVF or microinjection, oocytes were fixed for 24 h with 3:1 ethanol–acetic acid, stained with 1% Lacmoid in 45% acetic acid and assessed by phase-contrast microscopy. Normal fertilization was defined by the presence of two polar bodies (PB) with two pronuclei (PN). Thirteen replicate experiments were performed for each maturation condition and each mode of fertilization.

**Embryo culture (experiment 2)**

After IVM in control medium or in medium supplemented with FF, oocytes were fertilized by IVF or ICSI and allowed to develop in vitro for 6 days on Vero cell monolayers in Ménézo B2 Medium (Laboratoire C.C.D., Paris, France) supplemented with 15% fetal bovine serum (FBS, 10168-066 Gibco BRL). Embryonic development was evaluated daily using Nomarski optics ($\times$200–400) on a Zeiss Axiovert 135 M microscope. The embryo quality was graded as follows: Type a = blastomeres of equal size with <10% fragmentation; b = unequal blastomeres with 10–20% fragmentation; c = 20–40%; and d >40% fragmentation. When no further cleavage or change in appearance occurred during 24 h, embryos were removed from culture, fixed and stained as described previously and evaluated by phase-contrast microscopy. The experiment was performed twice and four times, respectively, using IVF after IVM in FF and control medium, and three times and twice, respectively, using ICSI after IVM in FF and control medium.

**Data analysis**

Results from the total number of oocytes evaluated in all experiments are presented. The statistical significance of the results was evaluated by the $\chi^2$-test with the Yates correction for continuity and by Fisher’s exact test. A comparison was made of the frequencies of matured oocytes, penetrated oocytes, 2PN oocytes and cleaved oocytes with a 2×2 contingency table to evaluate any influence of the addition of the FF in IVM versus the addition of the EMS, on the nuclear maturation, fertilization and embryonic development of compact-cumulus oocytes in both fertilization procedures (IVF or ICSI). Values with $P < 0.05$ were considered to be statistically significantly different.

**Results**

**Effects of follicular fluid on in-vitro maturation of equine oocytes**

In experiment 1, during the study period, 964 COC were harvested from the ovaries of 195 mares (average number of oocytes/ovary = 2.5). Of these COC, 635 (65.8%) showed a compact cumulus and were selected for culture. The maturation rates of oocytes cultured with either FF or EMS (control group) are shown in Table I. Lost, damaged or degenerate oocytes were excluded from the analysis. In the group of oocytes subjected to IVF, there was a significant difference ($P < 0.05$) in the metaphase II formation rates observed 28–30 h after IVM (68.5% in the presence of FF and 81.1% in control medium). In the group of oocytes undergoing ICSI, there was no significant difference in the metaphase II formation rates.

**Effects of follicular fluid on the fertilization of equine oocytes**

Table I also shows the percentages of penetrated, normally fertilized, polyspermic and cleaved oocytes. There was no significant difference in sperm penetration rates after IVF (11.5% when FF was present and 8.6% in control medium) or in normal fertilization rates (8.4 and 6% respectively). Only one oocyte was polyspermic (1/203, 0.5%) and no oocytes had incomplete fertilization. Five oocytes (two and three after IVM in FF and in controls respectively) cleaved to the 2-cell stage on the day after IVF. One hundred and sixteen out of 132 injected oocytes (87.8%) were intact after microinjection. A significantly higher proportion of oocytes showed signs of fertilization after maturation in the presence of FF than in control medium (45.8%, 39/85 versus 21.3%, 10/47 respectively; $P < 0.01$). A significantly higher proportion of oocytes ($P < 0.01$) were normally fertilized after FF maturation than in control medium (37.6% and 14.9% respectively). Two injected oocytes cultured in FF were digynic, having three pronuclei consequent to failed second polar body extrusion. Four oocytes underwent incomplete fertilization (two in each maturation group). Four oocytes (three and one from the FF and EMS treatments, respectively) cleaved to the 2-cell stage on the day after ICSI.

**Effects of follicular fluid on the development of equine embryos**

In experiment 2 (Table II) of 316 oocytes harvested, 253 had compact cumulus and were selected for IVM. One hundred and seventy-seven of them (69.9%) extruded the first polar body and were inseminated. A total of six of the 98 matured oocytes subjected to IVF cleaved (6.1%), two embryos from the group of 14 oocytes matured in FF (14.3%), one to the 16-cell stage and four embryos from the group of 84 control oocytes (4.8%), all of which blocked at the 4-cell stage. On day 2 of culture, one of the embryos of the IVF-FF group was Type a and the other was Type b; two of the embryos of the IVF control group were of Type a and the remaining two were of Type b. In the ICSI group, 17 of 79 matured oocytes cleaved (21.5%), 15 of which were from the group of 31 oocytes matured in FF (48.4%). On day 2 of culture, nine were Type a (blastomeres of equal size with <10% fragmentation), three were Type b (unequal blastomeres with 10–20% fragmentation), and three were Type c (20–40% fragmentation). Three embryos were blocked at the 4-cell stage and twelve reached the 16-cell stage. Six were non-surgically transferred into the uterus of recipient mares and a further six remained in culture. All of the six further cultured 16-cell embryos became morulae, and one developed to the blastocyst stage (16.7%). No pregnancies were obtained after transfers in recipient mares. Two embryos from the group of 48 control oocytes underwent further cleavage (4.2%), one to the 16-cell stage. Both embryos were Type b on day 2 of culture. Fisher’s exact test revealed significantly higher cleavage rates in the oocytes which were subjected to ICSI after culture in medium supplemented with FF when compared to the oocytes of the other three groups (IVF-FF versus ICSI-FF: $P < 0.05$; ICSI-FF versus ICSI-EMS: $P < 0.01$).

**Discussion**

In the present study, the effects of adding FF to IVM medium on the subsequent ability of the oocyte to sustain sperm head decondensation, male and female pronuclear formation and
Table I. Fertilization rates of equine oocytes matured in vitro in TCM-199 supplemented with 20% follicular fluid or oestrous mare serum and subjected to in-vitro fertilization (IVF) or to intracytoplasmic injection of a single spermatozoon (ICSI)

<table>
<thead>
<tr>
<th>Oocytes</th>
<th>IVF</th>
<th>ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Follicular fluid</td>
<td>Serum</td>
</tr>
<tr>
<td>Cultured</td>
<td>168</td>
<td>208</td>
</tr>
<tr>
<td>Analysed</td>
<td>127</td>
<td>143</td>
</tr>
<tr>
<td>Oocytes at metaphase II (% analysed)</td>
<td>87 (68.5)\textsuperscript{a}</td>
<td>116 (81.1)\textsuperscript{b}</td>
</tr>
<tr>
<td>Intact oocytes after ICSI (% MII)</td>
<td>78 (91.8)</td>
<td>38 (80.8)</td>
</tr>
</tbody>
</table>

Values are \(n\) (%).

Values with different superscripts differ between columns: \(\text{a, b} P < 0.05, \text{c, d} P < 0.01, \) Fisher’s exact test.

PB = polar body; PN = pronucleus.

Table II. Embryonic development from equine oocytes retrieved with compact cumulus, matured in vitro in TCM-199 supplemented with 20% follicular fluid or oestrous mare serum, subjected to in-vitro fertilization (IVF) or to intracytoplasmic sperm injection (ICSI) and cultured in vitro in Ménezo B2 medium on Vero cell monolayers

<table>
<thead>
<tr>
<th>Oocytes</th>
<th>IVF</th>
<th>ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Follicular fluid</td>
<td>Serum</td>
</tr>
<tr>
<td>Metaphase II inseminated</td>
<td>14</td>
<td>84</td>
</tr>
<tr>
<td>Cleaved (2–4-cell stage)</td>
<td>2 (14.3)\textsuperscript{a}</td>
<td>4 (4.8)\textsuperscript{a}</td>
</tr>
<tr>
<td>8–16-cell stage</td>
<td>1 (7.1)\textsuperscript{a}</td>
<td>0\textsuperscript{a}</td>
</tr>
<tr>
<td>Morula</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values with different superscripts differ between columns: \(\text{a, b} P < 0.05, \text{c, d} P < 0.01, \) Fisher’s exact test.

\*Six out of 12 embryos at the 16-cell stage were transferred into recipient mares, and are thus not included for calculation of further development. No pregnancy was obtained.

\text{16.7\%}.

In the group of oocytes subjected to IVF, the nuclear maturation rate observed in oocytes cultured in the presence of FF was significantly lower than the rate observed after culture in the control medium supplemented with EMS, and sperm penetration rates, normal fertilization rates and cleavage rates were also low after culture in either FF or control medium. In the group of oocytes subjected to ICSI, there was no significant difference in the metaphase II formation rate following culture in the presence of FF or in control medium. However, the normal fertilization rate and the cleavage rate of the oocytes matured in the presence of FF were significantly higher than those of the control oocytes (\(P < 0.01\), see Tables I and II). The proportion of normally fertilized oocytes in the ICSI-FF group, expressed as a percentage of the total population of fertilized oocytes, was considerably higher than in other groups (32/39, 82.1\%). Moreover, the normal fertilization rate was comparable to that previously achieved using oocytes with an expanded cumulus and matured in the presence of EMS (Dell’Aquila et al., 1997).

The use of frozen–thawed stallion semen together with the use of different procedures of sperm cell preparation for IVF and ICSI, has been previously discussed (Dell’Aquila et al., 1996, 1997), and our procedure has been shown to yield a mean normal 2PB 2PN fertilization rate of 10–20% (Dell’Aquila et al., 1996, 1997). Recent studies on IVF in the horse, evaluating the modification of these techniques, did not achieve higher fertilization rates (Kato et al., 1997a; Torner et al., 1997).

Results of embryo culture experiments in this study were encouraging. Only a limited number of studies have been carried out on the in-vitro production of equine embryos either by IVF (Grondahl et al., 1995; Li et al., 1995) or by ICSI.
(Guignot et al., 1996; Squires et al., 1996; Meintjes et al., 1996; Kato et al., 1997b). A significantly greater embryonic development of FF-supplemented and microinjected oocytes was achieved than in our previous study (Dell’Aquila et al., 1997). Development was also prolonged beyond the morula stage and one blastocyst out of six further cultured morulae (16.7%) was obtained. Due to very low rates of parthenogenetic activation observed in fixed oocytes (only two 1PN oocytes without sperm residues were observed in the group of EMS-supplemented and IVF-inseminated oocytes), the embryos obtained in this study can be considered to have resulted from normal fertilization. In programmes for in-vitro production of transferable embryos in farm animals, unlike humans, pronuclear evaluation is not possible without staining because of the presence of dark lipid granules in the cytoplasm. Therefore, the incidence of parthenogenesis is usually detected by fixing an aliquot of cultured oocytes (Gordon, 1995). Moreover, all embryos in this study resulted from oocytes showing two clearly separated polar bodies on the day after IVF or ICSI, a morphological feature which was always related to the presence of two pronuclei in the group of oocytes submitted to fixing and staining.

The failure of 16-cell embryos transferred into the uterus to achieve pregnancies was not at all surprising but, in preliminary attempts, the non-surgical transfer of healthy-looking 16-cell embryos was preferred. Previous attempts of surgical oviductal transfer, in our unit, using ICSI-derived embryos obtained from expanded cumuli oocytes, also did not result in pregnancies (unpublished observations). Few studies are available on this topic due to the limited results with IVF. Grondahl et al. (1995) discussed the unsuccessful surgical transfer of IVF-derived 8–16-cell embryos, while Squires et al. (1996) were the first to report a 75 day pregnancy after the non-surgical transfer of 10–12-cell embryos. Meintjes et al. (1996) reported the unsuccessful non-surgical transfer of morulae, and Kato et al. (1997b) carried out unsuccessful surgical and non-surgical transfers of 6–8-cell embryos.

The results obtained in this study indicate that, in the horse, when follicular fluid derived from preovulatory follicles containing an expanded-cumulus oocyte is added to the IVM medium, it may improve the maturity of oocytes retrieved from small follicles and showing a compact cumulus at the time of retrieval. These observations may be related to those of Hinrichs (1997) who examined the relationship among oocyte–cumulus morphology, follicular atresia, initial chromatin configuration and oocyte meiotic competence in the horse, and reported that oocytes with expanded cumuli had greater meiotic competence than those with compact cumuli; however, compact-cumulus oocytes gained meiotic competence during growth of the preovulatory follicle as well as during follicular atresia, probably following the withdrawal of a suppressive factor within the follicle. Homologous FF did not appear to improve nuclear maturation but, in association with the ICSI procedure, promoted the subsequent fertilization and development of compact-cumulus oocytes, thus permitting the rescue of such oocytes for in-vitro production of equine embryos. On the other hand, when conventional IVF was applied, FF supplementation did not influence oocyte penetrability. This finding may indicate that, in the horse, FF contains substances specifically involved in the improvement of ooplasmic maturity but without effect on those external investments of the oocyte (corona radiata, intercellular matrix, zona pellucida and oolemma) which are involved in sperm penetration. In pigs and in humans FF has been shown to inhibit the binding of spermatozoa to zona pellucida in vitro (Funahashi and Day, 1993b; Y ao et al., 1996).

Our data are in agreement with those obtained in previous studies on the effects of FF supplementation of IVM medium in other species. In pigs, FF induced oocyte maturation in vitro and improved the rate of male and female pronuclei formation (Naito et al., 1988; Yoshida et al., 1992a; Funahashi and Day, 1993a) and subsequent developmental capacity (Naito et al., 1989, Yoshida et al., 1992b). Larocca et al., (1993), in a direct comparison on the effects of FF and oestrous cow serum, reported that the presence of FF in culture medium during IVM-IVF of bovine oocytes increased the fertilization rate and percentage of morulae/blastocysts obtained. Kim et al. (1993, 1996) and Romero-Arredondo et al. (1994) also reported beneficial effects of the addition of follicular fluid to the maturation medium on the maturation and developmental ability of bovine oocytes. In humans, the addition of mature FF to IVM medium of immature oocytes from unstimulated ovaries was shown to increase both the fertilization rates and embryo quality (Cha et al., 1991). The use of immature FF-exposed oocytes in association with the ICSI technique may be relevant in the treatment of human infertility where both partners are affected.

Further studies are required to evaluate the mechanism by which equine FF acts on the oocyte. High concentrations of steroid hormones in the follicular fluid have been positively correlated with nuclear and cytoplasmic maturation of oocytes in stimulated women (Lobo et al., 1985) and in the rhesus monkey (Morgan et al., 1990). In humans, intrafollicular FSH was suggested to act synergistically with oestradiol to enhance cytoplasmic maturation, resulting in successful fertilization (Suchanek et al., 1994). In this study the effects of the addition of two serum and two FF samples were assessed and oestradiol levels in these samples were significantly different (97.61 and 379.25 pg/ml in the two serum samples; 1.5 and 5.3 µg/ml in the two FF samples).

In conclusion, our data confirm the benefit of the addition of preovulatory FF to IVM medium and provide new information on the fertilization and developmental competence of oocytes collected from excised ovaries in the mare. This information was only obtained using ICSI since efficient IVF procedures have not yet been defined in the horse. Our findings also add further information to recent investigations on the role (Palmer et al., 1997) and the chemical composition of equine follicular fluid (Duchamp et al., 1996; Gerard et al., 1996; Collins et al., 1997) and confirm that the low IVF rate in the horse may be due to incomplete maturation of the compact COC in conventional IVM systems which subsequently leads to abnormal sperm–oocyte interaction. Further investigations are necessary to elucidate possible mechanisms of spontaneous zona hardening occurring during IVM culture of equine oocytes.
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