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Equine assisted reproduction and embryo technologies

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Abstract

Assisted reproductive techniques in the horse have been only recently become available compared to other domestic species, in particular ruminants. The scarce availability of abattoir ovaries and the lack of interest from horse breeders and breed associations, and the anatomical and physiological differences have been the main reasons for this delay. Progressively though, the technology of oocyte maturation *in vitro* has been established especially after the application of ICSI to obtain *in vitro* fertilization. The parallel improvement of oocyte maturation conditions and embryo culture media has increased the rates of embryo development from *in vitro* matured and *in vitro* cultured ICSI embryos from 5-10% in the early studies to up to 26% in the latest under experimental conditions with abattoir derived oocytes. In 2003, the birth of the first cloned foal established the technology of somatic cell nuclear transfer. The largest set of data on non-surgical embryo transfer of *in vitro* produced embryos, from ICSI of *in vitro*-matured Ovum Pick Up (OPU) oocytes, and from somatic cell nuclear transfer, has been obtained in our laboratory. In the clinical context, where OPU and ICSI are applied for the treatment of female and or male infertility, the yield of embryos has been lower compared to experimental conditions. In conclusion, the basic procedures have been established for the use of assisted reproduction and somatic cell nuclear transfer to a degree suitable for clinical applications and the results have been replicated in several laboratories around the world.

Keywords: cryopreservation, embryo culture, ICSI, oocyte maturation, sexing, somatic cell nuclear transfer.

Introduction

Besides classical reproduction techniques such as artificial insemination and embryo transfer (Squires *et al.*, 2003), advanced reproductive biotechnologies like oocyte recovery and maturation, oocyte transfer, *in vitro* fertilization, embryo culture, embryo manipulation and nuclear transfer were first established in ruminants and served as a translational model both for the human and the horse.

The flow of knowledge from the animal field that has advanced human assisted reproduction in the past is now reverted and some information from the human field is benefiting the animals and in particular the horse with intracytoplasmic sperm injection (ICSI), pre-implantation genetic diagnosis, vitrification of oocytes and embryos, etc. As a matter of fact the scope for application of ART in the horse is very much similar to the human: to reproduce individuals by overcoming physiological, pathological or technical barriers to reproduction.

The progress in advanced assisted reproduction in the horse has been continuous although at an irregular pace compared with other domestic species and major developments occurred only in the last 12 years. In this paper we will review the recent progress in assisted reproduction in the horse with the main emphasis on the procedures of oocyte recovery and maturation, ICSI, *in vitro* embryo production, pre-implantation genetic diagnosis, cloning, and cryopreservation.

Oocyte recovery

Ovum Pick Up of matured oocytes

The use of assisted reproduction in the horse is limited to animals with fertility problems, both from female and male origin or those engaged in sporting activities to produce in most cases a limited number of embryos and offspring. As a consequence, a number of techniques, including laparotomy, colpotomy or blind aspiration through the paralumbar fossa, have been attempted to recover oocytes from live donors without compromising their reproductive and sporting abilities. Oocytes can be collected from the preovulatory follicle 24 h after administration of hCG to a follicle that has reached at least 35 mm and the donor showing signs of uterine edema. Only mild superovulatory treatment has benefited oocyte recovery (Altermatt *et al.*, 2009). Ovulation normally takes place 36-40 h after hCG. Therefore oocytes collected at 24 h or later have resumed meiosis, have an expanded cumulus that facilitate recovery but require 16 h of additional culture prior to transfer. Oocytes are more frequently recovered by ultrasound guided transvaginal follicular aspiration using a double lumen 12G needle (Carnevale *et al.*, 2005). After recovery from the donor mare the oocytes complete their maturation *in vitro* and then can be surgically transferred to inseminated recipients

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(Carnevale, 2004) whose preovulatory oocyte has been aspirated or can be subjected to ICSI for being transferred surgically to the oviduct of a synchronized recipient. In a clinical setting the success rate of establishing a pregnancy with oviduct transfer is in the range of 40% (Carnevale, 2004).

Ovum Pick Up of immature oocytes

Oocytes from antral follicles can be recovered as it is done in cattle (Galli *et al.*, 2001), defined as ultrasound guided transvaginal oocyte recovery or Ovum Pick Up (OPU). Similarly to its use in other species, this technology, developed in human medicine, started in the early nineties and has been subsequently refined (McKinnon *et al.*, 1998; Cochran *et al.*, 2000). This technology has been proven safe and repeatable in cattle and has the advantage of not requiring any hormonal stimulation of the donor. This aspect is of considerable importance in the horse because of the limitations of superovulation but also because it is difficult to handle large ovaries afterward for oocyte recovery. Oocyte recovery from immature follicles in the horse requires vigorous flushing and scraping of the follicle. There are several data reported in the literature for the recovery of immature oocyte recovery (Bogh *et al.*, 2003; Vanderwall *et al.*, 2006) but very limited data to assess of the developmental potential of such oocytes for producing horse embryos on a routine basis.

In our laboratory we have carried out a large study on the developmental capacity of immature oocytes recovered by OPU from both experimental and commercial donors within a commercial OPU and we currently offer this service to horse breeders (Colleoni *et al.*, 2007). The data reported in Table 1 refer to repeated OPU collections from donors of various breeds within a clinical OPU programme. Donors are monitored by ultrasound to select the best time for oocyte collection when there are several antral follicles and preferably the absence of a dominant follicle. This situation is particularly favorable during the transition season (no dominant follicle with many medium antral follicles). We do not collect normally oocytes from mares that have less than 5-7 follicles and we wait for more follicles to grow. The interval between collections is 2-4 weeks depending on the mares. Mares can be subjected to repeated collections without any side effect if the procedures are performed correctly (Mari *et al.*, 2005). We sedate the donor with detomidine and give epidural anesthesia to avoid the contraction of the rectum during ovary handling and catheterize the bladder. At the end of the procedure the donor is treated with antibiotic for 3 days. Other programs have reported fixed biweekly schedule (Jacobson *et al.*, 2010).

Table 1. Average embryo production from an OPU session at Avantea in a clinical setting. Donors are monitored by ultrasound scanning to select the appropriate moment to avoid the presence of pre-ovulatory follicles in favor of medium sized follicles (10-20 mm). Matured oocytes are subjected to ICSI and cultured for 7-8 days to the blastocyst stage when embryos are cryopreserved.

	Number	Rate (%)
Follicles	16.1	--
Oocytes	10.8	67.3
Matured oocytes	6.9	63.7
Cleaved oocytes	3.7	54.3
Blastocyst	0.5	7.4

Data from approximately 700 OPU sessions.

Oocyte collection from ovaries collected at the abattoir or after euthanasia

The collection of oocytes requires the dissection of the connective tissue enclosing the ovary, the incision of visible follicles and scraping of the inside follicle wall with a curette and extensive flushing to detach the cumulus-oocyte complexes (COCs). In the horse, two populations of COCs can be identified and oocytes with expanded cumulus mature normally and have normal developmental competence contrary to what is seen in other species. Table 2 presents the data from a large study conducted in our laboratory that gives an exact measure of the efficiency of oocyte collection from abattoir ovaries and the expected maturation rate from compact and expanded COCs.

Table 2. Maturation competence of horse oocytes derived from expanded or compact COCs.

No. ovaries	No. follicles (per ovary)	No. COCs expanded (per ovary)	No. COCs compact (per ovary)	No. COCs matured after IVM		No. COCs degen. after IVM	
				Expanded (%)	Compact (%)	Expanded (%)	Compact (%)
603	3204	590	1672	354	855	177	558
	5.3	1	2.8	(60.0%)[a]	(51.1%)[b]	(30.0%)	(33.4%)

Chi square test: values with different letters differ ($P < 0.05$). Galli *et al.* (2007).

Oocyte maturation in vitro and conventional *in vitro* fertilization

The first successful report of *in vitro* maturation of horse oocytes was that of Fulka and Okolski (Fulka and Okolski, 1981). The first embryo production from *in vitro* matured horse oocytes was reported in 1989 (Zhang *et al.*, 1989) when oocytes collected at the abattoir were matured *in vitro* and, after transfer to the oviducts of inseminated mares and recovery by uterine flushing 7 days later, had developed to the blastocyst stage. Oocytes are matured *in vitro* for 24-28 h at 38.5°C in 5% CO2. In our culture conditions, as shown in Table 2, the maturation rate ranges from 51.1 to 60% for compact and expanded COCs respectively. This difference is statistically significant and in agreement with other studies (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000; Hinrichs *et al.*, 2005) in which oocytes with expanded cumulus were found more capable to complete maturation than were oocytes with compact cumuli. It is interesting to note that in Table 2 the distribution of the degenerated oocytes is equal between the compact and expanded COCs groups. This is somehow surprising because, in other species, the presence of a compact and healthy cumulus cell layer is considered an indicator for good oocyte morphology. Since no lysis is observed with oocytes collected by OPU from live donors (Fig. 1a and b), this finding implies that the post mortem modifications occurring in the large equine ovaries are responsible for the degeneration of abattoir oocytes. Indeed, when equine oocytes were placed into maturation immediately after slaughter, the rate of maturation was higher and the rate of degeneration lower than those for oocytes recovered after transport of ovaries to the laboratory (Hinrichs *et al.*, 2005).

Interestingly, only two foals were reported as born from IVF and both were derived from *in vivo* matured oocytes collected by OPU from gonadotropin stimulated donors (Palmer *et al.*, 1991; Bezard, 1992). There is no published report of equine pregnancies derived from both *in vitro* maturation and conventional IVF. Failure of IVF remains a mystery but is probably related to inefficient sperm capacitation (Alm *et al.*, 2001), changes in the zona pellucida (Dell'Aquila *et al.*, 1999; Hinrichs *et al.*, 2002) or to incomplete *in vitro* maturation (Li *et al.*, 2001).

To date, a variety of oocyte maturation conditions has been evaluated using different maturation media comprising TCM199 (Willis *et al.*, 1991; Dell'Aquila *et al.*, 1997; Hinrichs and Schmidt, 2000; Galli *et al.*, 2002b; Lagutina *et al.*, 2005), B2 (Willis *et al.*, 1991) or DMEM/F12 (Galli *et al.*, 2007; Table 3), supplemented with different concentration of serum, hormones or follicular fluid. Recently a paper was published (McPartlin *et al.*, 2009) reporting high *in vitro* fertilization rates utilizing procaine for sperm capacitation, however there was no evidence of sperm penetration and the results were based only on the segmentation of the oocytes that could well be fragmentation or parthenogenetic activation. No embryo development was reported and the findings not replicated in other laboratories.

Table 3. Effect of maturation media on maturation, cleavage, and development rate of ICSI embryos.

Maturation medium	No. oocytes	No. degenerated	No. met. II (%)	No. injected	No. cleaved (% of injected)	No. blastocysts (% of injected)
TCM 199	434	105	205 (47.2%)[a]	191	111 (58.1%)[a]	23 (12.0%)[a]
DMEM/F12	338	71	159 (45.6%)[a]	140	108 (77.1%)[b]	37 (26.4%)[b]

replicates = 6

Chi Square test. Numbers within columns with different letters differ ($P < 0.05$). Galli *et al*. (2007).

Intracytoplasmic sperm injection (ICSI) and embryo culture

The clinical use of ICSI was developed several years ago to overcome male infertility in human assisted reproduction (Palermo *et al.*, 1992). The technique works so efficiently that it is now the preferred way of *in vitro* fertilization in humans even if it is suggested that sperm selection does not occur in a physiological way and it might carry abnormalities to the offspring. However when data are adjusted for age, twin pregnancies, infertility and other patient related factors ICSI offspring is not different from normal offspring (Palermo *et al.*, 2008, 2012). The application of ICSI to the horse (Fig. 1c), on the other hand, has overcome inefficient conventional IVF, irrespectively of the fertility of the semen used, resulting in the first pregnancy derived from an *in vitro* matured oocyte (Squires *et al.*, 1996), which was carried successfully to term. This success was followed by a period of variable results until the development of ICSI using the piezo drill, which removed most of the inconsistency of the technique due to a heterogeneous and thick zona pellucida difficult to penetrate with conventional ICSI pipettes, especially in *in vitro* matured oocytes with minimal damage to the oocytes. Moreover the advantage of ICSI is the ability to widen the choice of the stallions to be used, including those with poor sperm motility and reproductive performance *in*

vivo. In an original study, Lazzari *et al.* (2002a) compared the developmental capacity of *in vitro* matured oocytes fertilized by ICSI with frozen-thawed stallion semen of different motility and/or different fertility in the field. There was no difference in either cleavage or advanced embryo development rate among oocytes injected with sperm from stallions of good, poor and no fertility in the field, as long as a motile sperm was selected for ICSI (Table 4). These results indicate that ICSI can allow the use of semen with poor motility or no *in vivo* fertility, provided that a motile sperm cell is selected for injection. In another study (Choi *et al.*, 2006), oocytes injected with immotile sperm isolated from semen subjected to two freeze-thaw cycles were capable of blastocyst development. In a further study the same authors (Choi *et al.*, 2011a) have even used lyophilized sperm and activation with sperm extract to generate offspring.

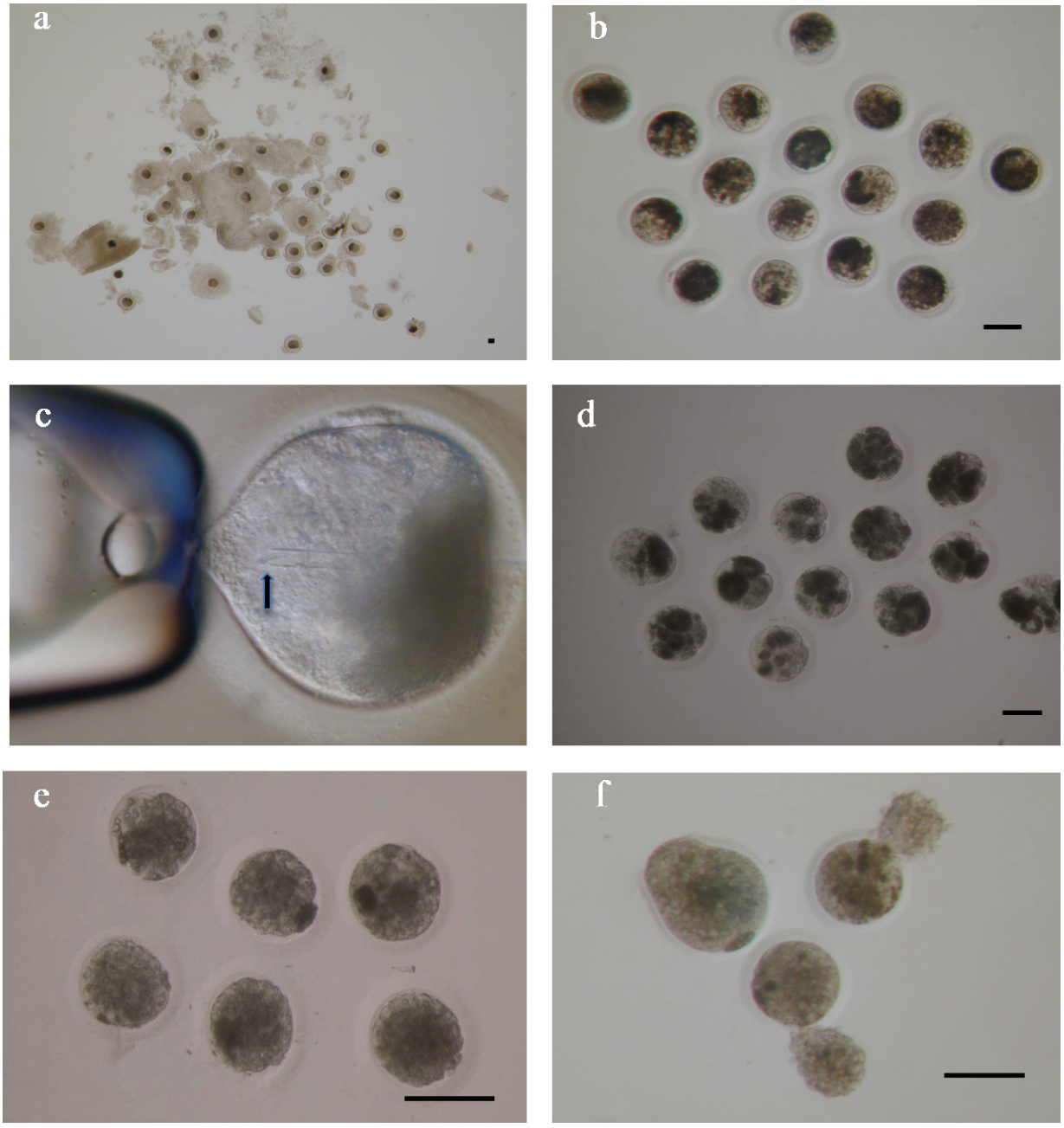

Figure 1. a) Oocytes collected from one OPU session: most of the oocytes display a compact cumulus. b) Denuded oocytes after *in vitro* maturation and selected for the presence of the first polar body ready for ICSI. c) Oocyte being injected with a spermatozoa indicated by the arrow. d) Cleaved embryos 48 h after ICSI. e) Embryos selected for freezing and or transfer on day 7 of development. f) When embryos are cultured *in vitro* for long time (up to day 10+) they begin the extrusion of cells from the hole made with the ICSI pipette.

Table 4. Cleavage and development of horse embryos following ICSI with semen of different motility and fertility post-thawing.

Group of stallions	No. met. II injected	No. Cleaved	Cleavage %	No. comp. morulae and blastocysts	% comp. morulae and blastocysts/injected
A	117	88	75.2[a]	42	35.9[a]
B	78	49	62.8[a]	21	26.9[a]
C	91	72	79.1[a]	33	36.3[a]
D	46	4	8.7[b]	0	0.0[b]

Chi square test. Numbers within columns with different letters differ (P < 0.05). Lazzari *et al.*(2002).

The progress of *in vitro* maturation and ICSI technology has increased efforts to design suitable culture systems for early cleavage stage embryos (Fig 1d). Many different culture conditions have been reported for preimplantation development of ICSI fertilized horse oocytes, including defined media such as G1.2 (Choi *et al.*, 2002), DMEM-F12 and CZB (Choi *et al.*, 2004) and modified SOF (Galli *et al.*, 2002b). In most of these systems, however, the blastocyst rates remained low, ranging from 4 to 16%. In contrast, the culture of presumptive zygotes following ICSI, *in vivo* either in the mare oviduct or in the surrogate sheep oviduct, allowed much higher development (Galli and Lazzari, 2001; Table 5).

A comparison between the published reports on *in vivo* culture of ICSI early cleavage stage embryos in the oviducts of mares (Choi *et al.*, 2004) or temporary recipient sheep (Galli *et al.*, 2002a; Lazzari *et al.*, 2002a) and *in vitro* culture in various culture media (see above) clearly demonstrated that the *in vivo* environment supports higher blastocyst development, being approximately 36% of injected oocytes in both the mare oviduct and sheep oviduct. Recently, an *in vitro* culture system has been developed using DMEM/F-12 medium under a mixed gas atmosphere that provides blastocyst development rates similar to those seen *in vivo* (27-38%) (Alm *et al.*, 2008; Jacobson *et al.*, 2010) (Fig 1f). However when cell number counts were compared (Tremoleda *et al.*, 2003) among *in vivo* produced embryos and those produced by *in vitro* culture in a modified SOF medium, both on day 7 of development, *in vitro* produced embryos had significantly lower cell numbers, resembling a day 5 *in vivo* embryo rather than a day 7.

Table 5. Oocyte recovery rate by OPU and effect of *in vitro* or *in vivo* sheep oviduct culture on embryo development.

	No. OPUs	No. oocytes (no. per OPU)	No. met. II (% of oocytes) (no. per OPU)	No. cleaved (% of injected) (no. per OPU)	No. comp. morulae/blastocysts (% of injected) (no. per OPU)
Sheep oviduct culture	20	60 (3.0)	46 (76.7%) (2.3)	41 (89.1%)[a] (2.1)	23 (50.0%)[a] (1.2)
In vitro culture	12	46 (4.1)	36 (73.5%) (3.0)	25 (69.4%)[a] (2.1)	5 (13.9%)[b] (0.4)

Student T test. Numbers within columns with different letters are significantly different (P < 0.05). Galli and Lazzari (2001).

Somatic cell nuclear transfer

The first equine clones, three mules and one horse, were reported in 2003 both from fetal cell (Woods *et al.*, 2003) and from adult cells (Galli *et al.*, 2003). The first horse, a filly, was obtained by nuclear transfer of adult somatic cells, using *in vitro* matured oocytes as recipients of the donor nuclei and *in vitro* culture to the blastocyst stage before non surgical transfer to recipient mares. In that study, the foal was born to the same mare that donated the cells used in the cloning procedure, representing an exclusive example of autologous pregnancy successfully gone to term. Other cloned foals were born (Lagutina *et al.*, 2005; Hinrichs *et al.*, 2006) by SCNT, demonstrating the reproducibility of this technology. Additionally, two cloned foals were born in 2005 in another laboratory confirming that the cloning technology is now established in horses (Hinrichs *et al.*, 2006) and that it can be done by using *in vitro* matured oocytes. More foals have been produced (Hinrichs *et al.*, 2007) in the same laboratory and in another laboratory (Gambini *et al.*, 2012). Other activities are undergoing in commercial laboratories but there is no published information about their activities and results. Development rate of SCNT embryos is greatly influenced by the cell line (Lagutina *et al.*, 2005) which is well known in other species and varies from 0 to 17%. Oocyte maturation *in vitro* is the only sustainable

source of oocytes for cloning and their quality is as critical as it is for ICSI. We have also tested the effect of changing maturation medium from TCM199 to DMEM-F12 on nuclear transfer (NT) embryo development (Table 6) and we have confirmed the positive results obtained with ICSI embryos. Enucleation of the oocytes and nuclear transfer can be obtained by the zona-free method followed by the electrical fusion of the somatic cells and the oocyte (Lagutina *et al.*, 2007) or by using the piezo-electric manipulator that is used both for enucleation and for the injection of the somatic cell after breaking its cell membrane (Hinrichs *et al.*, 2006). Activation of the reconstructed embryos is performed in our laboratory by using a combination of the two most common chemicals used in other species for induction of parthenogenetic development: 6-dimethylaminopurine (6-DMAP) and cycloheximide (Lazzari *et al.*, 2002b). Other workers (Hinrichs *et al.*, 2006) have used a combination of injection of sperm extract and culture in 6-DMAP to produce embryos resulting in successful foaling of cloned offspring. Although the development to term of cloned pregnancies is low as in other species, most of the pregnancy losses occur early in gestation (before day 50) thus creating less problems with the recipient management and the numbers of recipients required that can eventually be re-used. Moreover the foaling and foal survival are normal and we do not see the problems reported with cattle, for example of hydrops, placenta hyperplasia, and large offspring syndrome. Most of the foals are normal or require minor assistance at birth (Johnson *et al.*, 2010).

Table 6. Effect of maturation media on maturation, cleavage, and NT embryo development.

Maturation medium	No. oocytes	No. lysated	No. met. II (%)	No. NT embryos	No. cleaved (% of NT)	No. blastocysts (% of NT)
TCM 199	164	35	64 (39.0%)[a]	41	38 (92.7%)[a]	4 (9.8%)[a]
DMEM/F12	166	35	67 (40.4%)[a]	47	46 (97.9%)[a]	13 (27.7%)[b]

Chi Square test. Numbers within columns with different letters differ ($P < 0.05$). Galli *et al.* (2007).

Semen and embryo sexing and pre-implantation diagnosis

Sexing of semen in the horse has been done using flow cytometric cell sorting based on DNA quantitative differences between X and Y bearing sperm (Garner, 2006). The number of sperm required for a standard dose of artificial insemination cannot be obtained with the current technology, therefore, sexed semen can only be used for a low dose insemination with a fraction of the sperm that are normally used for artificial insemination (Lindsey *et al.*, 2002a, b). Results are much lower when using frozen sex sorted sperm (Clulow *et al.*, 2008). The low fertility, associated with loss of pregnancies (Gibb *et al.*, 2012), does not make it commercially viable for the industry also when used for embryo transfer programs. The natural way to use low number of sperm with poor viability would be ICSI. We have used sex sorted frozen thawed semen with ICSI (Colleoni *et al.*, 2009) and obtained very low cleavage but normal development (overall 10% of the efficiency obtained with the non sexed control semen), pregnancies and live foals. In another report no success was reported using sexed refrigerated semen for ICSI (Samper *et al.*, 2012). An alternative approach is the use of embryo biopsy and PCR for sex determination. Making the biopsy is complicated in the horse by the presence of the capsule. The biopsy is taken by aspiration with a micropipette driven by piezo electric micromanipulator without compromising the viability (Choi *et al.*, 2010). After recovery of the biopsy composed by few cells, it is subjected to WGA (whole genome amplification). This procedure generates sufficient DNA not only to perform sex determination (Peippo *et al.*, 1995; Bannasch *et al.*, 2007) but also for pre-implantation genetic diagnosis (Hinrichs and Choi, 2012).

Embryo cryopreservation

To date most of the equine embryos are transferred fresh or after cooling at 4°C for up to 24 h and very few are cryopreserved. The main obstacle to this development has been the poor success with embryos larger than 300 µm that are normally recovered at flushing. Collecting embryos earlier so that they are smaller than 300 µm significantly reduces the recovery rate. Smaller embryos stand very well classic slow freezing procedures with 10% glycerol but a freezing apparatus is required (Stout, 2012). Vitrification can also be used for cryopreservation; again smaller embryos survive better than larger ones (Eldridge-Panuska *et al.*, 2005), a freezing machine is not required, and kits for vitrification are available on the market. In an attempt to improve the survival of large embryos (Choi *et al.*, 2011b) it was noticed that collapsing the blastocele cavity, as it happens when taking a biopsy for sexing, improved survival, this is

probably due to the reduction of the large amount of fluid contained in the cavity that cannot be permeated by the cryoprotectant in normal conditions. In this respect *in vitro* produced embryos through ICSI can be selected at the appropriate stage of development, before they get too large, for slow freezing. Pregnancy rate have been in the range of 50 to 60%, making *in vitro* embryo production even more attractive for this advantage (Colleoni *et al.*, 2007).

Embryo transfer, pregnancies, and offspring

The true measure of oocyte developmental competence is the ability to generate viable offspring following the transfer, even though a percentage of failures can also be attributed to the recipient itself. Therefore, one of our research priorities, given also its practical application, was to show that the embryos produced with the techniques described above had normal developmental competence and therefore validated the steps involved including *in vitro* maturation, ICSI, embryo culture, and conventional cryopreservation in 10% glycerol. After 10 years of work in clinical practice using OPU and ICSI we can say that *in vitro* produced embryos have high survival rate after transfer with high pregnancy rates even after freezing and thawing. Pregnancy rates vary from 40 to 60% with a foaling rate between 35 to 50%. The transfer of *in vitro* produced embryos is done in recipients 5 days after ovulation since they are at the early blastocyst stage at day 7 of *in vitro* culture and this chronologically asynchronous transfer gives time to the embryo to "catch up" the delay that is present in development after *in vitro* culture. Pregnancy losses and perinatal mortality in our experience has been comparable to that obtained after artificial insemination. We never observed the phenotype of the LOS (large offspring syndrome) occasionally reported for ruminants.

Conclusions

Oocyte developmental competence is clearly the key requirement for all the technologies that have been described and the main bottleneck. Oocyte transfer, briefly described here, relies on *in vivo* matured oocytes and provides very high pregnancy rates but in small numbers. In addition, the animal welfare and economic issues involved, have given a strong thrust to the development of *in vitro* procedures for oocyte maturation. ICSI has allowed testing of the relationship between *in vivo* and *in vitro* fertility of stallions, demonstrating that even semen with poor fertility in the field can be successfully used for embryo production *in vitro* (Lazzari *et al.*, 2002a). However, sperm too play a significant role in determining embryo development and there are also in this case, as for the oocytes, extreme cases of infertility. Blastocyst production rate is influenced not only by culture environment, *in vivo* versus *in vitro*, but also by oocyte maturation conditions. The change of maturation medium from conventional TCM199 to DMEM-F12 has a pronounced effect on blastocyst rate in our laboratory, suggesting that probably there is still room for improvement in *in vitro* maturation conditions of equine oocytes. The culture environment, *in vivo* and *in vitro*, has been extensively tested and although the *in vivo* methods, either the mare oviduct or the surrogate sheep oviduct, have proven superior, the *in vitro* culture systems have improved considerably allowing high rates of blastocyst development. A common feature of all the *in vitro* culture systems used is that the embryos tend to be retarded with fewer cell numbers than expected. This finding has suggested that we should allow some time for the day 7-8 IVP embryos to catch up with normal development by transferring them into recipient mares 5 days after ovulation instead of 7 days. Pregnancy rates, following non-surgical transfer of ICSI embryos, have been acceptable for clinical use even after cryopreservation. Similarly, cloned embryos produced from *in vitro* matured oocytes are able to establish pregnancies after *in vitro* culture to the blastocyst stage followed by non-surgical transfer. All this work opens the way to the successful clinical application of assisted reproduction technologies in the horse. However, until the industry takes a more open attitude towards these technologies and allows registration of the animals obtained even by the more controversial techniques such as cloning (Church, 2006), many of these technologies will remain at the experimental stage.

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