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—Review—

Current Status of IVM/IVF/IVC Technology and Embryo Cryopreservation in the Equine Species

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There are many unique and interesting features of equine reproduction. For example, fertilized ova are retained at the oviductal ampullary-isthmus junction for as long as 120 hours post-ovulation, and late morula- to early blastocyst-stage embryos are in transit from the ampulla to the uterus for up to 132 hours post-ovulation [1–3]. Unfertilized ova and degenerating fertilized ova remain trapped in the ampullary-isthmus junction over several subsequent estrous cycles [4]. After the selective and rapid transport of equine embryos through the oviductal isthmus, the mucin-like embryonic coat, the so-called capsule, is deposited on the inner surface of the zona pellucida of early blastocysts coincident with expansion of the blastocoele [5, 6]. The capsule surrounds the embryo and maintains its spherical form until the fourth week of pregnancy [7, 8]. To investigate these two developmental phases of equine embryos (in the oviductal ampulla and in the uterus), the establishment of an *in vitro* model for early embryonic development would be desirable. However to date, the *in vitro* production of equine embryos has not been very successful [9]. There has been only one full-report of a foaling following IVF and this has been accomplished following *in-vivo* maturation of the oocyte [10]. More recently, there have been two foals produced by intracytoplasmic sperm injection (ICSI) at two separate laboratories [11].

The objective of this review is to describe the accumulated results of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of equine oocytes, as has also been done by Hinrichs [9]. In addition, recent progress in the cryopreservation of equine embryos is described.

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In Vitro Maturation

The source of mature oocytes is important for the success of equine embryo production *in vitro*. In cattle, superovulatory treatment combined with transvaginal ultrasound-guided ovum pick-up (OPU) has permitted the recovery of large numbers of mature oocytes from live animals. The first IVF foal was derived from mature oocytes recovered by a predecessor of this method-blind follicle aspiration through the flank [10], but the effectiveness of OPU is very limited in equine species because no regimen for the superovulation of mares has been established. Continuous FSH administration to cycling mares resulted in an increase in the number of multiple-ovulating mares, but no increase in embryo yield [12]. Moreover, the rate of recovery of equine oocytes from immature follicles by OPU was reported to be poor [13, 14]. The poor oocyte recovery from immature follicles in the mare may be related to the type of attachment of equine oocytes to the follicular wall [15].

An alternative approach to recovering equine oocytes is to use abattoir-derived materials. Since the parenchymatous zone containing follicles lies in the center of the ovary, most follicles are not visible on the ovarian surface (Fig. 1). The number of equine oocytes which can be recovered by follicle aspiration with needle and syringe, as done in cattle, was only 1.5 [16] to 2.7 oocytes per ovary [17]. The efficiency of oocyte recovery by aspiration can be increased by repeatedly flushing the follicles [18]. Oocyte recovery by dissection of individual follicles resulted in 3.1 [19] to 6.1 oocytes per ovary [20], but this method is labor-intensive and time-consuming. We have applied a technique for cutting the aspirated equine ovaries into slices 5 mm thick, followed by washing the slices with mPBS [21]. This combined method of follicle aspiration and slicing of the

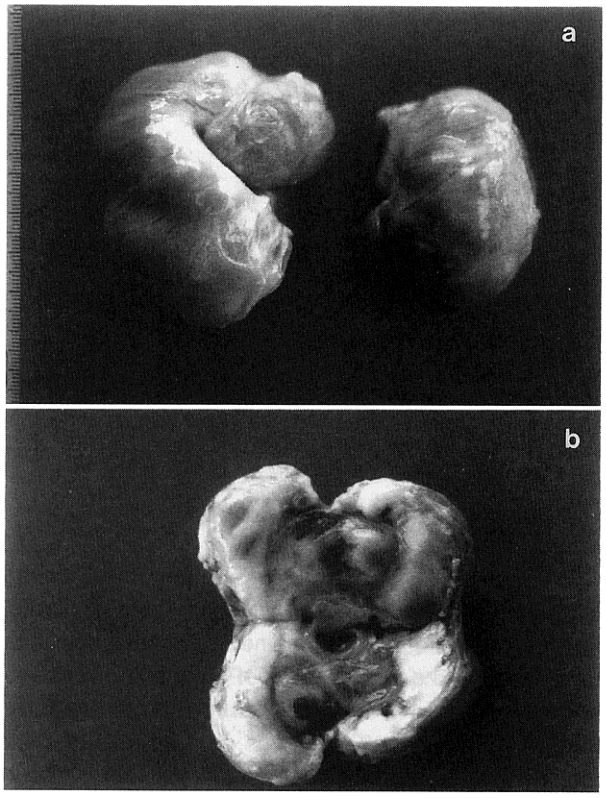

Fig. 1. Photographs showing typical equine ovaries (a: Exterior, b: Vertical section). Since the parenchymatous zone containing follicles lies in the center of the ovary, most follicles are not visible on the ovarian surface.

ovaries provided a recovery rate of 5.9 oocytes per ovary, and more than 80% of the oocytes surrounded with a compact cumulus cell layer were in the germinal vesicle (GV) stage [21]. The season of the year and breed of mare were found to be sources of variability in the rate of recovery of equine oocytes [22].

Immature equine oocytes can be successfully cultured to the metaphase-II (M-II) stage in the IVM system that is routinely used for bovine oocytes. Similar proportions of nuclear maturation (45–60%) have been reported from several laboratories [19–21, 23–25], except for a higher one (>80%) [18]. The time required for nuclear maturation of immature equine oocytes (32–36 h) was also similar in several laboratories [18–21, 24, 25], with the exception of Willis *et al.* (15 h) [23]. In our study [21], the proportion of M-II stage oocytes at 16 h of culture was below 10%, and a significant increase in oocytes at the M-II stage was observed between 16 and 24 h and again between 24 and 32 h of culture (Fig. 2). This time range required for nuclear maturation of equine oocytes *in vitro* (32–36 h) was similar to the time between hCG stimulation of preovulatory mares and the subsequent ovulation (36 h) [26]. The total time interval from slaughter at the abattoir to oocyte recovery at the laboratory was an important factor influencing the rate of nuclear maturation of equine oocytes *in vitro* [22]. It has been difficult to assess the quality of the IVM oocytes in the absence of a reliable IVF system. The ultrastructure of equine oocytes cultured for 15 h [27] had some different features from that of preovulatory oocytes reported by others [28, 29]. Even

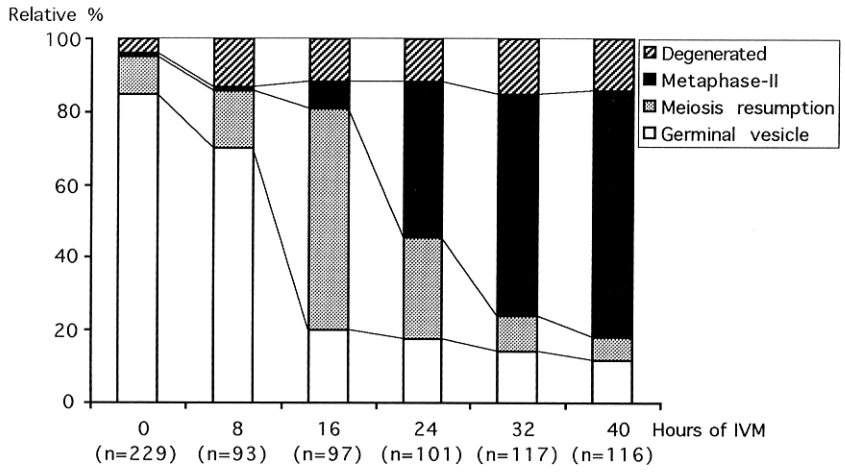

Fig. 2. Time course of *in vitro* maturation of equine cumulus-oocyte-complexes in TCM199 supplemented with 10% fetal bovine serum, 1 μg/ml estradiol 17β and 0.02 AU/ml FSH [21].

after prolonged maturation culture of equine oocytes, the migration of cortical granules to the oolemma has been found to be incomplete [30], suggesting an asynchrony between nuclear and cytoplasmic maturation.

For the purpose of mass production of experimentally valuable equine embryos, oocytes can be surgically transferred to the oviducts of bred recipient mares. Fertilization of the transferred oocytes *in vivo* and recovery of multiple embryos can be expected. This approach is also useful to assess the quality of equine IVM oocytes. When a total of 29 IVM oocytes were transferred to the oviducts of 4 bred recipient mares, 7 blastocysts were recovered from 2 recipients 8 days after the transfer [31]. Two of these 7 blastocysts were transferred to second recipients, resulting in the development of one fetus. This study was important in that it demonstrated the full developmental competence of equine oocytes that matured completely *in vitro*. A report of more successful equine oocyte transfer was from Hinrichs *et al.* [32]; they used the oocytes aspirated from follicles 24 h after hCG stimulation, followed by an additional maturation culture for 12 or 18 h. The *in vivo* + *in vitro* matured oocytes after oviductal transfer resulted in an overall pregnancy rate of 75% on Day 11–14.

In Vitro Fertilization

Protocols for successful equine IVF have not yet been established. A living foal has been born from the transfer of an equine IVF oocyte which had been recovered from preovulatory follicles of mares 34 h after hCG administration [10]. The limited success rate of equine IVF (4% [33], 17% [34], 26% [35], 30% [10], 33% [19], 40% [36]) has been considered to be due to the lack of an efficient *in vitro* capacitation system for stallion spermatozoa. The calcium ionophore A23187 was used for induction of capacitation of stallion spermatozoa in most of these studies. The stallion spermatozoa were treated either for 5–10 min with 6.0–7.14 μM ionophore A23187 in BSA-containing medium [10, 19] or for 1 min with 0.1 μM ionophore A23187 in BSA-free medium [33, 34]. Incubation of stallion spermatozoa with 5 mM caffeine for 4 h after ionophore treatment increased the rate of male pronucleus formation in the penetrated oocytes [34]. Treatment of stallion spermatozoa with 10 μg/ml heparin, as used for induction of sperm capacitation in bovine species, has resulted in no increase in acrosome-reacted spermatozoa [37]. Although stallion spermatozoa required incubation with more than 50 μg/ml heparin to induce the acrosome reaction [37], a recent study has indicated that 13–18% of equine IVM oocytes with intact cumulus cells could be penetrated by stallion spermatozoa in the presence of 1 μg/ml heparin [36]. In other studies, frozen-thawed [33] or fresh [34] stallion spermatozoa were washed by centrifugation on Percoll-gradient. The Percoll-washed stallion spermatozoa exhibited an increased binding to, and penetration of, the zona pellucida [38], and lysophospholipid-induced acrosome reaction (see next paragraph) of Percoll-washed stallion spermatozoa occurred more frequently than that of fresh ejaculated spermatozoa (Hochi, unpublished).

There has been some progress in developing techniques for capacitating stallion spermatozoa, but the success has been evaluated in terms of sperm penetration of zona-free hamster oocytes [16, 39, 40]. The rate of spontaneous acrosome reaction is often used as a criterion of capacitation in a sperm population, although a physiological acrosome reaction is unlikely to occur in the absence of the zona pellucida [41]. Since the hamster test detects whether the spermatozoa have acrosome reacted, the endpoint may be positive even if only a few spermatozoa were capacitated. A more reliable system to assess sperm capacitation in a sperm population involves assessing the ability of capacitated spermatozoa to display an acrosome reaction in response to an exogenous stimulus. Lysophosphatidylcholine (LPC) has been shown to induce the acrosome reaction only in capacitated bull spermatozoa, with no effect on non-capacitated spermatozoa [42]. Using this system, we compared the ability of various chemicals (10 μg/ml heparin, 0.5 mM hypotaurine, 5 mM caffeine, 0.1 μM ionophore A23187) to induce capacitation of stallion spermatozoa *in vitro* [43]. Ionophore A23187 had the most striking effect on inducing capacitation in stallion spermatozoa, followed by caffeine, hypotaurine and heparin. The use of zona-free equine oocytes for a sperm penetration assay, however, did not always reflect the extent of sperm capacitation. This may be explained by the short life span of capacitated/acrosome-reacted stallion spermatozoa.

For successful equine IVF, the qualitative aspects of equine IVM oocytes need to be improved. IVM of abattoir-derived GV stage oocytes may alter the zona pellucida and thus induce some dysfunction such as zona hardening [35, 44]. In addition, equine oocytes with compact cumulus cell layers cultured for 32 h for nuclear maturation have been found to have non-expanded cumulus layers inside and expanding cumulus layers outside (Hochi, unpublished). The projections of cumulus cells through the zona pellucida may still be intact in equine IVM oocytes, since the hyaluronidase treatment of IVM oocytes was not enough to remove all

the cumulus cells without the aid of mechanical pipetting [33]. Normal fertilization rates of equine IVM oocytes *in vitro* were higher after partial cumulus removal than in cumulus-intact oocytes (33 vs. 12%) [36]. When equine GV stage oocytes with expanded cumulus cell layers were cultured, they reached the M-II stage within 24 h of culture [20, 31]. Pronucleus formation in equine IVM oocytes with expanded cumulus cells following IVF was higher than in those with compact cumulus cells (17 vs. 4%) [45], so that extra-ooplasmic changes in equine oocytes during IVM, as well as nuclear and cytoplasmic maturation, must be studied further.

Assisted fertilization techniques have been applied as an alternative method for equine embryo production *in vitro*. We examined the effect of partial zona dissection (PZD) and partial zona removal (PZR) on *in vitro* fertilization of equine IVM oocytes [33]. As shown in Table 1, the rate of fertilization of PZR-treated equine oocytes (54%) was higher than those of zona-intact (1–3%) or PZD-treated (12%) oocytes, but lower than that of zona-free (84%) oocytes. Overall efficiency in producing monospermic fertilized oocytes was comparable in PZR-treated and zona-free oocytes (34 vs. 27%). The PZR procedure was superior to PZD, possibly because spermatozoa were able to cross the zona pellucida more easily. It has been reported that 79% of equine IVM oocytes with a zona drilled by acid Tyrode's solution have cleaved after IVF [44] but the penetration and polyspermy rates were not determined. These zona-manipulation techniques depend on the presence of capacitated and acrosome-reacted spermatozoa which can undergo fusion with the vitelline membrane of the oocytes. No intentional selection of spermatozoa is required for fertilization. In contrast, ICSI, widely used for treatment of male infertility in humans, does not require capacitation, acrosome reaction, or even motility of the injected spermatozoa. The first successful ICSI in horses was reported by Squires *et al.* [46], as 1 of 4 IVM oocytes resulted in development to a fetus up to Day 75 of pregnancy. More recently, it was reported by an another group that 52% of equine ICSI oocytes exhibited normal pronucleus formation but only 20% of the IVF oocytes did [45]. Male pronucleus formation was observed 10–20 h after PZR/IVF [33] or ICSI [47]. The timing of pronucleus formation was in accordance with observations *in vivo* [28] and *in vitro* [19, 34].

In Vitro Culture

Little information on the development of cleavage-stage equine embryos is available, mainly due to the shortage of experimental embryos. Foals from IVF [10] and ICSI [11, 46] resulted from the surgical transfer of very early cleaving embryos into the oviduct of recipient mares. To date, no pregnancies have been reported from IVMFC embryos developing to a stage when they were transferable to the mare's uterus.

Due to poor equine IVF protocols, most studies on *in vitro* culture have used embryos recovered from oviducts of bred mares. In an earlier study [26], cleavage of 1-cell embryos to the 14-cell stage was observed in Menezo's B2 medium supplemented with 15% fetal bovine serum. The earliest stages of embryogenesis are regulated by maternally inherited components stored within the oocytes, and the activation of the paternal genome coincides with a sensitive period in *in vitro* culture for most mammalian species [48]. It has been reported that paternal genome activation in the equine species occurs at the 8-cell stage [49, 50], and the *in vitro* developmental block of equine embryos is also

Table 1. Fertilizing ability of partially zona dissected and partially zona removed equine oocytes *in vitro*

IVM oocytes	No. (%) of oocytes		
	Examined	Penetrated	Monospermic
Intact cumulus	157	1 (1)[a]	1 (1)[a]
Denuded, zona-intact	152	5 (3)[a]	3 (2)[a]
Partially zona dissected	58	7 (12)[a]	4 (7)[a]
Partially zona removed	108	58 (54)[b]	37 (34)[b]
Zona-free	123	101 (82)[c]	33 (27)[b]

[a–c] Different superscripts within columns denote significant differences ($p<0.05$). Thawed and Percoll-washed stallion spermatozoa treated with 5 mM caffeine (3 hours) and 0.1 μM calcium ionophore A23187 (1 min) were mixed with the micromanipulated oocytes for 20 hours [33].

observed around the 8-cell stage [50, 51]. A proportion of 1- to 2-cell equine embryos (35%) could develop into blastocysts when cocultured with equine oviductal epithelial cells in an atmosphere of 5% CO_2 in air [51]. Coculturing equine embryos with oviductal tissue is therefore obviously effective in overcoming the developmental blocks and in supporting development to the blastocyst stage, whether the embryos are *in vivo*- or *in vitro*-derived. In other species, a cell-free culture system under an atmosphere of lowered oxygen concentration (5% O_2, 5% CO_2, 90% N_2) is an alternative approach to the production of transferable embryos *in vitro* [52]; but no data on the cell-free culture of *in vivo*-derived equine embryos are available.

For *in vitro*-fertilized embryos, the cleavage rate of equine oocytes at 24–48 h culture was around 25% [19, 35], but subsequent development of the cleaving embryos was not assessed. Our microfertilization technique for equine oocytes with a partially removed zona pellucida [33] provides an opportunity to test the developmental competence of IVM/IVF pronuclear-stage equine embryos. The effect of different glucose supplementation of the modified synthetic oviduct fluid (mSOF) plus 0.1% polyvinylalcohol (PVA) on the *in vitro* development of microfertilized equine embryos was examined under a lowered oxygen concentration [53], because Schini and Bavister [54] reported that glucose and phosphate are responsible for the developmental block of hamster embryos cultured in a lowered oxygen concentration, and Matsuyama *et al.* [55] reported that IVM/IVF bovine oocytes cultured in mSOF required increased glucose concentrations at around the fourth cell cycle to enable them to develop into blastocysts. As shown in Table 2, rates of cleavage of the oocytes varied from 17 to 45% in the different glucose supplementations, but increasing glucose concentration from 0.5 mM for Days 1–4 to 5.5 mM for Days 5–8 led to superior embryo development; 23% of cultured embryos had more than 8 cells and 4% had more than 50 cells and were presumed to be morulae. Their developmental competence after transfer into recipient mares was not assessed. The inability of the morulae to form blastocysts is consistent with the report that equine embryos at the late morula- to early blastocyst-stage, recovered non-surgically 144 to 156 h after ovulation, did not develop in TCM199 supplemented with 0.1% PVA [56]. In cattle, the first 24 h of IVC in the absence of glucose in mSOF enhanced blastocyst development of IVF embryos [57]. The glucose concentration in mSOF therefore significantly affected the early development of equine oocytes after IVM and IVF.

Improved *in vitro* development of microfertilized equine oocytes has been reported [44]; When the zona pellucida of equine IVM oocytes was drilled by acid Tyrode's solution before IVF, 79% of the oocytes cleaved and 36% developed into morulae or blastocysts in coculture with cumulus cells, but non-surgical transfer of the *in vitro*-produced equine embryos did not result in pregnancy.

Capsule Formation

An acellular embryonic capsule appears in the perivitelline space of blastocysts as expansion of the blastocoele occurs [5, 6]. The very thin capsule in Day 6 blastocysts is not visible beneath the zona pellucida by light microscopic observation, but its thickness increases to a visible level as blastocysts develop beyond Day 7 (Fig. 3). The zona pellucida is shed from the outside of the capsule around Day 8 of pregnancy, leaving the capsule to surround the conceptus and maintain its spherical form until Day 21–28 of pregnancy [7, 8].

Table 2. Effects of different glucose supplementations on the *in vitro* development of microfertilized equine oocytes

Glucose (mM):		No. (%) of zygotes			
Days 1–4	Days 5–8	Cultured	≥2-cells	>8-cells	Morulae
0	0	62	17 (27)	3 (5)[a]	0 (0)
0	0.5	60	22 (37)	3 (5)[a]	0 (0)
0	5.5	74	20 (27)	7 (9)[a]	0 (0)
0.5	5.5	123	55 (45)	28 (23)[b]	5 (4)
5.5	5.5	75	13 (17)	3 (4)[a]	0 (0)

[a, b] Different superscripts within columns denote significant differences ($p<0.05$). The presumptive zygotes were cultured in modified synthetic oviduct fluid + 0.1% PVA at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2 [53].

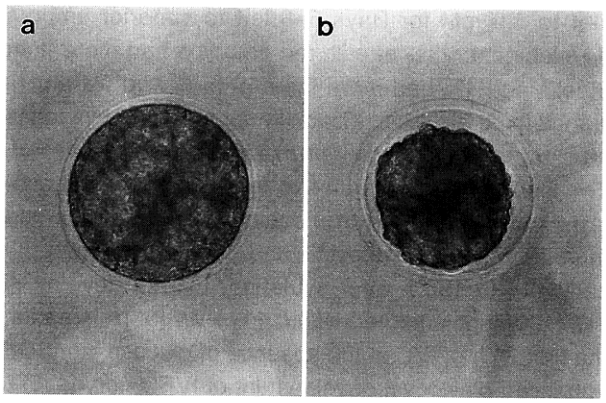

Fig. 3. Acellular embryonic capsule in the equine blastocysts. (a) Day-7 blastocyst, (b) The same blastocyst after exposure in a hyperosmotic solution. The capsule appears more clearly between the zona pellucida and trophoblast cells.

A physico-chemical investigation [5] indicated that the capsule is resistant to chemical (heat range 65 to 90°C, pH range 2.0 to 12.0, and reducing agents such as 1.0 M mercaptoethanol and 6.0 M urea) and enzymatic (collagenase Type I to III, or plasmin) solubilization. Under the same treatments, zonae pellucidae are completely solubilized. The glycoprotein of the capsule resembles those of the mucin glycoprotein family, as Gal, GalNAc, sulfated sugar, and sialic acid are major components of capsular carbohydrate whereas GlcNAc, Glu, and Man are minor components [6]. The capsular glycoproteins are O-glycosylated mainly on threonine residues, and SDS-PAGE analysis of solubilized capsules in Trypsin solution reveals a 21-kDa band [6]. Although the physiological role of the capsule during the early phase of pregnancy in horses remains to be determined, it is possible to speculate that the capsule serves as a protective barrier against any harmful environment for embryos in the uterus of mares.

Experiments on *in vitro* culture of mammalian embryos indicate that development of preimplantation embryos is not always autoregulated [58]. Therefore, the composition of the culture medium is crucial to their development *in vitro*. Development of equine embryos *in vitro* can be supported by Ham's F-10 [59], Ham's F-12 [60], or TCM199 [61, 62] to which serum of fetal origin has been added. When equine embryos recovered on Day 5–6 of pregnancy were cultured for 10 days, their diameter increased more than 1000 μm [60–62] but no capsule was produced [60]. Day-6 unexpanded blastocysts, in which capsule formation had been initiated in the mare uterus, hatched not only from the zona pellucida but also from the capsule *in vitro* [61, 62]. In these reports, *in vitro*-hatched equine embryos develop spherically despite the absence of the capsule. The inability of equine blastocysts to continue embryonic capsule production in serum-supplemented medium suggests that uterine factors are necessary for capsule formation.

In our study [56], two approaches have been attempted to prepare culture medium containing mare uterine components. Repeated use of TCM199 for uterine flushing on embryo recovery resulted in a culture medium with uterine components, including soluble proteins, but this culture medium was not effective in supporting *in vitro* development of Day-6 equine blastocysts. This negative result led us to collect uterine fluid by squeezing abattoir-derived uterine horns. Of five batches of uterine fluid, three batches supported *in vitro* development of equine blastocysts, but the other two did not and in no case could we find any sign of embryonic capsule formation *in vitro*. The poor capsule integrity of the cultured embryos may be explained by suboptimal culture conditions related to temperature and atmosphere composition. In another approach, Day 5–7 blastocysts were co-cultured with an oviductal cell monolayer, but capsule formation was not assessed [63]. A 18-19 kDa protein secreted by the endometrium of the mare has been detected in cultures of Day 11–19 equine capsule [64], suggesting the incorporation of this novel protein into the capsule during the late luteal phase. The Southern blot analysis of genomic DNA with the cDNA probe cloned for this protein suggested a single gene to be at least 4.5 kb in size [65].

Cryopreservation

The first successful report of embryo cryopreservation in horses was not published until 1982 [66], 10 years after Whittingham *et al.* [67] had reported the successful freezing of mouse embryos. The foaling rate after transfer of Day 6 equine blastocysts cryopreserved in 1.0 M glycerol was only 9% [66]. Eight years after Willadsen's report on the first two-step freezing of ovine and bovine embryos [68], an improved pregnancy rate (53%) was reported for equine embryos cryopreserved by two-step freezing [69]. In the latter report, unexpanded early blastocysts survived freezing better than expanded ones (pregnancy rates; 80 vs. 14%, respectively). From this and other studies [70], it is well established that smaller Day 6 embryos are more resistant to freezing injury than larger Day 7 embryos. The

size-dependent sensitivity of equine blastocysts to freezing injury has been explained by the different permeability to glycerol [71]. Development of the embryonic capsule during the expansion of equine blastocysts may be responsible for limiting permeation of the cryoprotectant (CPA).

The use of a more permeable solute, 1, 2-propanediol, resulted in the *in vitro* development of frozen-thawed equine blastocysts [72], but non-surgical transfer of blastocysts frozen-thawed in 1, 2-propanediol has not been successful [73]. DMSO also did not provide an adequate cryoprotective effect on equine blastocysts [66, 74]. Since permeation of ethylene glycol (EG) into unexpanded equine blastocysts was faster than that of glycerol [71, 75], the cryoprotective effect of EG on equine embryos was tested by non-surgical transfer [75] (*In vivo* survival of cryopreserved equine embryos in glycerol is generally less than 50%, except for one earlier report described above [69]). The pregnancy rate resulting from transfer of equine embryos cryopreserved in EG (25%) was comparable to those cryopreserved in glycerol (38%). Fracture damage of the blastocyst coverings (zona pellucida and capsule) was frequently observed in these post-thaw embryos. In contrast, when a small amount (0.1 M) of sucrose was added to the cryoprotective medium consisting of 1.8 M EG, all post-thaw embryos had intact coverings and developed *in vitro*. Such frozen embryos could be transferred directly into the uterus of recipient mares after in-straw dilution of the CPAs with physiological saline. The pregnancy rate following direct transfer of cryopreserved embryos (64%) was not different from that of non-frozen embryos (70%). The reproducibility of this technique needs to be determined with larger numbers of equine embryos for commercial embryo transfer programs.

Because of the lower permeability to CPAs of equine embryos compared with bovine embryos, vitrification of equine embryos has been considered to be difficult. The type of vitrification solution, the stage of development of the embryos, and the protocol for the addition and dilution of the CPAs are important variables for successful vitrification. Based on the results of freezing and vitrification of embryos from other domestic species [76–79], an EG-based vitrification solution (EFS), originally described by Kasai *et al.* [80], was tested. This solution consists of a highly permeating chemical (EG, 7.2 M), a low-toxicity macromolecule that promotes vitrification (Ficoll-70, 0.0026 M), and a disaccharide that causes osmotic dehydration of the embryo (sucrose, 0.3 M). Application of the original protocol [80] (direct exposure to EFS; as used for *in vitro*-produced bovine blastocysts

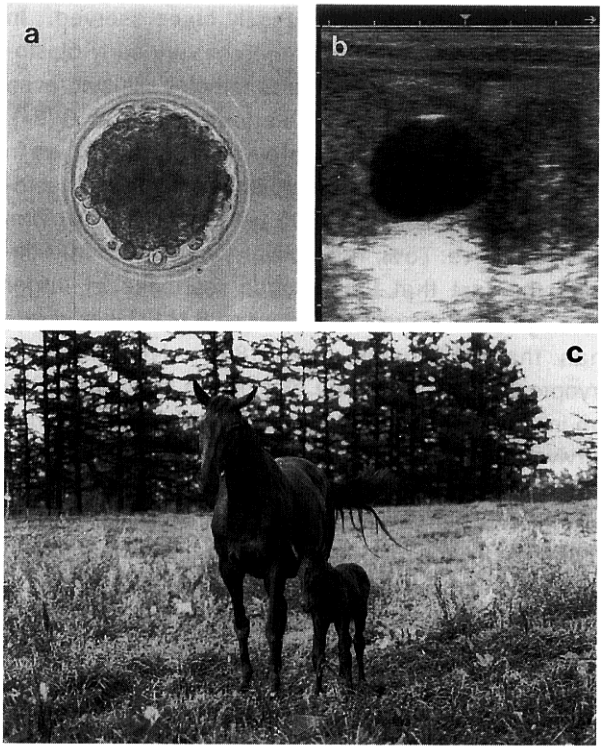

Fig. 4. The first pregnancy and new-born foal, derived from transfer of an equine embryo following vitrification and warming. (a) Post-warm Day-6 embryo transferred, (b) Ultrasonographic image of the Day-15 conceptus 2 cm in diameter, (c) A native-pony foal and the Thoroughbred recipient mare.

[76, 81]) to unexpanded equine embryos failed to yield any surviving embryos after vitrification and warming, but 57% of post-warm embryos exposed to the EFS in a stepwise manner were capable of developing *in vitro*. The exposure of embryos to 3.6 M EG until their re-expansion has been achieved may allow sufficient permeation into embryonic cells by EG. Cells equilibrated with a low concentration of EG may easily increase their intracellular EG level through further dehydration during brief exposure to EFS, and the concentrated intracellular EG may allow the cytoplasm to be vitrified. Non-surgical transfer of 5 unexpanded early equine blastocysts following vitrification resulted in 2 pregnancies and the delivery of foals (Fig. 4). This first report on vitrification of equine embryos [82] was published in 1994, 9 years after the first successful vitrification in mice [83].

Understanding the mechanism responsible for low post-thaw survival of larger equine embryos could be the key to extending the developmental stages of the

embryos that can be successfully cryopreserved. In fact, the recovery of late morulae or very early blastocyst-stage embryos from mares remains difficult, even when recovery attempts are performed on Day 6 [84]. With vitrification, equine blastocysts 200 to 300 μm in diameter survived cryopreservation as well as blastocysts that were less than 200 μm in diameter (*in vitro* survival; 88 vs. 75%, respectively). This was true despite the fact that the osmotic response of larger blastocysts during pretreatment with 3.6 M EG was slower than that of smaller ones [85]. Nevertheless, cryopreservation of equine blastocysts larger than 300 μm in diameter either by conventional freezing (*in vitro* survival; 22% [71]) or vitrification (*in vitro* survival; 25% [85]) is still in its infancy. Recent progress in overcoming this obstacle has been made using conventional freezing after step-down equilibration [86]. In this method, large (>300 μm) equine blastocysts were exposed in turn to 2.0 M glycerol, 4.0 M glycerol and 2.0 M glycerol + 0.3 M galactose, and then subjected to two-step freezing in 1.0 M glycerol. Survival *in vitro* (83%) and *in vivo* (40%) was achieved with the cryopreserved embryos.

Conclusion

Stallion spermatozoa appear to have difficulty penetrating the zona pellucida of equine oocytes [10, 19, 33–36]. Since developmental competence of IVM oocytes following *in vivo*-fertilization has been unsatisfactory [31], further research on IVF in this species should focus on improving extra-ooplasmic (zona pellucida and cumulus cells) and cytoplasmic maturation of oocytes as well as the *in vitro* capacitation of spermatozoa. On the other hand, the slow progress of equine IVMFC has required the application of assisted reproductive technologies such as zona-manipulation (PZD, PZR, zona-drilling) [33, 44] and ICSI [11, 45–47], which have resulted in embryo development and the birth of a few foals. These studies may be helpful in understanding the factors associated with unusual aspects of gamete interaction in the equine species.

Equine embryos at the unexpanded blastocyst-stage have been successfully cryopreserved by slow-freezing [66, 69, 75] and by vitrification [82, 85, 86]. The developing embryonic capsule in the perivitelline space of equine blastocysts is one factor which may affect the permeation of cryoprotectants. Cryopreservation of larger expanding blastocysts with a well developed embryonic capsule remains a challenge and the fundamental role of the equine capsule during the early stage of pregnancy remains to be determined.

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