

# Why doesn't conventional IVF work in the horse? The equine oviduct as a microenvironment for capacitation/fertilization

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## Abstract

In contrast to man and many other mammalian species, conventional *in vitro* fertilization (IVF) with horse gametes is not reliably successful. The apparent inability of stallion spermatozoa to penetrate the zona pellucida *in vitro* is most likely due to incomplete activation of spermatozoa (capacitation) because of inadequate capacitating or fertilizing media. *In vivo*, the oviduct and its secretions provide a microenvironment that does reliably support and regulate interaction between the gametes. This review focuses on equine sperm–oviduct interaction. Equine sperm–oviduct binding appears to be more complex than the presumed species-specific calcium-dependent lectin binding phenomenon; unfortunately, the nature of the interaction is not understood. Various capacitation-related events are induced to regulate sperm release from the oviduct epithelium and most data suggest that exposure to oviduct secretions triggers sperm capacitation *in vivo*. However, only limited information is available about equine oviduct secreted factors, and few have been identified. Another aspect of equine oviduct physiology relevant to capacitation is acid–base balance. *In vitro*, it has been demonstrated that stallion spermatozoa show tail-associated protein tyrosine phosphorylation after binding to oviduct epithelial cells containing alkaline secretory granules. In response to alkaline follicular fluid preparations (pH 7.9), stallion spermatozoa also show tail-associated protein tyrosine phosphorylation, hyperactivated motility and (limited) release from oviduct epithelial binding. However, these ‘capacitating conditions’ are not able to induce the acrosome reaction and fertilization. In conclusion, developing a defined capacitating medium to support successful equine IVF will depend on identifying as yet uncharacterized capacitation triggers present in the oviduct.

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## Introduction

Conventional *in vitro* fertilization (IVF) involves the penetration of a mature oocyte by a capacitated spermatozoon. The birth of Louise Brown in 1978 heralded the first successful conventional human IVF procedure in clinical practice (Stephoe & Edwards 1978) and today, IVF is a well-established assisted reproductive technique in human medicine. In farm animals, conventional IVF has also become widespread, as illustrated by the 9930 IVF calves recorded to have been born in 2012, that is, 6.8% of all births (Perry 2013). Similarly, in pigs and various laboratory animals, dozens of offspring are produced by conventional IVF each year (Galli *et al.* 2003, Betteridge 2006). By contrast, despite the birth of two IVF foals in 1991 following incubation of oocytes with calcium ionophore treated spermatozoa (Palmer *et al.* 1991, Bézard *et al.* 1992), no reliable IVF protocol has yet been established for equine gametes (Choi *et al.* 1994, Dell'Aquila *et al.* 1997a,b, Alm *et al.*

2001, Hinrichs *et al.* 2002, Mugnier *et al.* 2009a). An overview of the published equine IVF studies is shown in Table 1, which illustrates the disappointing success rates, varying on an average between 0% and 33%. Using calcium ionophore A23187 (Li *et al.* 1995, Alm *et al.* 2001), heparin (Li *et al.* 1995, Alm *et al.* 2001), zona pellucida proteins (Graham 1996), caffeine (Graham 1996), lysophospholipids (Graham 1996) and progesterone (Tremoleda *et al.* 2003), to trigger sperm activation, reported fertilization rates are very low. On the other hand, it has been demonstrated that both zona pellucida drilling and partial removal of the zona pellucida improve sperm penetration rates (Choi *et al.* 1994, Li *et al.* 1995, Dell'Aquila *et al.* 1996), although the associated high rates of polyspermy render these approaches unusable in practice.

In 2009, McPartlin *et al.* reported very promising fertilization/cleavage rates (61%) after co-incubating equine oocytes with stallion spermatozoa treated with

**Table 1** Overview of equine conventional IVF results.

Oocyte maturation	No. of oocytes	Sperm treatment	% fertilized oocytes with 2 pronuclei	% cleaved oocytes	Reference
<i>In vivo</i>	113	Ca Ionophore A23187	14 <sup>a</sup>		Palmer <i>et al.</i> (1991)
<i>In vivo</i>	173	Ca Ionophore A23188	17 <sup>a</sup>	13 <sup>a</sup>	Bézar <i>et al.</i> (1992)
<i>In vitro</i>	57	Caffeine/Ca Ionophore A23187	4		Choi <i>et al.</i> (1994)
<i>In vitro</i>	232	Heparin	12–33*	0–1*	Dell'Aquila <i>et al.</i> (1996)
<i>In vitro</i>	206	Heparin	4–17*	0	Dell'Aquila <i>et al.</i> (1997a)
<i>In vitro</i>	203	Heparin	6–8*	2–3*	Dell'Aquila <i>et al.</i> (1997b)
<i>In vitro</i>	349	Heparin/Ca Ionophore A23187	11–30*		Alm <i>et al.</i> (2001)
<i>In vitro</i>	815	Ca Ionophore A23187	0–24*		Hinrichs <i>et al.</i> (2002)
<i>In vitro</i>	54	Ca Ionophore A23187	33	24	Zhang <i>et al.</i> (1990)
<i>In vitro</i>	370	Progesterone	0		Tremoleda <i>et al.</i> (2003)
<i>In vitro</i>	385	Heparin/Ca Ionophore A23187/BSA	0–40 <sup>b,*</sup>		Roasa <i>et al.</i> (2007)
<i>In vitro</i>	994	Ca Ionophore A23187	0–8*		Mugnier <i>et al.</i> (2009b)
<i>In vitro</i>	21	Caffeine	0		Mugnier <i>et al.</i> (2009a)
<i>In vitro</i>	74	Procaine	40–84 <sup>c,*</sup>		McPartlin <i>et al.</i> (2009)
		10% follicular fluid	19 <sup>c</sup>		Lange-Consiglio and Cremonesi (2011)
<i>In vitro</i>	154	Procaine	38–62*		Ambruosi <i>et al.</i> (2013)

<sup>a</sup>Birth of an IVF foal. <sup>b</sup>High fertilization rate was effect from one specific ejaculate. <sup>c</sup>Fertilized and cleaved oocytes. \*Depending on different conditions tested.

procaine to induce hyperactivated motility (McPartlin *et al.* 2009). Similar results were subsequently reported by Ambruosi *et al.* (2013). However, seven years later, this technique has still not been validated by reports of blastocyst formation or the birth of IVF foals. Moreover, we recently demonstrated that procaine directly affects equine oocytes, inducing cytokinesis via a pH-dependent pathway (Leemans *et al.* 2015a). These findings have now been confirmed by Goudet *et al.* (2016). In this respect, both McPartlin *et al.* (2009) and Ambruosi *et al.* (2013) classified fertilization as the presence of at least two pronuclei or cleavage after an 18h gamete co-incubation followed by an additional 24h culture. However, this methodology would not allow definitive differentiation of true fertilization from parthenogenetic activation of an unfertilized oocyte. These results emphasize the need to assess the direct effect of chemical capacitation triggers on co-incubated oocytes and to evaluate fertilization more accurately, that is, by documenting sperm penetration (sperm mid-piece or tail visualization) and second polar body extrusion, rather than pronucleus formation alone.

Theoretically, the obstacles to equine IVF could reside at the level of either inadequate oocyte maturation or incomplete sperm capacitation. In this respect, it has been demonstrated that whereas IVF using *in vivo* matured oocytes is not successful (Palmer *et al.* 1991), *in vitro* matured oocytes transferred to the oviduct of an inseminated mare yield similar embryo development rates to spontaneous ovulations (Hinrichs *et al.* 2002). The latter observation confirms that *in vitro* matured oocytes are capable of being fertilized, which in turn

suggests that equine capacitation or fertilization media lack one or more of the oviductal factors required for the spermatozoa to penetrate the zona pellucida and enter the oocyte. Moreover, there is little evidence that irreversible zona pellucida hardening resulting from premature cortical granule release triggered by suboptimal *in vitro* maturation conditions, or failure of oviduct-dependent changes in zona pellucida penetrability, play a role in the failure of equine IVF (Dell'Aquila *et al.* 1999). In short, the failure of equine IVF is most likely attributable to the inability of the spermatozoa to penetrate the zona pellucida as a result of inadequate activation (capacitation). In this respect, Tremoleda *et al.* (2003) found that while equine IVF conditions supported the binding of stallion spermatozoa to the zona pellucida they failed to induce either the acrosome reaction or characteristics typical of capacitation.

The high fertilization rates that occur after conventional insemination *in vivo* are generally accepted to be attributable to a capacitation-supporting oviductal environment (McPartlin *et al.* 2009). In this respect, the oviduct and its secretions provide an optimal environment for gamete maturation and interaction, including (1) transport of both male and female gametes to the site of fertilization, (2) final gamete maturation, (3) fertilization, (4) early embryonic development and (5) transport of the embryo to the uterus. It is hoped that incubating equine gametes with oviduct cells and their secretions *in vitro* will improve gamete preparation and activation to undergo fertilization and embryonic development (Li *et al.* 2001). More specifically, in-depth knowledge is required on how stallion spermatozoa

are activated and acquire fertilizing capacity in the oviductal microenvironment, as a means of identifying the capacitation triggers missing in current capacitating/fertilization media. To this end, this review focuses on equine sperm–oviduct interactions.

### Equine oviduct models for studying sperm–oviduct interactions *in vitro*: monolayers, explants and apical plasma membranes

Establishing an *in vitro* equine oviduct model that faithfully recreates *in vivo* conditions would bring a working conventional equine IVF protocol within reach. While interactions between spermatozoa and the oviductal environment have been studied extensively in many mammals, little data is available for horse, in part because of the scarcity of equine reproductive tissues available to study this interaction. In addition, *in vivo* studies are challenging because invasive surgery or laparoscopy is required to access the oviduct. Several oviduct cell models have been developed, including oviduct epithelial cell monolayers (Thomas *et al.* 1995b, Dobrinski *et al.* 1999), explants (Nelis *et al.* 2014) and apical plasma membrane preparations (Smith & Nothnick 1997) (Fig. 1).

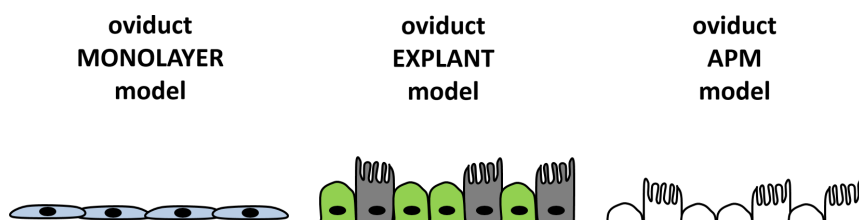
Oviduct explants have several advantages over monolayers for studying sperm–oviduct interactions: (1) In cattle (Thibodeaux *et al.* 1992, Walter 1995) and horses (Thomas *et al.* 1995b, Dobrinski *et al.* 1999), proliferating oviduct epithelial cells grown in monolayers ‘de-differentiate’ exhibiting a reduction in cell height, and loss of cilia, secretory granule abundance and bulbous protrusions. In this respect, monolayers do not accurately reflect the *in vivo* situation (Thibodeaux *et al.* 1992, Walter 1995, Reischl *et al.* 1999), (2) Explants of bovine (Harvey *et al.* 1995, Walter 1995, De Pauw *et al.* 2002, Rottmayer *et al.* 2006) and equine (Nelis *et al.* 2014) oviduct epithelium maintain vigorously beating cilia, an important marker of cell viability. Nelis *et al.* (2014) showed that, even after 6 days in culture, equine oviduct epithelial explants still contained beating cilia and numerous microvilli, and confirmed this using transmission electron microscopy. Moreover, the epithelial cells lining the explants maintained a highly differentiated morphology, even at the ultrastructural level, characterized by numerous mitochondria and rough endoplasmic reticulum, similar to freshly harvested oviduct epithelium. (3) The secretion of

insulin-like growth factor-2 (IGF2) from bovine oviduct explants was significantly higher than from monolayers, indicating better preservation of cell function (Winger *et al.* 1997). (4) Explants can be used as soon as 6–12 h after harvesting, whereas monolayers require several days of culture before they can be used (Rottmayer *et al.* 2006). (5) Bull sperm heads bind preferentially to the cilia or deeper regions of ciliated epithelial cells of explants rather than to secretory epithelial cells (Pollard *et al.* 1991, Petrunkina *et al.* 2001). In addition, both human (Baillie *et al.* 1997) and bovine (Sostaric *et al.* 2008) spermatozoa bind preferentially to explants compared to monolayers. (6) We found that stallion spermatozoa bound to oviduct explants undergo capacitation-related events like tail-associated protein tyrosine phosphorylation, confirming that the explants mimic some important *in vivo* functions (Leemans *et al.* 2014). In conclusion, explants are preferred for assessing the effect of sperm–oviduct epithelium binding on sperm physiology because they are the only current model in which oviduct epithelial secretory activity is maintained within physiological ranges (Nelis *et al.* 2014).

While the above properties favour the use of the more ‘*in vivo*-like’ oviduct explants, it is difficult to reliably quantify sperm–oviduct binding to explants because of their invaginated and irregular surface. Therefore, sperm-binding studies are often performed using equine oviduct monolayers, which have a flat surface (Thomas *et al.* 1995b, Dobrinski *et al.* 1999). Alternatively, accurate quantification of sperm binding can be performed using oviduct apical plasma membranes coated onto nitrocellulose (Leemans *et al.* 2016). However, when using oviduct explants and apical plasma membranes in parallel, we were unable to identify significant differences in sperm binding, even though the number of spermatozoa bound per mm<sup>2</sup> varied more using the explants with their irregular surface (Leemans *et al.* 2016).

### Sperm–oviduct binding: establishing a sperm reservoir

After entering the oviduct during the peri-ovulatory period, spermatozoa bind to the oviduct epithelium to establish a sperm reservoir in the caudal isthmus and uterotubal junction (Boyle *et al.* 1987). In the mare, spermatozoa in the reservoir may need to survive relatively long periods, given the prolonged



**Figure 1** Different *in vitro* equine oviduct models to study various fertilization events. Each model has its advantages and disadvantages but depending on the aim of the study one model is more preferable than the other.

duration of oestrus. It was hypothesized previously that the interaction between oviduct epithelial cells and spermatozoa serves various aims: (1) to provide an environment suitable for sperm storage and survival until ovulation; (2) to select a viable, morphologically normal and motile sperm population; (3) to prepare spermatozoa to capacitate and acquire the ability to fertilize a mature oocyte; and finally (4) to prevent polyspermic fertilization (Suarez 2002).

In cattle and horses, only viable spermatozoa with an intact acrosome and plasma membrane bind to the oviduct epithelium (Ellington *et al.* 1993c, Thomas *et al.* 1994a,b, Gualtieri & Talevi 2000, Sostaric *et al.* 2008). *In vitro*, stallion spermatozoa bind to oviduct epithelial explants via the apical region of the sperm head (Thomas *et al.* 1994b). When using oviduct monolayers, sperm binding properties are not affected by cycle stage (Lefebvre & Samper 1993) whereas, for equine oviduct explants, a higher sperm–oviduct binding capacity is observed when material is harvested during the immediate pre- and post-ovulatory periods, compared with later in the luteal phase (Thomas *et al.* 1994a). The latter study also showed that sperm tail-beating was higher when bound to oviduct explants harvested at the follicular stage than at other cycle stages. In addition, sperm–oviduct explant binding was better for isthmic than ampullary oviduct epithelial explants (Thomas *et al.* 1994a). It has also been demonstrated that direct membrane contact between stallion spermatozoa and oviduct cells *in vitro* results in (1) better maintenance of motility compared to spermatozoa incubated in the absence of oviduct cells (Ball *et al.* 1993, Ellington *et al.* 1993b, Ellington *et al.* 1999) and (2) a lower intracellular calcium concentration resulting in a delay in capacitation and prolonged viability of the bound spermatozoa (Dobrinski *et al.* 1996b, Dobrinski *et al.* 1997). An analogous scenario to the isthmic sperm reservoir *in vivo* might ensure the prolonged presence of a competent sperm subpopulation for activation and release at the time of fertilization.

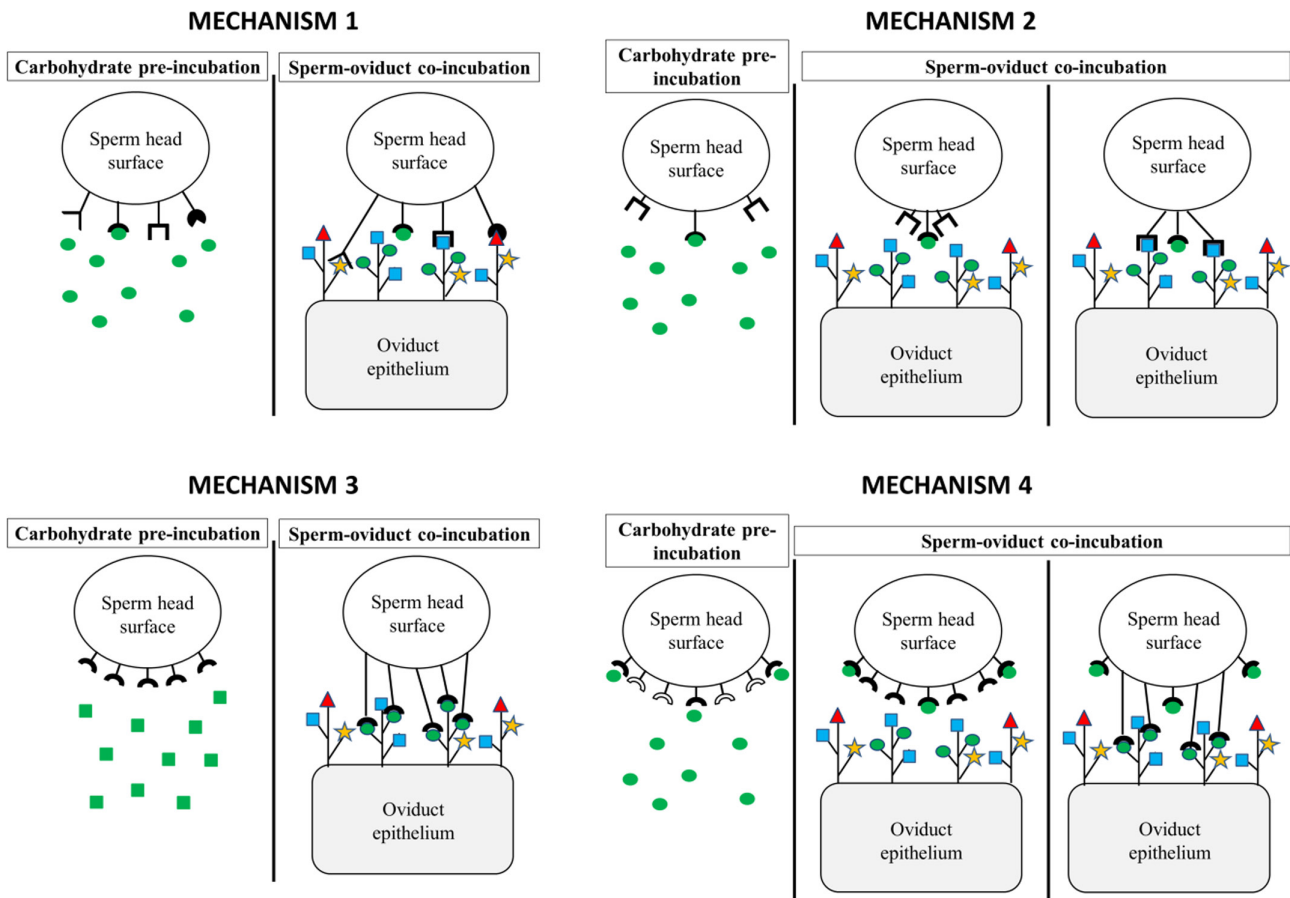
### Key biochemical factors in equine sperm–oviduct binding

In the mammalian oviduct, proteins present on the (outer) plasma membrane of non-capacitated spermatozoa recognize carbohydrate moieties on the surface of the plasma membrane of oviduct epithelial cells (Suarez 2001, 2002, Sostaric *et al.* 2005). In horses, it has been reported that both fetuin and asialofetuin block *in vitro* sperm–oviduct binding, with the latter doing so more potently. When the sialic acid is removed from fetuin, D-galactose is the principal carbohydrate exposed at the end of the oligosaccharide chains (Lefebvre *et al.* 1995). Indeed, a competitive inhibitory effect of D-galactose on sperm–oviduct binding *in vitro* has been demonstrated

using an oviduct monolayer model (Lefebvre *et al.* 1995, Dobrinski *et al.* 1996a), while galactose-binding proteins were detected on the surface of stallion spermatozoa by Sabeur and Ball (2007). Galactosyl residues have also been shown to be highly expressed in the isthmic part of the equine oviduct (Ball *et al.* 1997), suggesting their involvement as ligands for sperm adhesion. These residues were less highly expressed in the ampulla where cycle-related differences were observed, characterized by maximum galactosyl expression during oestrus (Ball *et al.* 1997). Using an oviduct explant or apical plasma membrane model, we were unable to identify D-galactose as key molecule in equine sperm–oviduct interaction (Leemans *et al.* 2016). Similarly, immunohistochemistry failed to reveal expression of D-galactose on the equine oviduct epithelial plasma membrane, whereas other galactosyl carbohydrates were present, which supported the findings of a previous study (Desantis *et al.* 2004). Although various carbohydrates, including galactosyl moieties, were highly expressed on the epithelium of oviduct explants, we were unable to competitively inhibit sperm–oviduct epithelium binding using any of a variety of candidate mono- and polysaccharides, including D-galactose, and lectins. We did, however, find that, in contrast to some other mammalian species (Suarez 2001), equine sperm–oviduct explant binding was neither dependent on calcium nor on disulphide (S–S) covalent bonds (Leemans *et al.* 2016). In short, it appears that equine sperm–oviduct epithelium interaction *in vivo* is not based on a single lectin or S–S covalent bond.

One of the major seminal plasma proteins associated with the sperm plasma membrane is horse seminal plasma protein-7 (HSP-7). This protein is the equine homologue of the porcine spermadhesin, AWN (Topfer-Petersen *et al.* 2005). As a group, the HSP-7 interact with carbohydrates expressed on the oviduct epithelium and may, therefore, be involved in sperm reservoir formation. SP-1 and SP-2 (previously HSP-1 and HSP-2), which are members of the fibronectin-2 protein family, have also been identified in equine seminal plasma and are homologues of bovine seminal plasma proteins involved in establishing the bovine sperm oviduct reservoir (Ekhlasi-Hundrieser *et al.* 2005). Nevertheless, the role of HSPs in equine sperm–oviduct binding has yet to be fully elucidated.

Unfortunately, the nature of the oviduct receptor(s), which is/are responsible for equine sperm–oviduct binding is still unknown. Moreover, the specificity of equine sperm–oviduct binding, both *in vitro* and *in vivo*, is questionable. *In vitro* studies have suggested lectin interactions between spermatozoa and oviduct epithelial cells, which might represent either the totality or only a portion of the pre-ovulatory sperm-binding reaction. If the latter is true, a strong degree of non-specific binding is present *in vivo*. This hypothesis was supported by studies that showed spermatozoa to have a



**Figure 2** Four potential mechanisms that may be involved in equine sperm–oviduct binding that hypothesize why this interaction cannot be competed with (pre)incubation of one of the carbohydrates or lectins: (1) multiple protein–carbohydrate interactions are involved (‘aspecific’ binding nature), only the carbohydrate or lectin of choice partly and insufficiently blocks the interaction; (2) a primary–secondary carbohydrate ligand–receptor binding where a primary carbohydrate binding allows a secondary binding with another carbohydrate ligand expressed on the oviduct explant epithelium once the secondary binding is established; (3) not only the type of carbohydrate but also the carbohydrate conformation/isomer might be important; and (4) the lectin binding is dependent on multivalency and only cooperative lectin binding allows firm binding. (Pre)labelling of spermatozoa with the monomeric form of the carbohydrate involved in sperm–oviduct binding will not compete with the cooperative sperm–oviduct type of lectin binding. Only a small portion of the exposed lectins with high affinity on the sperm head is covered by the carbohydrate and a bigger portion has lower affinity and can only bind in cooperative manner to multivalent lectin binding sites at the oviduct epithelium. These sites remain operative after (pre)labelling with monomeric carbohydrates and thus sperm–oviduct binding cannot be competed.

similar binding capacity for tracheal epithelium, another type of ciliary epithelium (Hunter 2003, 2011).

Several hypotheses can be generated for the mechanism(s) of equine sperm–oviduct binding: (1) various carbohydrate–receptor interactions play a role; (2) a secondary binding response is triggered by interaction with a primary carbohydrate receptor; (3) not only the type of carbohydrate, but also the conformation/isomer might be important; and (4) carbohydrates on both the oviduct and sperm cell surface might cooperate in sperm–oviduct binding. Carbohydrate-binding proteins typically have very shallow, solvent-exposed binding sites and make relatively few direct contacts with their target ligands. Thus, a key phenomenon in the specificity of such interactions is ‘multivalency’, that is multiple protein–carbohydrate interactions cooperating

in a recognition event to achieve the necessary increased functional affinity (reviewed by del Carmen Fernandez-Alonso *et al.* 2012). As such, multiple receptors need to be arranged in a specific order to bind efficiently to multiple saccharide ligands (Kiessling & Pohl 1996, Gabius *et al.* 2011). An overview of the different hypotheses is given in Fig. 2. In conclusion, the interaction between carbohydrates and their receptors is a very complex area, and in-depth studies are required to elucidate their role in equine sperm–oviduct binding.

#### How are sperm cells released from oviduct epithelial cell binding?

At the late pre-ovulatory stage, a select group of spermatozoa must be released from oviduct epithelium

binding and migrate to the site of fertilization at the ampullary–isthmic junction. Theoretically, various mechanisms could contribute to sperm release: (1) a decrease in the availability of oviductal sperm-binding sites, (2) release in response to capacitation-related changes in the sperm plasma membrane, (3) secretion of molecules that compete for sperm-binding sites, (4) spermatozoa detaching as a result of acquiring hyperactivated motility, (5) spermatozoal contact with disulphide reductants, and (6) two or more of these mechanisms combine to trigger sperm cell release.

In horses, very little data is available on the mechanisms involved in releasing spermatozoa from oviduct epithelium binding. It has, however, been demonstrated that the intracellular calcium concentration in unbound stallion spermatozoa is many times higher than that in bound spermatozoa (Dobrinski *et al.* 1996b, Dobrinski *et al.* 1997), as it is in cattle spermatozoa (Gualtieri *et al.* 2005). In order to be released from oviduct epithelium binding, it is thought that sperm cells have to undergo capacitation. In this respect, stallion spermatozoa co-cultured with oviduct epithelial cell explants showed signs of capacitation, whereas spermatozoa cultured in similar conditions but without oviduct cells did not (Leemans *et al.* 2014). Similar findings were acquired using an oviduct monolayer model (Ellington *et al.* 1993a). The capacitated sperm population also showed an increased affinity for zona pellucida binding (Ellington *et al.* 1993a). Conversely, after previous exposure to calcium ionophore significantly fewer stallion spermatozoa are able to bind to oviduct monolayers. Moreover, oestradiol and heparin have been reported to trigger sperm release from oviduct epithelial cell monolayers (Thomas *et al.* 1995a) but using oviduct explants, we were unable to confirm this finding in case of heparin and disulphide reductants such as D-penicillamine (Leemans *et al.* 2016). Nevertheless, currently used equine capacitating media, which contain bicarbonate, calcium and albumin, do not trigger sperm release from oviduct explants (Leemans *et al.* 2016). Recently, we demonstrated that, of a range of potential physiological triggers, only heat-inactivated, charcoal-treated, 30 kDa filtered or triple treated follicular fluid (pH 7.9) was able to induce hyperactivated motility and release of stallion spermatozoa bound to oviduct explants. However, while a significant proportion of bound spermatozoa showed hyperactivated motility combined with tail-associated protein tyrosine phosphorylation, only a small number of spermatozoa detached from the oviduct epithelium (Leemans *et al.* 2015b).

One obvious question is how many sperm cells need to be released from the oviductal reservoir to fertilize the oocyte at the moment of ovulation. A low sperm:egg ratio *in vivo* is essential to prevent polyspermy (Hunter 1996, 2012). The number of spermatozoa detaching from the epithelium and progressing to the site of fertilization in

the peri-ovulatory period should, therefore, be strongly regulated by the oviduct. Just before ovulation, when an oocyte (or a small number of oocytes) will be released into the oviduct, only a small number of sperm cells should capacitate and detach from the oviduct epithelium, to ensure a low sperm:egg ratio at the isthmic–ampullary junction and reduce the risk of polyspermic fertilization (Smith & Yanagimachi 1991, Hunter 1993, 2005, 2008, Suarez 2008). This hypothesis may explain the *in vitro* finding of very limited release of stallion spermatozoa from oviduct epithelium in hyperactivating, capacitating conditions. The stringent regulation of sperm release during the peri-ovulatory period *in vivo* is in sharp contrast to the progressively increasing post-ovulatory release of sperm cells, when it is too late to influence fertilization (Hunter 1996, 2005, 2012, Coy & Aviles 2010, Coy *et al.* 2012).

### Is sperm binding to oviduct epithelium in the isthmic sperm reservoir indispensable for capacitation/fertilization?

The caudal isthmus of the oviduct has been identified as a sperm reservoir in mares before ovulation (Bader 1982, Thomas *et al.* 1994a, Scott *et al.* 1995). However, it is less clear whether spermatozoa need to interact with oviduct epithelial cells *in vivo* to acquire fertilizing capacity. Indeed, when spermatozoa are directly introduced into the equine oviductal ampulla via the infundibulum, pregnancy rates are similar to those obtained after artificial insemination into the uterine body (McCue *et al.* 2000), suggesting that neither the uterine body nor the isthmus are obligatory stopping points on the route to capacitation/fertilization. On the other hand, stallion spermatozoa may need to interact with cells or fluids in the ampulla to capacitate/fertilize, whereas in many other species such as man, cattle and pig, sperm capacitation and fertilization can be achieved *in vitro* in the absence of any oviduct epithelia.

Exposing stallion spermatozoa *in vitro* to modified Whitten's medium containing both bicarbonate (25 mM; pH=7.4) and albumin significantly decreased the density of sperm binding to the oviduct epithelium, in association with a massively increased incidence of sperm head-to-head agglutination (Leemans *et al.* 2016). In the pig, sperm head-to-head agglutination is considered an early indicator of *in vitro* capacitation, signalling the removal of anti-agglutinin from the sperm surface (Lindahl & Sjoblom 1981, Harayama *et al.* 1999, Harayama *et al.* 2000). Head-to-head agglutination in stallion spermatozoa might similarly indicate (1) the removal of sperm surface bound de-capacitation factors (seminal plasma proteins) and (2) early plasma membrane changes.

Intracellular alkalization and tail-associated protein tyrosine phosphorylation are other important hallmarks

of capacitation. In this respect, *in vitro* oviduct-bound stallion spermatozoa showed higher rates of intracellular alkalization and protein tyrosine phosphorylation than unbound spermatozoa. Initially, we hypothesized that interaction between the sperm cells and the oviduct *in vitro* was required to switch on intracellular capacitation processes. However, the triggering of capacitation in oviduct explant-bound spermatozoa is more likely due to the release of 'pro-capacitating factors' from the secretory oviduct cells. Indeed, a similar protein tyrosine phosphorylation response was reported for stallion spermatozoa suspended in alkaline medium (Gonzalez-Fernandez *et al.* 2012, Aalberts *et al.* 2013). We recently demonstrated that the secretory cells in oviduct explants contain large alkaline vesicles (Leemans *et al.* 2014). Although discharge of these vesicles only marginally raised the pH of the incubation medium, they might nevertheless create an alkaline gradient around each oviduct explant. Moreover, *in vivo* any alkaline secretions would be diluted to a lesser extent by oviductal fluid and may therefore produce an alkaline local microenvironment sufficient to induce sperm protein tyrosine phosphorylation without sperm binding to the oviduct. In conclusion, a local increase in the bicarbonate concentration might be essential for stallion sperm capacitation (Liu *et al.* 2012).

Overall, the data suggest that exposure to oviduct secretions, secreted primarily by ampullary epithelium, may be more significant to the induction of capacitation in stallion spermatozoa than sperm–oviduct binding. We therefore suggest that *in vivo* sperm–oviduct binding and establishment of an oviductal sperm reservoir, is facultative rather than obligatory and depends on when, with respect to ovulation, the non-capacitated sperm cells enter the oviduct:

- If non-capacitated sperm cells arrive in the early pre-ovulatory period, a significant sperm population will bind to the oviduct epithelium in the isthmus and form a sperm reservoir. These spermatozoa will be stored in the oviduct until capacitation triggers are generated just before ovulation.
- If non-capacitated sperm cells arrive in the peri-ovulatory oviduct, the sperm cells immediately contact oviduct secretions containing the capacitation triggers and lose their oviduct-binding affinity. The fertilizing sperm cell(s) is/are not stored since fertilization should occur as soon as possible.

## What is the role of oviductal fluid in capacitation/fertilization in the horse?

### Composition of oviductal fluid

Ovarian steroids, that is oestrogens and progesterone, regulate the composition of oviduct fluid that is produced by selective serum transudation, active biosynthesis

and secretion from the oviduct secretory cells (Hunter & Nichol 1988, Leese 1988). Additionally, follicular and peritoneal fluid enter the oviduct lumen. Oviduct cell secretory activity is region-dependent; the ampulla exhibits the highest secretory activity, the infundibulum intermediate and the isthmus has the least secretory activity (sheep: (Buhi *et al.* 1991); pig: (Buhi *et al.* 1992); horse: (McDowell *et al.* 1993)). The volume of oviduct fluid is also cycle-dependent with the largest volume measured during the follicular phase under oestrogen influence (cattle: (Malayer *et al.* 1988, Buhi *et al.* 1989); pig: (Buhi *et al.* 1989); mare: (Campbell *et al.* 1979)).

To date, only very few oviduct secreted factors that might modulate gamete preparation for fertilization have been studied. Oestrogen-dependent oviduct secretory glycoprotein (OSG) or oviductin is a unique oviduct protein conserved across many mammalian species. OSG enhances sperm–oocyte binding and penetration through the zona pellucida, and also plays a role in early embryonic development (cattle: (Boice *et al.* 1990); sheep: (Murray 1993); pig: (Kouba *et al.* 2000)). However, when searching the equine genome Mugnier *et al.* (2009a) were only able to find an OSG-like gene that included stop codons, which they concluded might be a pseudogene. Nevertheless, it is possible that the failure to identify a functional OSG gene was due to species-specific differences in the sequence and incomplete annotation of the equine genome, rather than true absence of this gene in the horse.

Osteopontin, atrial natriuretic peptide A (ANP A) and deleted in malignant brain tumor 1 (DMBT1), have been specifically identified as oviduct secretory proteins in the horse. When stallion spermatozoa were capacitated with calcium ionophore, co-incubation of mature equine oocytes with either equine or porcine oviduct epithelial explants or monolayers increased equine IVF rates (9 vs 0% in controls). Although there was no significant effect of osteopontin or ANP A on fertilization, osteopontin slightly improved the IVF rates (Mugnier *et al.* 2009a). Moreover, Ambruosi *et al.* (2013) reported increased monospermic fertilization rates when mature equine oocytes were pre-incubated with DMBT1 and subsequently fertilized by procaine-capacitated spermatozoa.

There are further unidentified oviduct secreted proteins with affinity for the sperm plasma membrane or that affect final maturation of the equine oocyte (Battut *et al.* 1995). However, little is known about their function in the equine oviduct (Goudet 2011).

Besides proteins, many other factors including electrolytes (e.g. calcium, bicarbonate), lipids (including steroids) and carbohydrates contribute to the composition of oviductal fluid to create optimal fertilization conditions. The exact effect on capacitation/fertilization is poorly understood. The ion concentrations in the oviductal fluid of mares tend to be similar to those in serum (Aguilar & Reyley 2005), with the exception of magnesium, which

is present in concentrations 2–5 times higher than in plasma (Campbell *et al.* 1979) and much higher than in other species (Aguilar & Reyley 2005).

### Acid–base balance of oviductal fluid

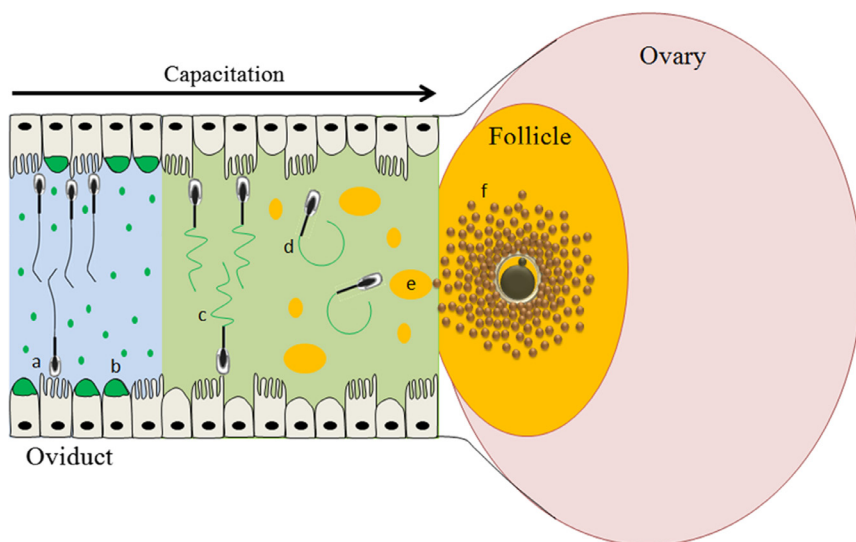
The acid–base balance in both the male and female reproductive tracts is finely modulated (Pastor-Soler *et al.* 2005, Lishko *et al.* 2012). The male reproductive tract and more specifically the epididymis contains acidic luminal fluid (pH  $\approx$  6.5; 3–4 mM bicarbonate) (Levine & Marsh 1971, Acott & Carr 1984, Carr *et al.* 1985, Rodriguez-Martinez *et al.* 1990), which renders mature spermatozoa quiescent. As a result of bicarbonate secretion in the cauda epididymis, the pH increases slightly and the spermatozoa acquire progressive motility. During ejaculation, spermatozoa are mixed with seminal plasma resulting in a further increase of the pH to 7.2 (25–30 mM bicarbonate) (Rodriguez-Martinez *et al.* 1990). However, seminal plasma also contains de-capacitation factors that inhibit capacitation. In contrast, female reproductive tract luminal fluids, that is the uterus and in particular the oviduct, tend to be alkaline (pH  $\approx$  7.6–7.8) (Vishwakarma 1962, Murdoch & White 1968, Maas *et al.* 1977). In the uterus, spermatozoa are still mixed with seminal plasma whereas upon entering the oviduct the sperm cells are separated from the seminal plasma de-capacitation factors (reviewed by Liu *et al.* (2012)).

It has also been suggested that in the peri-ovulatory period, the luminal fluids of the mare's genital tract also exhibit an increased, slightly alkaline pH. The following findings support this theory: (1) When untreated stallion semen was introduced into the uterus of oestrous mares and retrieved 30 min later, the pH increased from 7.47 to 7.85 (Gonzalez-Fernandez *et al.* 2012). (2) We showed that the number of alkaline secretory vesicles in the epithelium of oviduct explants decreased

significantly during sperm binding, and suggest that this generates an alkaline local microenvironment that stimulates capacitation *in vitro* (Leemans *et al.* 2014). (3) In response to these alkaline conditions (pH 7.9), stallion spermatozoa show (a) a decreased affinity for oviduct epithelium binding and increased propensity for head-to-head agglutination (Leemans *et al.* 2016), (b) a gradual increase in intracellular pH associated with increased protein tyrosine phosphorylation of proteins in the sperm tail (Gonzalez-Fernandez *et al.* 2012, Aalberts *et al.* 2013, Leemans *et al.* 2014), and (c) hyperactivated motility which facilitates a limited release of spermatozoa from oviduct binding (Leemans *et al.* 2015b). To date, however, the pH of equine oviduct fluid has not been measured during the peri-ovulatory period secretory phase of the oviduct epithelium. Nevertheless, we have strong evidence that an alkaline pH in the mare's oviduct during the late pre-ovulatory period is important for preparing stallion spermatozoa for fertilization.

### Does follicular fluid have an additional effect on capacitation/fertilization?

At ovulation, the majority of the follicular fluid passes through the fimbriae into the peritoneal cavity, but a small proportion enters the infundibulum of the oviduct (Ginther *et al.* 2008). This small volume of follicular fluid might nevertheless influence the oviductal microenvironment because it contains factors capable of activating or capacitating spermatozoa present in the oviduct, as already demonstrated in man (Fetterolf *et al.* 1994a,b, Yao *et al.* 2000), cattle (Sostaric *et al.* 2005), hamster (Yanagimachi 1969) and rabbit (Harper 1973a,b). In this respect, progesterone has been designated as a key factor in the capacitation of human spermatozoa (Harper *et al.* 2004, Publicover *et al.* 2007, Lishko *et al.* 2011, Strunker *et al.* 2011), while in cattle



**Figure 3** Three hypotheses explaining which *in vivo* biological oviduct factors are involved in stallion sperm capacitation – sperm release – fertilization. The missing capacitation/fertilization factors might be originating from (1) a mix of alkaline oviduct secretions and follicular fluid components, (2) the oocyte anchored with cumulus cell secretions and (3) oviduct epithelium secretions. (A: Sperm–oviduct binding, B: secretion of the alkaline vesicles of the secretory epithelial cells, C: induction of hypermotility and tail-associated protein tyrosine phosphorylation, D: release of sperm from oviduct epithelium, E: a small amount of follicular fluid enters the oviduct after ovulation and F: the oocyte anchored with cumulus cells will be shedded in the oviduct as well).



glycosaminoglycans have been identified as the key activators of capacitation (Sostaric *et al.* 2005). In horse, little is known about capacitation factors in follicular fluid, although progesterone has been proposed as a likely candidate (Cheng *et al.* 1998a,b, Rath *et al.* 2003).

We initially hypothesized that a mix of oviductal secretions and follicular fluid components must induce sperm capacitation in the equine peri-ovulatory oviduct (Hypothesis 1) because we had shown that the combined effect of an elevated environmental pH, external calcium and unidentified follicular fluid factor(s) (heat-resistant, hydrophilic factor <30 kDa) induced hyperactivation, tail-associated protein tyrosine phosphorylation and sperm release from the oviduct epithelium (Leemans *et al.* 2015b). However, the physiological relevance of hypothesis 1 is questionable because pre-ovulatory follicular fluid is not alkaline (pH  $\pm$  7.2) (Leemans *et al.* 2015b). Instead, capacitation in the narrow and tortuous oviductal lumen might be induced by oviductal fluid alone, while the unidentified follicular fluid factor(s) might also be secreted by cumulus cells anchored to the oocyte (Hypothesis 2) and the oviduct epithelium (Hypothesis 3) (Fig. 3). In the latter scenario, the fact that we observed protein tyrosine phosphorylation only in sperm bound to oviduct explants (Leemans *et al.* 2014) might reflect a dilution gradient of pro-capacitating factors in which the secretory epithelial cells generated the appropriate alkaline local microenvironment whereas the concentration of the unidentified follicular fluid factor(s) may have been too low to induce the other capacitation events such as hyperactivated motility and plasma membrane fluidity. It is also noted that co-incubation of stallion spermatozoa with cumulus–oocyte complexes did not trigger any capacitation-related parameters (unpublished data).

### Further considerations

Since the *in vitro* oviduct models used have their specific limitations, and *in vivo* collection of oviduct fluid is very challenging, a 3D oviduct model may provide a valuable *in vitro* alternative. Recently, nanotechnologies such as microfluidic automation, that is the automated routing, dispensing, mixing and separation of fluids through microchannels, via 3D prints and inserts have been introduced in attempts to more closely mimic an *in vitro* oviduct model that better recapitulates physiological function. For example in pig, IVF of porcine oocytes in microchannels resulted in a higher incidence of monospermic fertilization than in oocytes fertilized in the traditional microdrop system, despite comparable penetration and male pronucleus formation rates (Clark *et al.* 2005). In horse, further research could usefully focus on cultivating oviductal cells in microfluidic channels. The switch from a static to a more dynamic model should generate a more robust oviduct epithelium cell model in terms of morphological, ultrastructural

and physiological features. Currently, a 3D oviduct culture system is being developed in horse and in cow (Dr Gadella and Dr Stout, personal communication), in which oviduct epithelial cells maintain or re-establish their *in vivo*-like differentiation status and function. Preliminary observations have already shown that spermatozoa can bind to the zona pellucida and subsequently acrosome react, which is followed by partial penetration of the zona pellucida. This has been achieved in a medium that was conditioned by epithelial cells, but in the absence of any exogenous capacitation factors. A basolateral microperfusion system that allows creation of the peri-ovulatory ovarian steroid endocrine shift to induce changes in the secretion of epithelial cells in an oviduct culture insert is currently being designed. The applicability of such systems to aid equine IVF is a viable topic for future study, but it will almost certainly deepen our understanding of gamete–oviduct interaction, including sperm capacitation, fertilization and early embryo development. Ultimately, after identifying capacitation triggers, a redefined capacitating medium incorporating the primary capacitating/fertilization triggers might then be established and finally enable successful IVF in the horse.

### Conclusion

For more than 25 years, co-incubation of mature oocytes with capacitated spermatozoa has been the standard method for producing *in vitro* embryos in several species including man, cattle, pigs and many laboratory animals. However, conventional IVF is still not reliably successful using equine gametes. Inadequate capacitation of stallion spermatozoa under *in vitro* conditions is almost certainly the major obstacle to the failure of equine IVF. We hypothesize that one or more oviduct-derived factor(s) is/are indispensable to adequate triggering of capacitation to enable stallion sperm cells to penetrate an oocyte. The key capacitating factor composition of oviduct fluid is usually mimicked *in vitro* to establish an efficient capacitation/fertilization medium. The effect of increased local pH on capacitation strongly suggests that equine sperm capacitation/fertilization media require a higher pH than for other mammalian species. However, even these capacitating conditions do not induce the acrosome reaction and allow successful equine IVF. In the future, the role of oviduct fluid components on equine IVF should be further elucidated by new technologies such as 3D oviduct printing and microfluidic automation.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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