

Update on mammalian sperm capacitation: how much does the horse differ from other species?

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Abstract

In contrast to various other mammalian species, conventional *in vitro* fertilization (IVF) with horse gametes is not reliably successful. In particular, stallion spermatozoa fails to penetrate the zona pellucida, most likely due to incomplete activation of stallion spermatozoa (capacitation) under *in vitro* conditions. In other mammalian species, specific capacitation triggers have been described; unfortunately, none of these is able to induce full capacitation in stallion spermatozoa. Nevertheless, knowledge of capacitation pathways and their molecular triggers might improve our understanding of capacitation-related events observed in stallion sperm. When sperm cells are exposed to appropriate capacitation triggers, several molecular and biochemical changes should be induced in the sperm plasma membrane and cytoplasm. At the level of the sperm plasma membrane, (1) an increase in membrane fluidity, (2) cholesterol depletion and (3) lipid raft aggregation should occur consecutively; the cytoplasmic changes consist of protein tyrosine phosphorylation and elevated pH, cAMP and Ca²⁺ concentrations. These capacitation-related events enable the switch from progressive to hyperactivated motility of the sperm cells, and the induction of the acrosome reaction. These final capacitation triggers are indispensable for sperm cells to migrate through the viscous oviductal environment, penetrate the cumulus cells and zona pellucida and, finally, fuse with the oolemma. This review will focus on molecular aspects of sperm capacitation and known triggers in various mammalian species. Similarities and differences with the horse will be highlighted to improve our understanding of equine sperm capacitation/fertilizing events.

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Introduction

Conventional *in vitro* fertilization (IVF), that is co-incubation of mature oocytes with capacitated sperm in a petri dish, is a standard procedure for producing embryos *in vitro* in a range of species, including man (Stephoe & Edwards 1978), the cow (Perry 2013), the pig and various laboratory animals (Galli *et al.* 2003, Betteridge 2006). By contrast, conventional IVF has to date been very poorly successful in the horse. Equine IVF is hampered by the inability of stallion spermatozoa to penetrate the zona pellucida *in vitro* (Tremoleda *et al.* 2003). In theory, this failure could be attributed to deficiencies in either *in vitro* prepared sperm and/or *in vitro* matured oocytes. Given that *in vitro* matured oocytes placed in the oviduct can be fertilized *in vivo* (Hinrichs *et al.* 2002), while *in vivo* matured oocytes harvested from pre-ovulatory follicles cannot be fertilized *in vitro* (Palmer *et al.* 1991), the inability of sperm cells to penetrate the zona pellucida *ex vivo* is most likely

the result of deficient capacitation, that is activation of spermatozoa (Tremoleda *et al.* 2003). Although current equine IVF conditions do support the binding of stallion sperm to the oocyte's zona pellucida, this does not result in subsequent initiation of the acrosome reaction mandatory to penetrate the oocyte (Tremoleda *et al.* 2003). In contrast to sperm cells from other mammalian species, stallion sperm incubated under typical IVF conditions, that is in medium containing HCO₃⁻, Ca²⁺ and albumin, also fail to display other capacitation-related characteristics, such as hyperactivated motility, increased plasma membrane fluidity and protein tyrosine phosphorylation (Tremoleda *et al.* 2003) (Fig. 1).

Capacitation is the maturation process that sperm cells undergo after ejaculation, during their passage through the female genital tract (Suarez 2002). These biochemical changes enable the sperm to bind to and penetrate the zona pellucida and subsequently fuse with the oocyte (Fig. 1) (Yanagimachi 1994). The sperm

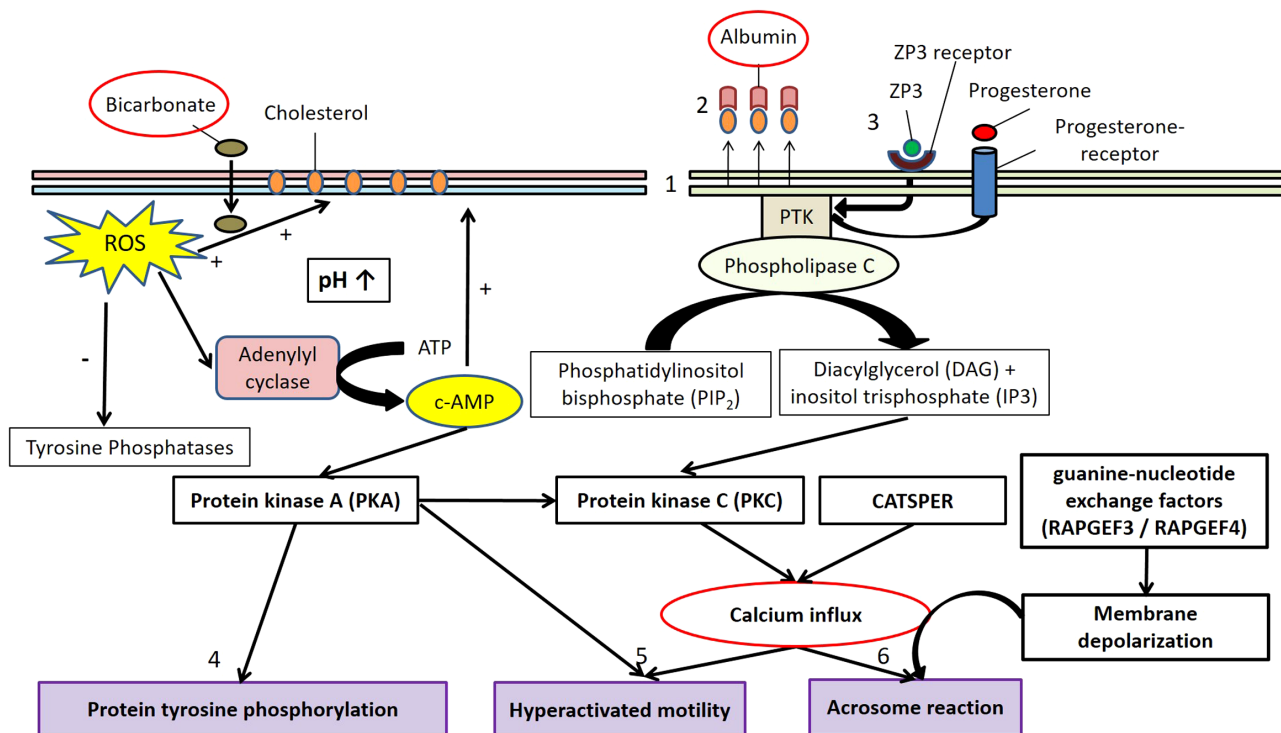


Figure 1 Pathways involved in sperm capacitation, with emphasis on the horse. When spermatozoa undergo capacitation, the following changes occur: (1) increase in membrane fluidity, (2) cholesterol depletion, (3) aggregation of lipid raft receptors, (4) protein tyrosine phosphorylation, (5) hyperactivated motility and (6) the acrosome reaction. PTK, protein tyrosine kinase; ROS, reactive oxygen species; ZP3, ZP protein 3; ZP3-receptor, ZP protein 3-receptor; +, activation; -, inhibition.

plasma membrane fuses with the outer acrosomal membrane, resulting in the exocytotic event known as the acrosome reaction that involves the release of lytic enzymes which locally dissolve the zona pellucida. The sperm cells also acquire hyperactivated motility in order to 'drill' through the zona pellucida (Yanagimachi 1994). Equids are one of the exceptions, in which capacitation does not appear to progress as described above. Using an oviduct epithelial explant model, we identified physiological triggering of three important capacitation parameters, namely increased intracellular pH (Leemans *et al.* 2014), protein tyrosine phosphorylation in the sperm tail (Leemans *et al.* 2014, 2015b) and hyperactivated motility (Leemans *et al.* 2015b). However, this approach was not sufficient to induce the acrosome reaction and fertilization during co-incubation with oocytes. Hence, our understanding of sperm activation in the oviduct, the ability of these spermatozoa to achieve fertilizing competence and, in particular, the intracellular pathways involved in this process needs to improve. This review focuses on what is currently known about capacitation pathways in sperm cells of non-equine species, the provision of fuel to support the highly energy-dependent capacitation events and how much these events differ to capacitation-related events observed in stallion spermatozoa.

Energy metabolism in stallion spermatozoa to support energy-demanding, fertility-related processes such as capacitation

Sperm capacitation and other fertility-related pathways require large quantities of energy. To fuel these crucial capacitation events, individual sperm cells must generate relatively large amounts of ATP. In various mammalian species including man and the mouse, sperm motility and the maintenance of membrane integrity rely predominantly on ATP generated by non-aerobic glucose metabolism via glycolysis (Mukai & Okuno 2004, Storey 2008, du Plessis *et al.* 2015), as demonstrated by the fact that mitochondrial oxidative chain uncouplers did not affect sperm motility or membrane integrity in sperm suspended in glucose-containing media. By contrast, the proportion of membrane intact and motile stallion sperm, and the velocity of that motion, in glucose-enriched media were all significantly reduced after exposure to mitochondrial uncouplers (Gibb *et al.* 2014, Davila *et al.* 2016). Inhibiting complex IV or ATP synthase, two proteins on the inner mitochondrial membrane critical to electron flow during oxidative phosphorylation, had similar effects on stallion sperm (Davila *et al.* 2016). Moreover, subsequent uncoupling of mitochondria or inhibition of mitochondrial respiration led to a massive

drop in cytoplasmic ATP concentrations in stallion spermatozoa. Overall, this indicates that maintenance of stallion sperm membrane integrity and motility, two important requirements for fertility, are highly dependent on aerobic production of ATP (Gibb *et al.* 2014, Plaza Davila *et al.* 2015). As a consequence of the requirement for stallion sperm to generate mitochondrial ATP aerobically, increased cytoplasmic concentrations of reactive oxygen species (ROS), such as superoxide anions and their reduced form, hydrogen peroxide, are observed during stallion sperm incubation and activation. In turn, this cytoplasmic ROS accumulation leads to increased production of metabolic by-products, and the combination of accumulating ROS and cytotoxic lipid aldehydes (acrolein, 4-hydroxynonenal and malondialdehyde) ultimately leads to massive lipid peroxidation and DNA damage and thereby accelerated cell death (Gibb *et al.* 2016). To counteract the adverse effect of endogenous ROS production, and enable ROS homeostasis (Jones 2006) or defense against oxidative stress, stallion seminal plasma contains relatively high concentrations of antioxidants such as catalase (Ortega Ferrusola *et al.* 2009), superoxide dismutase (Ball *et al.* 2000, Ortega Ferrusola *et al.* 2009), glutathione (Hemachand & Shaha 2003, Jones 2006) and ergothioneine (Mann 1975). Moreover, mitochondrial aldehyde dehydrogenase 2 has been identified in stallion sperm cells and proposed to serve as a detoxifier of aldehydes generated either endogenously or exogenously (Gibb *et al.* 2016). However, ROS defense mechanisms will eventually become exhausted and are not thus completely protective against sperm cell degeneration.

In this aspect, an interesting observation has been made with respect to stallion sperm oxidative stress and fertility. Spermatozoa from matings that did result in conception (and therefore considered 'more fertile') had lower percentages of membrane intact and higher percentages of cells displaying ROS-induced damage than spermatozoa from matings that did not result in conception (Gibb *et al.* 2014). It has been proposed that this can be explained by the fact that fertile stallions produce metabolically more active sperm cells that exhibit higher levels of oxidative phosphorylation. This will result in higher concentrations of ROS, leading to increased lipid peroxidation and, in turn, impaired sperm motility and membrane integrity in more fertile stallions. This has led to the suggestion that spermatozoa from highly fertile stallions 'live fast and die young'. It should also be borne in mind that a modest degree of cytoplasmic ROS production plays an important physiological role in sperm capacitation. These aspects are discussed below.

How to trigger capacitation *in vitro*?

In vivo, capacitation is initiated when a sperm cell is exposed to the environment within the female genital

tract, that is the uterus and oviduct, close to the time of ovulation (reviewed by Leemans *et al.* (2016a)); capacitation events can be mimicked *in vitro* by incubating sperm cells in medium containing HCO_3^- , Ca^{2+} and albumin, after performing density gradient centrifugation (e.g. Percoll) to separate the sperm cells from the seminal plasma. HCO_3^- , Ca^{2+} and albumin are three capacitation factors known to induce the sperm changes required for the acquisition of fertilizing potential across many species (mice: Visconti *et al.* 1995a,b; hamster: Visconti *et al.* 1999b; man: Osheroff *et al.* 1999; pig: Flesch & Gadella 2000). In addition, species-specific factors have been identified. In cattle, for example, heparin-like molecules such as glycosaminoglycans are an essential capacitation trigger (Parrish *et al.* 1988). Unfortunately, the species-specific capacitation triggers required to establish a repeatable, effective equine IVF system have yet to be identified. In various studies of stallion sperm capacitation, 'capacitating medium' has included the three general capacitation triggers (HCO_3^- , Ca^{2+} and albumin) even though it is known that full capacitation is not accomplished; non-capacitating medium, lacking these three triggers, is used as control medium (McPartlin *et al.* 2008, Leemans *et al.* 2016b). In Table 1, an overview of molecules frequently used as capacitation triggers in mammals, including the horse, is shown.

Capacitation events

Although capacitation was described for the first time in 1951 (Chang 1951, Austin 1952), the process is still not completely understood. It is known that capacitation involves a series of changes including (1) the removal of seminal plasma and surface-adhered decapacitation factors from the sperm plasma membrane, (2) reorganization of the sperm plasma membrane and (3) activation of intracellular pathways (Fig. 1).

After the removal of seminal plasma, a rapid increase in membrane fluidity (within 10 min) is induced by an increasing intracellular HCO_3^- concentration and the activation of second messenger systems, including soluble adenylyl cyclase and a rise in intracellular Ca^{2+} (pig: Flesch & Gadella 2000, Gadella *et al.* 2008; horse: Rathi *et al.* 2003). The activation of soluble adenylyl cyclase and the concomitant production of cAMP results in depletion of cholesterol from the sperm plasma membrane via a cholesterol acceptor such as albumin (>1 h), which is followed by a slower series of functional membrane changes in which lipid ordered microdomains aggregate at the apical ridge of the sperm head (>1 h) (pig: van Gestel *et al.* 2005a). These microdomains contain functional zona pellucida-binding protein complexes (pig: van Gestel *et al.* 2007) and the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which play an important role in the induction of the acrosome

Table 1 Overview of capacitation triggers and their *in vitro* capacitation effect in different mammalian species.

Capacitating trigger	Capacitation effect	Species	Reference
Ca ²⁺	Membrane fluidity	Mouse	Visconti <i>et al.</i> (1995a,b)
	Protein tyrosine phosphorylation	Man	Osheroff <i>et al.</i> (1999)
	Hyperactivated motility	Pig	Flesch & Gadella (2000)
	Acrosome reaction	Cow	Byrd (1981), Breininger <i>et al.</i> (2010)
		Horse	McPartlin <i>et al.</i> (2008)
HCO ₃ ⁻	Membrane fluidity	Mouse	Visconti <i>et al.</i> (1995a,b)
	Protein tyrosine phosphorylation	Hamster	Visconti <i>et al.</i> (1999b)
	Hyperactivated motility	Man	Osheroff <i>et al.</i> (1999)
	Acrosome reaction	Pig	Flesch & Gadella (2000)
		Cow	Breininger <i>et al.</i> (2010)
		Horse	Rathi <i>et al.</i> (2003), McPartlin <i>et al.</i> (2008)
		Mouse	Visconti <i>et al.</i> (1995a,b)
Albumin	Cholesterol depletion	Pig	Flesch & Gadella (2000)
		Cow	Byrd (1981)
		Horse	McPartlin <i>et al.</i> (2008)
		Mouse	Visconti <i>et al.</i> (1999a)
		Pig	van Gestel <i>et al.</i> (2005b)
Methyl β-cyclodextrin	Cholesterol extraction	Horse	Bromfield <i>et al.</i> (2014)
		Mouse	Cheng <i>et al.</i> (1998a,b)
		Pig	Lishko <i>et al.</i> (2011)
Heparin	Membrane fluidity	Cow	Parrish <i>et al.</i> (1988), Gualtieri <i>et al.</i> (2005), Breininger <i>et al.</i> (2010)
	Hyperactivated motility		
	Protein tyrosine phosphorylation		
Progesterone	Acrosome reaction	Horse	Cheng <i>et al.</i> (1998a,b)
	Hyperactivated motility	Man	Lishko <i>et al.</i> (2011)
Ca ²⁺ ionophore A23187	Acrosome reaction	Mouse	Tateno <i>et al.</i> (2013)
		Man	Bielfeld <i>et al.</i> (1994), Liu <i>et al.</i> (2011)
		Pig	
		Cow	Birck <i>et al.</i> (2009)
		Horse	Fraser <i>et al.</i> (1995)
		Mice	Balao da Silva <i>et al.</i> (2013), Tateno <i>et al.</i> (2013)
		Horse	Graham (1996)
Lysophospha-tidylcholine c-AMP and caffeine	Hyperactivated motility	Horse	Graham (1996)
	Acrosome reaction	Cow	Breininger <i>et al.</i> (2010)
	Protein tyrosine phosphorylation	Horse	Pommer <i>et al.</i> (2003)
		Pig	Funahashi & Nagai (2001)
		Cow	Breininger <i>et al.</i> (2010)
ROS	Hyperactivated motility	Horse	Baumber <i>et al.</i> (2003)
	Protein tyrosine phosphorylation	Horse	Gonzalez-Fernandez <i>et al.</i> (2012)
		Cow	Marquez & Suarez (2007)
Alkaline medium pH	Protein tyrosine phosphorylation	Guinea pig	Mujica <i>et al.</i> (1994)
Procaine	Hyperactivated motility	Horse	McPartlin <i>et al.</i> (2009), Leemans <i>et al.</i> (2015a)

reaction (pig: Tsai *et al.* 2010, 2012). Simultaneously, the production of cAMP enables the activation of protein kinase A which is required to phosphorylate the tyrosine residues on sperm proteins (Rathi *et al.* 2003, Ijiri *et al.* 2012, Signorelli *et al.* 2012). In various species, this cAMP-dependent protein tyrosine phosphorylation, especially in the sperm tail, has been associated with the acquisition of hyperactivated sperm motility and is considered a marker for some essential elements of the capacitation process (mouse: Visconti *et al.* 1995a,b; pig: Flesch *et al.* 1999, Harayama *et al.* 2012).

Plasma membrane changes

Spermatozoa have a highly polarized morphology with a heterogenic surface differentiated into at least four surface membrane domains: the apical ridge and the pre-equatorial, equatorial and post-equatorial surface

areas, respectively. Each of these regions plays a specific role in fertilization: (1) the apical ridge facilitates zona pellucida-binding (rabbit: O'Rand & Fisher 1987), (2) the apical ridge, together with the pre-equatorial surface area, is involved in the acrosome reaction while (3) the equatorial surface area initiates binding to the oolemma and the subsequent fertilization fusion. These events occur in the region of the sperm head where the sperm plasma membrane, including the lipid rafts (microdomains), covers the acrosome and plays an essential role in the dynamics of sperm capacitation (Gadella *et al.* 2008).

Cholesterol depletion

A redistribution of phospholipids in the surface of the sperm head is an essential step in the capacitation process. HCO₃⁻/soluble adenylyl cyclase/cAMP mediate

an increase in membrane fluidity, detected as enhanced merocyanine 540 binding (Gadella & Harrison 2000, 2002), that enables the redistribution of cholesterol from the equatorial area to the apical part of the sperm plasma membrane, while seminolipids move retrogradely (pig: Gadella *et al.* 1994, 1995). Both molecules are considered to be plasma membrane stabilizers, which prevent membrane fusion prior to capacitation. The HCO_3^- -mediated repacking of the sperm surface lipids is also essential in enabling the efflux of cholesterol which further increases plasma membrane fluidity by allowing the aggregation of lipid rafts, another essential step in the reorganization of the plasma membrane.

In vitro, the extraction of cholesterol from the sperm plasma membrane can be achieved by the oxidation of membrane sterols (Boerke *et al.* 2013), or active cholesterol transporters that deliver free cholesterol to the hydrophobic pocket of albumin (Flesch *et al.* 2001). Brouwers *et al.* (bull: 2011) showed that the production of ROS is an essential step in oxysterol formation. ROS production at low levels during sperm capacitation was first demonstrated in 1993, and indicates that sperm capacitation should be considered an oxidative process dependent on the active generation of ROS (man: de Lamirande & Gagnon 1993). This process may also help to regulate protein tyrosine phosphorylation, another important capacitation marker, by increasing cAMP production (Aitken *et al.* 2004, Aitken 2011) and suppressing tyrosine phosphatase activity (Takakura *et al.* 1999). Since oxysterols are more hydrophilic than intact cholesterol, they can more easily move through the plasma membrane to bind to sterol acceptor molecules like albumin. Indeed, the presence of bovine serum albumin in capacitating medium is essential because of its unique ability to scavenge hydrophilic oxidation products and facilitate cholesterol extraction (Boerke *et al.* 2008). An alternative macromolecule not of animal origin (important in terms of risk of disease transmission) is methyl β -cyclodextrin. Whereas albumin extracts only 20% of the cholesterol from the non-raft sperm plasma membrane fraction, methyl β -cyclodextrin extracts 50% of the cholesterol from the entire sperm plasma membrane, including the lipid rafts (pig: van Gestel *et al.* 2005b). However, methyl β -cyclodextrin might have a deteriorative effect on sperm cells, even in low concentrations. A deteriorative effect of methyl β -cyclodextrin on mouse oocytes has also been reported (Buschiazzo *et al.* 2013). In stallion sperm, however, standard capacitating conditions, that is inclusion of Ca^{2+} , HCO_3^- and bovine serum albumin (individual or in combination), does not facilitate cholesterol removal from the sperm plasma membrane. Indeed, while HCO_3^- induces an increase in ROS, this is abolished by the addition of Ca^{2+} or albumin (Macias Garcia *et al.* 2015). These observations suggest a species-specific difference in the regulation of plasma membrane changes during stallion sperm capacitation.

Aggregation of lipid rafts

As demonstrated in pig spermatozoa, increased membrane fluidity and cholesterol depletion are followed by a redistribution of the laterally segregated molecules, that is lipid ordered microdomains aggregate at the apical ridge area of the sperm head (Boerke *et al.* 2008). The proteins and lipids which make up these microdomains also show a capacitation-dependent change to include higher proportions of cholesterol, sphingomyelin, gangliosides and phospholipids with saturated long-chain acyl chains and lipid-modified proteins such as GPI-anchored proteins (Simons & Toomre 2000, van Gestel *et al.* 2005a). Moreover, caveolin-1 and flotillin-1 are lipid raft-specific markers (van Gestel *et al.* 2005a) that accumulate in these microdomains along with functional zona pellucida-binding protein complexes. In man, angiotensin-converting enzyme and protein disulfide isomerase A6 were identified in the microdomains as proteins able to interact with heat shock protein A2, which plays a pivotal role in the remodeling of the sperm surface during capacitation and the recognition of human oocytes (Bromfield *et al.* 2016). In pigs, it has been shown that isoforms of AQN-3 (spermadhesin), P47 (porcine homologue of SED-1), fertilin β and peroxiredoxin 5 are key proteins regulating the primary binding between capacitated spermatozoa and the zona pellucida (van Gestel *et al.* 2007). However, it is not known whether these proteins reside in microdomains.

Hyperactivated motility

General sperm motility

A specific cytoplasmic pH is crucial to allow sperm motility. For de-membranated sperm, maximal percentages of motile sperm were recorded between pH 7.0 and 8.1 for bull (Ho *et al.* 2002), pH 7.8 for human (Giroux-Widemann *et al.* 1991) and between pH 7.5 and 8.0 for ram (Ishijima & Witman 1987) spermatozoa. For comparison, the maximum percentage of motile stallion spermatozoa was observed in medium at pH 7.0 (Loux *et al.* 2014). An appropriate cytoplasmic pH enhances symmetrical flagellar, and therefore, progressive sperm motility, which requires the activation of dynein ATPases on phosphorylated dynein molecules and their interaction with Ca^{2+} which, in turn, results in the sliding of the adjacent outer axonemal doublet microtubules (Shingyoji *et al.* 1977). When the doublets slide along one another, the sliding force is translated into a bend in the sperm tail (Tash 1989). The normal flagellar waveform requires an asynchronous phosphorylation and dephosphorylation of the dynein arms along the complete axonemal length (Wargo & Smith 2003). In mammals, sperm motility is mostly initiated and maintained by ATP, Ca^{2+} and HCO_3^- -driven cAMP-dependent phosphorylation of flagellar proteins

(Tash & Means 1983, White & Aitken 1989, Ho *et al.* 2002). Like HCO_3^- , Ca^{2+} also directly regulates soluble adenylyl cyclase, which results in cAMP generation and activation of protein kinase A (Fig. 1) (Liguori *et al.* 2004, Hess *et al.* 2005). An important downstream target of cAMP in the sperm flagellum is serine/threonine kinase protein kinase A (Visconti *et al.* 1997). The phosphorylation of serine/threonine activates protein kinase A which results in the downstream phosphorylation of tyrosine kinases whose targets are primarily located in the sperm tail (man: Leclerc *et al.* 1996; mouse: Si & Olds-Clarke 2000). On the other hand, dynein dephosphorylation is evoked by the calmodulin-dependent protein serine/threonine phosphatase, calcineurin, which is necessary to balance the cAMP-driven serine/threonine kinases in the sperm tail. The resulting net phosphorylation represents the sperm motility status well (Tash & Bracho 1994). If serine/threonine phosphatase activity is dominant, spermatozoa are mainly immotile whereas serine/threonine kinase activity correlates with increased motility (man and Rhesus monkey: Smith *et al.* 1996; bull: Vijayaraghavan *et al.* 1996).

Relationship between Ca^{2+} , pH and hyperactivated sperm motility?

Sperm cells must acquire hyperactivated motility to (1) detach from their oviduct epithelial cell binding and leave the oviductal reservoir (Suarez & Pacey 2006), (2) migrate through the viscous lumen of the oviduct (Suarez & Dai 1992) and (3) penetrate the cumulus matrix and zona pellucida of the mature oocyte in order to fuse with the oolemma (Stauss *et al.* 1995). In many species, hyperactivated motility is characterized by a highly asymmetrical and high-amplitude flagellar beating pattern that gives rise to a whip-like motion of the sperm tail which, *in vitro*, results in circular, figure of eight or zigzag swimming trajectories (Yanagimachi 1994, Ishijima *et al.* 2006). The onset and maintenance of hyperactivated motility is associated with an influx of Ca^{2+} into the cytosol of the sperm tail (Suarez *et al.* 1993, Suarez 2008). Ho *et al.* (2002) showed in de-membranated bull sperm that a symmetrical, progressive sperm movement was maintained when the intracellular Ca^{2+} concentration was ~ 50 nM. When hyperactivated motility was initiated, the intracellular Ca^{2+} concentration increased to 400 nM. By contrast, Loux *et al.* (2014) reported that the degree of hyperactivated motility was inversely related to the cytoplasmic Ca^{2+} concentration in stallion spermatozoa. Using a similar de-membranated sperm model, they demonstrated that an increasing Ca^{2+} concentration did not induce hyperactivated motility in stallion spermatozoa at any pH. Moreover, cytoplasmic alkalization induced both a hyperactivated motility response and cytoplasmic Ca^{2+} rise in intact sperm cells (Loux *et al.* 2013). Loux *et al.* (2013) observed an intracellular pH increase from 7.1

to 7.3–7.4 in hyperactivating conditions. A pH effect on the motility of de-membranated stallion spermatozoa was not apparent. However, an extremely low Ca^{2+} concentration (27 pM) was sufficient to maintain motility in de-membranated stallion sperm at pH 7.4 (Loux *et al.* 2014). Moreover, procaine which is considered a potent inducer of hyperactivated motility in intact stallion spermatozoa, acts independently to extracellular Ca^{2+} influx (Loux *et al.* 2013). Overall, a species-specific relationship between Ca^{2+} sensitivity and hyperactivated motility is apparent in the horse.

In vitro, Ca^{2+} ionophores such as A23187 or ionomycin can induce and maintain hyperactivated motility in mouse spermatozoa for several hours (Suarez *et al.* 1987, Tateno *et al.* 2013). Besides the reliable induction of the acrosome reaction in stallion spermatozoa, Ca^{2+} ionophore A23187 exposure results in a complete loss of sperm motility and membrane integrity within 1 h (Christensen *et al.* 1996, Rathi *et al.* 2001). It is possible that these adverse effects of Ca^{2+} ionophore on sperm motility and membrane integrity are related to the fact that stallion sperm, in contrast to other mammals, are dependent on mitochondrial ATP (as discussed above). In this respect, excessive mitochondrial Ca^{2+} concentration can lead to mitochondrial failure and initiate cell death (Contreras *et al.* 2010). Moreover, Ca^{2+} ionophores uncouple oxidative phosphorylation by inhibiting mitochondrial ATPase activity (Humes & Weinberg 1980, Krumschnabel *et al.* 1999). As mentioned above, other inhibitors that uncouple oxidative phosphorylation adversely affect stallion sperm motility and membrane integrity (Gibb *et al.* 2014, Davila *et al.* 2016). This is because production of mitochondrial ATP to support stallion sperm motility and membrane integrity is severely compromised soon after exposure. This is a cause for concern with respect to a capacitation induction protocol, because sperm viability and motility would need to be conserved over a period of hours during *in vitro* fertilization. Other pharmacological agents such as caffeine (Ho & Suarez 2001b), thimerosal (Ho & Suarez 2001b, Marquez & Suarez 2004) and thapsigargin (Ho & Suarez 2001b, Ho & Suarez 2003) also trigger an intracellular Ca^{2+} rise and initiate asymmetrical flagellar beating in mouse spermatozoa. The effect of these molecules on the cytoplasmic Ca^{2+} concentration and hyperactivated motility have yet to be studied in the horse.

In mammals such as man, cattle and mice, CATSPER channels present on the principal piece of the sperm tail's need be activated to induce hyperactivated motility. There are four CATSPER genes coding for proteins that are structurally similar to subunits of conventional voltage-gated cation channels. When a mouse single CATSPER gene knock-out was created, males were infertile because the spermatozoa were not able to achieve hyperactivated motility (Carlson *et al.* 2005, Qi *et al.* 2007). The general trigger of these CATSPER

channels is alkaline depolarization evoked by a change in the oviductal ionic environment, which results in an elevated pH of the oviduct fluid (Carlson *et al.* 2003, Navarro *et al.* 2008). Contact between spermatozoa and the alkaline oviductal environment increases the intracellular pH and activates the CATSPER channels. In stallion sperm, CATSPER1 mRNA has been identified, and CATSPER1 protein was localized to the principal piece of the sperm tail. However, analysis of the equine CATSPER1 protein revealed species-specific differences in the structure in the pH sensor region (Loux *et al.* 2013). This indicated that, despite the presence of a CATSPER channel in stallion sperm, the relationship between hyperactivated motility and Ca^{2+} influx is likely to be weak.

A clear increase in oviduct pH and, as a consequence, in sperm cytoplasmic pH likely to activate the CATSPER channels, was observed in *Rhesus* monkeys approaching ovulation (7.1–7.3 to 7.5–7.8) (Maas *et al.* 1977). In the mouse, a Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchange controls the intracellular pH of the sperm (Zeng *et al.* 1996), while in man, an outflow of H^+ is initiated by activating a voltage-gated proton channel (Lishko *et al.* 2010). To maximize the subsequent Ca^{2+} entry through the CATSPER channels, both a pH-sensitive efflux of K^+ by KSPER (Navarro *et al.* 2007) and an activation of Cl^- channels by closing Na^+ channels might support capacitation-related sperm plasma membrane hyperpolarization (mouse: Hernandez-Gonzalez *et al.* 2007). Hyperpolarization also plays a central role in the acrosome reaction. In human spermatozoa, it has been demonstrated that CATSPER channels can be alternatively activated by progesterone and, to a lesser extent, prostaglandins (Lishko *et al.* 2011). Other Ca^{2+} metabolism-related pathways identified in the mammalian sperm tail, include the following: (1) a transient receptor potential Ca^{2+} channel, which may affect sperm motility and re-sequestration of Ca^{2+} into sperm stores (Castellano *et al.* 2003), (2) cyclic-nucleotide-gated Ca^{2+} channels (Wiesner *et al.* 1998) and (3) voltage-gated Ca^{2+} channels (Benoff 1998, Darszon *et al.* 2006). However, the role of the various Ca^{2+} regulatory pathways in the physiological activation of hyperactivated motility is unclear.

Besides the extracellular Ca^{2+} influx through the CATSPER channels, there are also intracellular Ca^{2+} stores, in the so-called redundant nuclear envelope (RNE) located at the base of the sperm tail, which are able to provide Ca^{2+} to the sperm cytoplasm (Ho & Suarez 2001b, 2003). Inositol 1,4,5-triphosphate (IP_3)-gated channels on the RNE membranes trigger Ca^{2+} release to the sperm cytoplasm whereas calreticulin, a Ca^{2+} binding protein, sequesters Ca^{2+} in the RNE (bull: Ho & Suarez 2001b, 2003; man: Naaby-Hansen *et al.* 2001). In the mouse, it has been shown that ryanodine receptors on the RNE membrane also play a role in intracellular Ca^{2+} release (Trevino *et al.* 1998).

As such, both Ca^{2+} sources contribute to initiation and maintenance of high Ca^{2+} concentrations during sperm hyperactivation (Ho & Suarez 2001b, 2003). More specifically, the Ca^{2+} -induced Ca^{2+} release from the RNE amplifies the intracellular Ca^{2+} rise resulting from CATSPER activation (Costello *et al.* 2009, Olson *et al.* 2010). Furthermore, this activation of CATSPER and IP_3 -gated channels is clearly involved in induction of the acrosome reaction (Quill *et al.* 2003, Ren *et al.* 2001). So far, a role of intracellular Ca^{2+} stores in the induction of hyperactivated motility in stallion spermatozoa is purely theoretical.

Hypermotility is induced by HCO_3^- and Ca^{2+} -driven pathways

So far, it has not been elucidated how sperm cells modify the beat of their tail, at the molecular level. It is known that the Ca^{2+} rise and the subsequent onset of hyperactivated motility changes the sliding of the microtubules (Lindemann & Lesich 2010). Furthermore, it has been demonstrated in primates (Mahony & Gwathmey 1999), pigs (Harayama *et al.* 2012) and rodents (Si & Okuno 1999) that hyperactivated motility is associated with a highly increased cAMP-dependent tyrosine phosphorylation of the flagellar proteins. The generator of tyrosine phosphorylation events, protein kinase A, is connected to the fibrous sheath of the sperm tail by A-kinase-anchoring proteins, and it has been suggested that these proteins play an important role in hyperactivated sperm motility (hamster: Si 1999). Moreover, A-kinase-anchoring proteins could attach protein kinase A to specific subcellular regions in close proximity to motility-related targets in the axoneme (Carrera *et al.* 1994, Mandal *et al.* 1999). A-kinase-anchoring protein 3 and A-kinase-anchoring protein 4 play a central role in activating tyrosine kinases to cause extensive tyrosine phosphorylation of sperm tail proteins (hamster: Si 1999; man: Ficarro *et al.* 2003). This altered tail protein tyrosine phosphorylation status is required for the acquisition of hyperactivated sperm motility (hamster: Si 1999; man: Leclerc *et al.* 1996). Changes in A-kinase-anchoring protein-mediated protein targeting might also play an essential role in this process.

Independent of protein kinase A, Ca^{2+} is involved in pathways regulating sperm motility. Calmodulin, located in the principal piece of the sperm tail (Schlingmann *et al.* 2007), is a Ca^{2+} -binding protein essential to the Ca^{2+} -dependent modulation of mammalian sperm motility (Ho & Suarez 2001a, Ignatz & Suarez 2005). It has been suggested that this pathway progresses in parallel with cAMP/protein kinase A activity, although the two act independently (Litvin *et al.* 2003, Schlingmann *et al.* 2007). Binding of Ca^{2+} to calmodulin activates Ca^{2+} /calmodulin-dependent kinases (Marin-Briggiler *et al.* 2005) which phosphorylate a specific axonemal protein, resulting in hyperactivated motility

(Suarez 2008). Calmodulin kinases present in the flagellum of bull (Ignatz & Suarez 2005) and human (Marin-Briggiler *et al.* 2005) sperm were associated with hyperactivated motility. On the other hand, phosphatase activities are also directly involved in axonemal function because they reverse the latter effect by regulating dynein ATPase activities (Tash *et al.* 1988, Carrera *et al.* 1996) (Fig. 2).

Is hyperactivated motility linked to protein tyrosine phosphorylation during stallion sperm capacitation?

As indicated above, in various mammalian species it has been shown that hyperactivated motility is associated with a marked increase in cAMP/protein kinase A and Ca²⁺/calmodulin-dependent kinase activity that induces tyrosine phosphorylation of the flagellar proteins that, in turn, alter the sliding of the microtubules within the sperm axoneme (mouse: Visconti *et al.* 1995a,b; pig: Fleisch *et al.* 1999, Harayama *et al.* 2012). Surprisingly, hyperactivated motility and protein tyrosine phosphorylation can also occur independently (mouse: Olds-Clarke 1989; cow: Marquez & Suarez 2004). This latter observation certainly seems to be true for stallion sperm. For example, type 10 sAC induced protein tyrosine phosphorylation in stallion sperm, without initiating hyperactivated motility (McPartlin *et al.* 2011). An increased PKA activity and protein tyrosine phosphorylation without hyperactivation was also observed after incubating stallion spermatozoa *in vitro* with membrane-soluble cAMP analogues or a phosphodiesterase inhibitor (caffeine) (Pommer *et al.* 2003). ROS (Baumber *et al.* 2003) and modified-Whitten's capacitating medium with higher alkalinity

(pH=7.8–8.0) (Gonzalez-Fernandez *et al.* 2012, 2013) also induce protein tyrosine phosphorylation in the absence of, or only a mild, hyperactivated motility response. Similarly, binding of stallion sperm to late pre-ovulatory stage oviduct epithelium induces protein tyrosine phosphorylation without hyperactivated motility. This protein tyrosine phosphorylation was regulated primarily by a high pH within oviductal secretory cells (Leemans *et al.* 2014). When stallion sperm are incubated in capacitating conditions at pH 7.4, a Ca²⁺-mediated inhibitory effect on protein tyrosine phosphorylation occurs due to the formation of a Ca²⁺/calmodulin complex that supports sperm phosphatase activity (Gonzalez-Fernandez *et al.* 2012). Under alkaline-capacitating conditions (pH=7.8–8.0) however, Ca²⁺/calmodulin-dependent kinases dominate and play a downstream role in protein kinase A-dependent protein tyrosine phosphorylation. As such, Ca²⁺-induced protein tyrosine kinases activate protein tyrosine phosphorylation in stallion spermatozoa downstream of protein kinase A (Gonzalez-Fernandez *et al.* 2013).

Interestingly, after cryopreservation a premature induction of protein tyrosine phosphorylation is observed, commonly referred to as 'cryocapacitation' (Bailey *et al.* 2000, Watson 2000). The increased osmolality associated with cryopreservation may induce a ROS-dependent increase in protein tyrosine phosphorylation in stallion sperm (Aitken *et al.* 1983, Burnaugh *et al.* 2010). After cryopreservation, sperm cells display an increased intracellular Ca²⁺ concentration, increased ROS generation and a reduced antioxidant capacity. Recently, Ortega-Ferrusola *et al.* (2017) examined the effect of cryopreservation on both apoptosis and

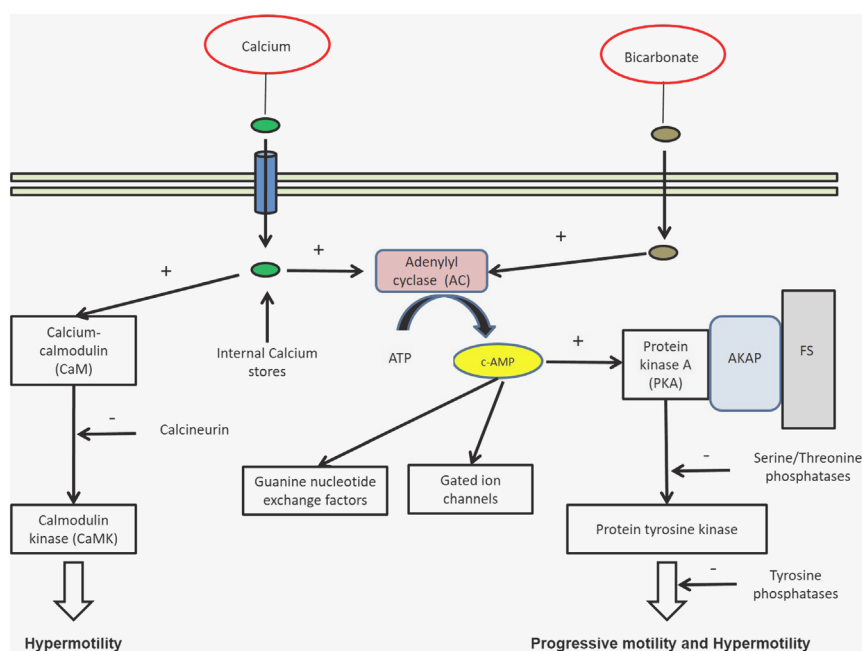


Figure 2 Schematic representation of signaling pathways involved in the regulation of mammalian sperm (hyper)motility. Progressive motility is regulated by low-level activity of the AC/cAMP/PKA pathway, while hyperactivated motility is induced when the latter pathway is activated to a much higher level, in combination with the activation of the calmodulin kinase (CaMK) pathway. Image modified from Turner (2006).

premature capacitation-like changes simultaneously, to investigate the reduced longevity of frozen/thawed stallion sperm. In general, cryopreservation induced an increase in caspase 3 activity, translocation of phosphatidylserine from the inner to outer layer of the plasma membrane lipid bilayer, a reduction in mitochondrial membrane potential and an increased cytoplasmic Na^+ concentration, all of which indicate the occurrence of an apoptotic-like process in frozen/thawed stallion sperm cells. Additionally, a small subpopulation of cryopreserved sperm showed capacitation-like characteristics after thawing, such as increased membrane fluidity and protein tyrosine phosphorylation. To avoid the negative effects of cooling and cryopreservation on stallion sperm, Gibb *et al.* (2015) recently described a promising approach to store stallion spermatozoa at room temperature. Taking into account that stallion spermatozoa rely heavily on mitochondrial ATP production which generates ROS (as discussed previously), combined supplementation of sperm storage medium with 10 mM pyruvate and 50 mM L-carnitine was found to counteract the adverse effects of ROS production during room temperature storage. Much improved preservation of sperm total and progressive motility after 72 h incubation and significantly higher acetyl-L-carnitine concentrations were observed using this antioxidant-enriched sperm storage medium.

In contrast to other mammalian species, no intracellular Ca^{2+} rise and activation of hyperactivated motility is observed when stallion sperm are exposed to progesterone or prostaglandin E_1 (Loux *et al.* 2013). Other hyperactivation triggers for stallion sperm have been described, but do not support protein tyrosine phosphorylation in the sperm tail. For example, three parameters of progressive motility (straight line velocity, straightness and linearity) were decreased when stallion sperm was incubated in 10% follicular fluid, indicating the acquisition of hyperactivated motility (Lange-Consiglio & Cremonesi 2012). Similarly, Leemans *et al.* (2015b) demonstrated the induction of hyperactivated motility when stallion spermatozoa were exposed to an alkaline follicular fluid fraction, while procaine also effectively induced hyperactivated motility in stallion spermatozoa; however, both did so in the absence of protein tyrosine phosphorylation (McPartlin *et al.* 2009, Leemans *et al.* 2015a).

Acrosome reaction

The Ca^{2+} -dependent release of the acrosomal contents is an essential step in mammalian fertilization because it facilitates penetration through the acellular glycoprotein coat of the oocyte. After traversing the zona pellucida, the sperm cell will fuse with the oolemma of the mature oocyte (Yanagimachi 1994). *In vivo*, the acrosome reaction takes place in the female genital tract, at the site of fertilization. The acrosome reaction is a multipoint

membrane fusion event between the sperm plasma membrane and the outer acrosomal membrane (Vigil 1989, Tsai *et al.* 2010), which results in the generation of mixed vesicles containing both plasma membrane and outer acrosomal membrane material. The remaining unfused acrosomal membranes, that is the equatorial area of the outer acrosome and the sperm plasma membrane connected to the inner acrosomal membrane covering the apical part of the nucleus, subsequently function as the sperm plasma membrane surface (Kim & Gerton 2003, Vjugina & Evans 2008). This re-designed outer sperm membrane contains a hairpin structure that is able to bind to the oolemma to enable gamete fusion and oocyte activation (Yanagimachi 1994). In boar sperm, it has been demonstrated that soluble N-ethylmaleimide-SNARE protein interactions play a fundamental role in the fusion of the sperm plasma membrane and the outer acrosomal membrane, because a *trans* ternary-soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein complex is formed during capacitation. Key factors involved in this process are syntaxin 1B and VAMP 3 from the plasma membrane, and SNAP 23 from the outer acrosomal membrane (Tsai *et al.* 2010). Additionally, the entry of Ca^{2+} (*in vitro* by use of Ca^{2+} ionophores; *in vivo* after ZP binding) is required to facilitate the conversion to *cis*-soluble N-ethylmaleimide-SNARE complexes which will result in acrosomal exocytosis and penetration of the zona pellucida (Roggero *et al.* 2007).

In mice, one of the main molecules triggering the acrosome reaction in capacitated spermatozoa is zona pellucida glycoprotein 3 (ZP3) present on mature oocytes (Bleil & Wassarman 1990, Yanagimachi 1994, Arnoult *et al.* 1999). More recently, it has been shown that only contact with the intercellular matrix of the cumulus cell complex was able to induce the acrosome reaction and allow mouse spermatozoa to pass through the zona pellucida (Inoue *et al.* 2011, Jin *et al.* 2011). In many other mammals, including the horse, it has been demonstrated that capacitated, acrosome-intact spermatozoa initiate zona pellucida binding (Yanagimachi 1994), although stallion spermatozoa showed a low incidence of acrosome reaction after 1 h of *in vitro* binding to the zona pellucida (Ellington *et al.* 1993, Cheng *et al.* 1996, Meyers *et al.* 1996). Progesterone, present in follicular fluid or cumulus cell secretions, is another factor that might be responsible for inducing the acrosome reaction in horse spermatozoa (Saaranen *et al.* 1993, Cheng *et al.* 1998a, Lange-Consiglio & Cremonesi 2012) (Fig. 1). Interestingly, the progesterone-induced acrosome reaction did not proceed in a protein kinase A, but rather in a protein kinase C and protein tyrosine kinase-dependent manner (Rathi *et al.* 2003), which is very similar to the zona pellucida-mediated induction of the acrosome reaction. Indeed, Breitbart and Naor (1999) showed that zona pellucida protein 3 activates a sperm protein tyrosine

kinase coupled to phospholipase C (PLC), which in turn stimulates protein kinase C by generating diacylglycerol (DAG) from phosphatidylinositol-bisphosphate (PIP₂) (Fig. 1). How the generation of diacylglycerol eventually results in the onset of the acrosome reaction is not yet known. By contrast, when stallion spermatozoa are incubated *in vitro* in HCO₃⁻-enriched conditions, the acrosome reaction is mainly supported by a protein kinase A instead of a protein tyrosine kinase and protein kinase C-dependent pathway (Rathi *et al.* 2003) (Fig. 1), indicating that progesterone and HCO₃⁻ induce the acrosome reaction in different ways. *In vitro*, the acrosome reaction can also be reliably induced by non-physiological triggers, such as Ca²⁺ ionophore in combination with HCO₃⁻ (Rathi *et al.* 2001).

In mice and pigs, it has been demonstrated that several neurotransmitter receptor/ion channels are present in sperm cell membranes. At least two of these channels, the glycine receptor/Cl⁻ channel and a nicotinic acetylcholine receptor, are important for the ZP-initiated acrosome reaction, but not for the progesterone-initiated acrosome reaction (mouse: Meizel 2004; pig: Melendrez & Meizel 1995). In general, the glycine receptor/Cl⁻ channel is involved in inhibiting neurotransmission in the central nervous system, where glycine inhibits transmission by increasing Cl⁻ influx through the glycine receptor/Cl⁻ channel to induce hyperpolarization (Betz & Becker 1988). However, in some neurons, neutrophils, and glycerol kinase pseudogene 3 cells, glycine appears to cause depolarization by activating the glycine receptor/Cl⁻ channel (presumably stimulating Cl⁻ efflux), resulting in an increased cytoplasmic Ca²⁺ concentration (Boehm *et al.* 1997, Tapia *et al.* 1997, Weaver *et al.* 1998). The latter has also been demonstrated in sperm cells which came into contact with zona pellucida proteins. The depolarizing effect was evident as Ca²⁺ influx through voltage-gated Ca²⁺ channels (Arnoult *et al.* 1996, Florman *et al.* 1998). This event initiated the onset of the acrosome reaction. In the horse, the potential role of a glycine receptor/Cl⁻ channel and/or a nicotinic acetylcholine receptor in the onset of the acrosome reaction has not been studied. McPartlin *et al.* (2011) showed that the cAMP-driven activation of guanine-nucleotide exchange factors (RAPGEF3/RAPGEF4) induced a sperm membrane depolarization in capacitated stallion spermatozoa. Depolarization-dependent Ca²⁺ influx subsequently initiated acrosomal exocytosis. However, the activation of these factors did not play any role in the activation of protein kinase A or in protein tyrosine phosphorylation (Fig. 1).

Equine IVF: an update

Worldwide, only two IVF foals have been born, both more than 25 years ago in 1990–1991 (Palmer *et al.* 1991, Bézard *et al.* 1992). The success of IVF in these cases was attributed to *in vitro* co-incubation of equine

oocytes with calcium ionophore-treated stallion spermatozoa. Unfortunately, neither this nor other equine IVF protocols have proven to be reproducible. Indeed, subsequent attempts have yielded extremely poor equine IVF results, with reported cleavage rates varying from 0 to 33% (Zhang *et al.* 1990, Choi *et al.* 1994, Li *et al.* 1995, Dell'aquila *et al.* 1996, 1997a,b, Alm *et al.* 2001, Hinrichs *et al.* 2002, Tremoleda *et al.* 2003, Roasa *et al.* 2007, Mugnier *et al.* 2009a,b, Lange-Consiglio & Cremonesi 2012). A detailed overview of the published equine IVF studies was produced by Leemans *et al.* (2016a).

During the last decade, the utility of procaine in equine IVF media has been a topic of discussion. As mentioned previously, procaine is a potent inducer of hyperactivated motility in stallion spermatozoa (McPartlin *et al.* 2009, Leemans *et al.* 2015a). Indeed, procaine-induced hyperactivation of stallion spermatozoa can even be induced in non-capacitating medium in the absence of external Ca²⁺ (Loux *et al.* 2013), with CATSPER channels apparently not participating in procaine-induced hyperactivation (Loux *et al.* 2013). Initial reports further suggested that procaine promoted equine *in vitro* fertilization by inducing hyperactivated motility while other requisite sperm capacitation events, such

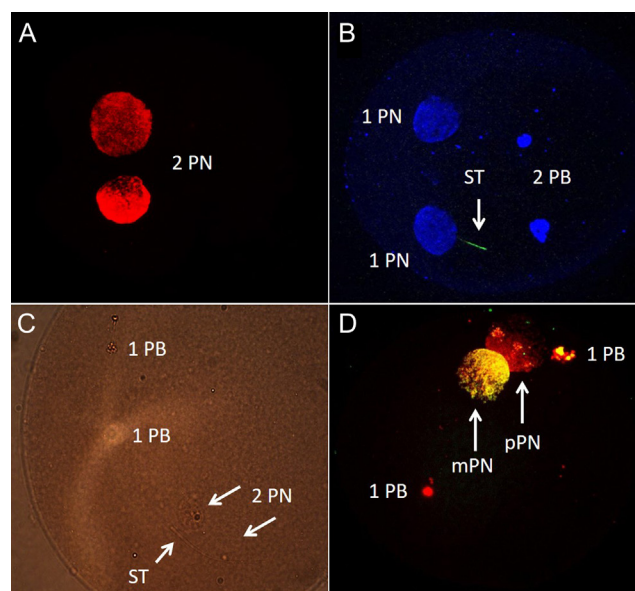


Figure 3 Methods to distinguish fertilization from parthenogenesis. (A) Previously the presence of 2 pronuclei (2 PN), evaluated using nuclear stains such as propidium iodide, was considered adequate to indicate fertilization of equine oocytes. However, this method cannot exclude parthenogenesis. Therefore, the additional presence of the sperm tail (ST) and the second extruded polar body (PB) shown by (B) MitoTracker Green FM pre-labeling combined with Hoechst post-fixation (Leemans *et al.* 2015a; published with permission from Oxford University Press (permission number 4450310125499)); (C) lacmoid post-fixation, should be demonstrated; or (D) paternal (pPN) and maternal (mPN) pronuclei in equine zygotes should be distinguished by different histone 3 methylation (H3K9me3) patterns.

as protein tyrosine phosphorylation, were proposed to have been triggered by other medium constituents or a high pH (McPartlin *et al.* 2009, Ambruosi *et al.* 2013). Cleavage rates exceeding 60% of co-incubated oocytes were reported. However, none of these cleaved oocytes developed to the blastocyst stage. Leemans *et al.* (2015a) subsequently demonstrated that, although procaine did indeed trigger sperm hyperactivation, procaine-induced oocyte cytoplasmic cleavage also occurred in the absence of sperm. Moreover, gamete fusion induced oocyte activation, indicated by cytoplasmic Ca²⁺ oscillations, was not observed in the presence of procaine. It transpired that procaine acted further downstream in the oocyte-activation process, and only induced cytokinesis-like events via pH-dependent depolymerization of F-actin, whereas nuclear activation was not triggered. Moreover, the procaine concentration used to induce sperm hyperactivated motility and oocyte cytokinesis was DNA toxic (Leemans *et al.* 2015a). Rather than true fertilization, procaine-induced DNA fragmentation with degeneration of the cleaving blastomeres occurring at the 8–16 cell stage, that is stage of genome activation in horse embryos (Brinsko *et al.* 1995).

As a result of the initial misinterpretation, more stringent evaluation methods are clearly needed to confirm fertilization in horse oocytes. For various mammals, the presence of two pronuclei after 20–24 h gamete co-incubation, as visualized by nuclear chromatin stains such as Hoechst or propidium iodide, is considered valid proof of fertilization because of the low incidence of parthenogenesis (around 5%) in current farm animal IVF systems (Beek *et al.* 2012, Heras *et al.* 2014, 2015). However, the presence of two pronuclei is not sufficient to distinguish normal fertilization from parthenogenesis in the horse. Using techniques such as pre-labeling sperm with MitoTracker Green FM/Hoechst, and lacmoid post-fixation, Leemans *et al.* (2015a) were able to differentiate fertilization (two polar bodies within the perivitelline space, two pronuclei and a sperm tail within the ooplasm) from parthenogenesis (one polar body, one or two pronuclei and no sperm tail) (Fig. 3). Recently, a staining technique to differentiate the paternal from the maternal pronucleus in equine zygotes produced by intracytoplasmic sperm injection (ICSI) was established (Heras *et al.* 2015), based on different histone 3 methylation (H3K9me3) patterns. This staining technique could, however, not be used in the procaine experiments because the procaine-exposed oocytes did not form normal pronuclei but instead produced condensed DNA fragments (Fig. 3).

Conclusion

For more than 25 years, co-incubation of mature oocytes with capacitated sperm has been the standard method for producing *in vitro* embryos in several species including

man, cattle, pigs and many laboratory animals. In the horse, however, conventional IVF still does not work. Inadequate capacitation of stallion spermatozoa under *in vitro* conditions is probably the major obstacle. It appears that several factors required to induce capacitation in other mammalian species are not involved in equine sperm capacitation. However, biological and chemical capacitation triggers have been identified to facilitate tail-associated protein tyrosine phosphorylation and hyperactivated motility in stallion sperm. However, since the acrosome reaction cannot be induced reliably using these factors, further research should focus on capacitation-related changes that prepare the sperm plasma and outer acrosomal membranes for fusion. It will be necessary to verify that 'improved' capacitation conditions support (1) cholesterol depletion from the sperm plasma membrane, (2) lateral redistribution of lipid rafts in the apical region and ultimately (3) the zona pellucida-induced acrosome reaction. It is likely that the induction of appropriate sperm plasma membrane changes will support the acrosome reaction. Only fully capacitated stallion spermatozoa will be able to penetrate the cumulus and zona pellucida barriers to accomplish fertilization.

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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