Sperm surface changes and physiological consequences induced by sperm handling and storage

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Abstract

Spermatozoa interact with their immediate environment and this contact remodels the sperm surface in preparation for fertilisation. These fundamental membrane changes will be critically covered in this review with special emphasis on the very specific surface destabilisation event, capacitation. This process involves very subtle and intricate modifications of the sperm membrane including removal of suppression (decapacitation) factors and changes in the lateral organisation of the proteins and lipids of the sperm surface. Processing of sperm for assisted reproduction (storage, sex-sorting, etc.) subjects spermatozoa to numerous stressors, and it is possible that this processing overrides such delicate processes resulting in sperm instability and cell damage. To improve sperm quality, novel mechanisms must be used to stabilise the sperm surface during handling. In this review, different types of membrane stress are considered, as well as novel surface manipulation methods to improve sperm stability.

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Introduction

Spermatozoa interact with, and are altered by, their immediate environment and the plasma membrane serves as the two-way communication device. During transit in the male reproductive tract, at ejaculation, and upon entry to the female tract, spermatozoa encounter a host of fluids, epithelial and immunocompetent cells (Amann & Hammerstedt 1993) from the specialised regions of the epididymis, the accessory sex glands, and the oviduct. The complex and varied protein complement of these fluids prepares the sperm surface for fertilisation. Countless experiments have been undertaken to determine the mechanics of this remodelling process (although they are usually performed under *in vitro* capacitating/IVF conditions) and the results are the subject of constant review (Flesch & Gadella 2000, Gadella 2008, Tsai & Gadella 2009, Ikawa *et al.* 2010, Reid *et al.* 2011). The aim of the current review is to overview surface alterations that are important for sperm survival and fertilisation competence and discuss how this knowledge could be implemented to compensate for current shortcomings in sperm processing.

Physiological alterations of the sperm surface

Modification of the sperm surface in the testis and epididymis

Round germ cells are transformed into fertile spermatozoa by various morphological and surface-modifying

events (Gatti *et al.* 2004). Morphological transformation occurs in the testis where round germ cells undergo division, differentiation and meiosis to form haploid, elongated spermatids in a process known as spermatogenesis. The sperm surface is then further modified in the epididymis by sequential exposure to a highly dynamic protein environment created by regionalised secretion and absorption of proteins across the epididymal epithelium. The varying complement of molecules found in the specialised regions of the epididymal tract has been described in numerous species (pig (Dacheux *et al.* 1989, Syntin *et al.* 1996, Guyonnet *et al.* 2009), bull (Belleannee *et al.* 2011), stallion (Fouchecourt *et al.* 2000), rat (Vreeburg *et al.* 1992, Suryawanshi *et al.* 2011) and human (Baker *et al.* 2005, Li *et al.* 2010, 2011)) including details of all the proteins present (proteome), secreted (secretome) or transcribed (transcriptome), and this information has greatly expanded knowledge of the role of epididymal proteins in sperm maturation (reviewed by Gatti *et al.* (2004) and Dacheux *et al.* (2005, 2009)).

These proteins may form weak associations with the sperm surface, such as those reported for certain isoforms of clusterin (Howes *et al.* 1998), whereas others, such as human epididymal protein (Kirchhoff *et al.* 1998) or its porcine homologue (Okamura *et al.* 1999), form strong glycosylphosphatidylinositol (GPI) anchors. Proteins may also be removed or modified by

proteolytic processing, and some have been reported in the surrounding fluid in active enzymatic form (e.g. angiotensin converting enzyme (Gatti *et al.* 1999, Metayer *et al.* 2001)).

Many potential mediators of sperm–egg communication are acquired, or modified, in the epididymis, demonstrating the significant contribution of this organ to successful fertilisation (see Fig. 1). Examples identified through biochemical investigation include GPI-anchored proteins (bull: SPAM1 (Morin *et al.* 2010); mouse: PH20 (Thaler & Cardullo 1995) and rat: 2B1 glycoprotein (Seaton *et al.* 2000)), proteins showing carbonyl reductase activity (hamster: P26h (Montfort *et al.* 2002) and pig (van Gestel *et al.* 2007)), cysteine-rich secretory proteins (mouse and rat: CRISP1 (Roberts *et al.* 2006, Busso *et al.* 2007, Da Ros *et al.* 2008)) as well as the secreted protein containing N-terminal Notch-like

type II EGF repeats and C-terminal discoidin/F5/8 C domains type 1 (SED1 in human and rodents (Ensslin & Shur 2003, Shur *et al.* 2006, Copland *et al.* 2009)) and its porcine and bovine homologues, p47 and lactadherin, respectively (Ensslin *et al.* 1998, van Gestel *et al.* 2007), and in the pig the spermadhesin AQN3, which is secreted in part from the cauda epididymis and mainly from the seminal vesicles (van Gestel *et al.* 2007). Integral membrane proteins of testicular origin such as proteins of a disintegrin and metalloprotease (ADAM) family have also been described to function in sperm–oocyte interaction and fertilisation (Evans 2001). Further insight into these putative sperm–egg receptors has been gained by the use of knockout mice models. Some well-known gene products (e.g. the ADAM protein fertilin β) were shown to be largely dispensable for *in vivo* fertilisation (for review, see Ikawa *et al.* (2010)) or have

Figure 1 Physiological sequence of surface changes rendering spermatozoa fit to fertilise the oocyte. (A) When spermatozoa leave the testis, they are morphologically complete but lack the capacity to fertilise the oocyte as they are immotile and contain remnants of the cytosolic bridges that synchronise spermatogenic cells until spermiation (the cytoplasmic droplet). (B) In the epididymis, spermatozoa (1) acquire motility characteristics, (2) lose the cytoplasmic droplet and (3) undergo some final chromatin condensation. The sperm surface undergoes various molecular alterations. The most important being the adsorption of proteins (green) that are involved in sperm–zona binding (4) and surface stabilising factors. (C) At ejaculation, spermatozoa are mixed with seminal plasma from the accessory sex glands. Seminal plasma glycoproteins (orange) adhere tightly to the sperm surface and efficiently stabilise the cell during transport through the lower portion of the female genital tract (5). (D) When spermatozoa reach the upper portion of the female genital tract (the isthmus of the oviduct), they are triggered to capacitate. The mechanisms are unknown but it is thought that migration of spermatozoa through the uterus and the uterotubal junction causes the removal of decapacitation factors (orange) and that spermatozoa respond to female-derived stimulatory signals (Holt & Fazeli 2010). *In vitro*, this response is elicited after density gradient washing (which strips decapacitation factors originating from the epididymis and accessory sex glands from the sperm surface) and incubation with capacitation factors such as bicarbonate and albumin. This treatment elicits a number of parallel surface changes: (6) cholesterol (black) is oxidised and removed from the sperm surface by albumin (blue), (7) epididymal proteins (green) aggregate into membrane rafts at the apical ridge of the sperm head, (8) a partial lipid scrambling takes place in the same area (9) and seminolipids migrate towards the equatorial area of the sperm head. At the cytosolic site, the apical ridge of the sperm head is now efficiently and stably docked to the outer acrosomal membrane (10). Functionally, the apical head area is now prepared for fertilisation as it contains protein complexes capable of interacting with the zona pellucida or the extracellular matrix of the cumulus mass. It is also likely that ROS formation is an essential part of this signalling system and that it may continue even after capacitation and, in the long-term, induces sperm deterioration (similar to Fig. 2C). At the sperm tail, an increase in the pH of the fluid environment evokes proton extrusion and signal transduction cascades provoke tyrosine phosphorylation events, which generate hyperactivated motility, probably by enhancement of anaerobic ATP generation (11). For further details and references, see section ‘Physiological alterations of the sperm surface’.

alternate mechanisms, such as ADAM3, which was shown to promote sperm migration through the uterotubal junction (Yamaguchi *et al.* 2009). Furthermore, new candidate genes essential for sperm–egg fusion were discovered (e.g. *IZUMO1* (Inoue *et al.* 2005)).

The sterols and fatty acids of the sperm membrane also undergo significant remodelling during epididymal transit, causing dramatic alterations in membrane architecture. The extent of these changes is highly species specific (reviewed by Saez *et al.* (2011)). In ram, rat and mouse spermatozoa, cholesterol content decreases (~50%) during epididymal transit and the ratio of unsaturated fatty acids increases. However, few changes have been observed in the boar. These lipid modifications increase membrane fluidity and are thought to prime signalling pathways that regulate capacitation because testicular and caput spermatozoa are not responsive to stimuli that capacitate caudal spermatozoa (Yanagimachi 1994, Shadan *et al.* 2004).

From the information above, it is evident that the proteins and lipids of the plasma membrane are remodelled in the epididymis to prime spermatozoa to undergo capacitation, interact with the zona pellucida and fuse the oolemma (see Fig. 1). In general, it is proposed that spermatozoa from the cauda epididymis are now fit to fertilise.

Seminal plasma and the sperm surface

Upon ejaculation, spermatozoa are mixed with another set of surface remodelling components derived from the accessory sex glands and are deposited in the female tract. In the oviduct, spermatozoa undergo a final biochemical and structural modification, collectively termed capacitation, which culminates in the acrosome reaction and fertilisation. To attempt to synchronise capacitation with the arrival of spermatozoa at the oocyte, molecules present on the surface of the spermatozoa, and in its immediate environment, act in concert to both stimulate and inhibit the onset and progression of this process (Bedford 2004). An early capacitation-related event is the loss, modification and redistribution of molecules on the sperm surface (Gadella *et al.* 1995). Entities that coat the sperm membrane and are removed during capacitation are termed decapacitation factors (Bedford & Chang 1962, Yanagimachi 1994) as their presence is thought to stabilise the sperm membrane and keep it in a non-capacitated state (see Fig. 1). These factors are present in the epididymal fluid and seminal plasma and their mechanisms of action are highly complex and species dependent.

Nixon *et al.* (2006) showed that CRISP-1 was released from the mouse sperm membrane following capacitation and that its subsequent re-addition inhibited in a dose-dependent fashion, a range of capacitation indicators including tyrosine phosphorylation of the sperm plasma

membrane and the ability of spermatozoa to undergo the acrosome reaction and bind to the zona pellucida. The mechanism of action of CRISP-1 is unknown but it shares structural similarity with toxin molecules capable of blocking ion channels, and it is thought that it may inhibit the uptake of ions, such as Ca^{2+} , required for capacitation (Koppers *et al.* 2011). Other decapacitation factors identified by Nixon *et al.* (2006) include plasma membrane fatty acid binding protein, decapacitation factor 10, and phosphatidylethanolamine binding protein 1. Although, the latter is likely to be a receptor (Gibbons *et al.* 2005) for a well-known 40 kDa glycoprotein characterised as a decapacitation factor by Fraser (1984) over two decades ago. Binding of this 40 kDa decapacitation factor to spermatozoa stimulates a calmodulin-sensitive Ca^{2+} -ATPase, which helps to maintain low intracellular Ca^{2+} levels (Adeoya-Osiguwa & Fraser 1996). A mouse seminal vesicle auto-antigen has also been described as a potential candidate to maintain low intracellular levels through targeted regulation of membrane sphingolipids and plasma membrane Ca^{2+} -ATPase (Lu *et al.* 2010).

Decapacitation factors of seminal plasma origin are best described in production animals (e.g. ram, bull and boar) where they have been shown to prevent or reverse capacitation of spermatozoa under conditions of handling-induced stress (Topfer-Petersen *et al.* 1998, Manjunath *et al.* 2007, Maxwell *et al.* 2007, Kirkwood *et al.* 2008, Muino-Blanco *et al.* 2008). Further discussion of these agents is provided in section 'Novel sperm surface manipulation methods'.

***In vitro* capacitation induces sperm surface alterations that enable the spermatozoon to fertilise the egg**

The capacitation process has been observed *in vitro* through studies that attempt to mimic conditions in the oviduct. *In vivo*, spermatozoa undergo extreme selection because only a small number of competent spermatozoa are able to cross the uterotubal junction and interact with the epithelium of the oviduct (Sostaric *et al.* 2008, Suarez 2008, Holt & Fazeli 2010, Ikawa *et al.* 2010). It is not well understood how these cells achieve capacitated properties but it is believed that initiation of the process precedes release from oviductal epithelial cells (Lefebvre & Suarez 1996, Fazeli *et al.* 1999) and may be triggered by specific oviductal fluid factors that are released on the initiation of ovulation (Overstreet *et al.* 1978, Hunter 2008, Sostaric *et al.* 2008). This final maturation enables the spermatozoon to recognise and penetrate the cumulus mass and zona pellucida, fertilise the oocyte by fusing with the oolemma and initiate embryonic development (Rodriguez-Martinez 2007, Brussow *et al.* 2008).

These conditions are mimicked *in vitro* by washing ejaculated spermatozoa through a preparative density gradient, which causes a partial stripping of more loosely

associated extracellular coating material (Martins *et al.* 2003, Caballero *et al.* 2009) and may also remove decapacitation and stabilising factors (Fraser 1984). In some species, additional factors are included, such as negatively charged polysaccharides (e.g. heparin with bull spermatozoa (Parrish *et al.* 1988)), to further enhance stripping of extracellular sperm coating material, possibly mimicking the surface effects elicited by glycosaminoglycans that are present in oviductal fluids (Parrish *et al.* 1989, Coy *et al.* 2008, Killian 2011).

Washing allows selection of a sperm population with the highest density, representing cells with signs of superior maturation (higher condensation of chromatin and removal of cytoplasmic droplets). Typically, such cells show considerably lower rates of intrinsic morphological and functional abnormality and reduced signs of peroxidative and/or apoptotic-like response; which in part relates to the removal of immature spermatozoa but also to reduced contamination of leukocytes that actively produce reactive oxygen species (ROS; Sakkas *et al.* 2000, Aitken *et al.* 2007, Fariello *et al.* 2009). Further selection can be achieved using a swim-up procedure to select the most motile sub-fraction of sperm cells, and these procedures are common practice for IVF (for review, see Mortimer (2000)).

The spermatozoa can now respond to a capacitation-supportive medium, which resembles that of oviductal fluid but is more extreme than normal physiological surroundings. For example, the spermatozoa are exposed to a higher temperature (39 °C instead of 35 °C) and artificially raised levels of known capacitating agents (for review, see Gadella & Visconti (2006)).

Incubation with bicarbonate and calcium ions triggers adenylate cyclase/cAMP/protein kinase A and tyrosine kinase signalling pathways (Visconti *et al.* 1995), which induces a partial scrambling of aminophospholipids (Elliott & Higgins 1983, Gadella & Harrison 2000). Fatty acid-free BSA is also often included in capacitation-supportive medium because it extracts sterols (Flesch *et al.* 2001) from the sperm surface, further enhancing membrane fluidity. These changes cause small lipid-ordered domains, which initially reside over the entire acrosomal region of the sperm plasma membrane, to aggregate in lipid rafts at the apical plasma membrane (see Fig. 1). These lipid rafts are enriched in cholesterol, glycosphingolipids (Lin & Kan 1996, Visconti *et al.* 1999, Flesch *et al.* 2001, Selvaraj *et al.* 2009) and seminolipids (Gadella *et al.* 1994, 1995), and this lipid aggregation creates lateral heterogeneity of sperm membrane proteins (Lucero & Robbins 2004). Certain proteins, such as flotillin and caveolin (Anderson 1998, van Gestel *et al.* 2005a), are well known to be permanently associated with lipid rafts, but since these structures aggregate in the exact area of the sperm head where initial zona recognition occurs (Cross 2004), they would be expected to also contain zona binding proteins. Indeed, lipid rafts isolated from *in vitro*-capacitated

mouse (Nixon *et al.* 2009), human (Nixon *et al.* 2011) and porcine (van Gestel *et al.* 2005a, 2007) spermatozoa were highly enriched in zona binding proteins. Therefore, it is likely that *in vitro* capacitation leads to delicate sperm surface alterations, which allow the formation of a multi-protein complex involved in zona recognition (for review, see Gadella (2010)).

Furthermore, it was recently demonstrated that during *in vitro* capacitation, porcine sperm cells show a characteristic redistribution of SNARE proteins into the same area where the aggregation of membrane rafts and zona binding proteins occurs (Tsai *et al.* 2007, 2010). SNARE proteins are involved in the execution of the acrosome reaction (Mayorga *et al.* 2007, Tomes 2007), but rather than inducing membrane fusion, this redistribution caused a very stable docking of the apical plasma membrane with the outer acrosomal membrane. This interaction involved the formation of trans trimeric SNARE complexes of SNAP23, VAMP3 and syntaxins (Tsai *et al.* 2010), which were stabilised by complexins (Zhao *et al.* 2007) and probably other factors (PS Tsai, IA Brewis & BM Gadella, unpublished results).

Subsequent binding with the zona pellucida, or cumulus oophorus complex surrounding it (Jin *et al.* 2011, Yanagimachi 2011), invokes a rise in intracellular Ca^{2+} (Arnoult *et al.* 1996, Breitbart 2002), which triggers a calcium-sensitive conformational change from a *trans*- to *cis*-SNARE complex (for model, see Tsai *et al.* (2010)) resulting in multiple-point fusions, or the acrosome reaction. Thus, this prior docking is thought to localise multiple-point fusions at the apical sperm head, where primary binding takes place, leaving the equatorial region of the sperm head intact for subsequent fertilisation fusion with the oocyte (for review, see Gadella & Evans (2011)).

Taken together, this research suggests that spermatozoa undergo complex ergonomic changes during capacitation. Processing of spermatozoa for assisted reproduction subjects the cells to numerous stressors and it is not known how these disturb the delicate processes outlined above. Stressors associated with storage and sex-sorting, and their consequent alterations of the sperm surface, are discussed in the next section and are visually outlined in Fig. 2.

Sperm surface alterations during sperm handling

Cryopreservation

Long-term storage permits the banking of gametes from high-merit animals, the possibility to check the health status of samples and to internationally distribute superior genetics (Vishwanath 2003, Bailey *et al.* 2008). Cryopreservation is possible because of the fortuitous discovery of the cryoprotective properties of glycerol (Polge *et al.* 1949), and requirements for cooling, freezing and thawing of spermatozoa were

Figure 2 Effects of sperm handling on the sperm surface. (A) Spermatozoa destined for *in vitro* processing are usually obtained by artificial vagina or electroejaculation (at this point, they have similar surface properties to Fig. 1C). Subsequent processing for technologies such as sex-sorting and cryopreservation affect the sperm surface. (B) Dilution, washing steps and shear forces (flow cytometry) strip decapacitation factors (orange) from the sperm surface. This normally occurs during physiological capacitation (see Fig. 1D) but physical stripping of proteins from the sperm surface does not result in a capacitation-associated aggregation of micro-domains to the apical ridge area. Instead, local micro-aggregation of proteins (1) is shown by capacitation markers (e.g. Merocyanine 540). In addition, changes in temperature, buffers, osmolytes and pressure (sorting) may cause changes in sperm surface properties. This can include either one or more of the following: phase separation of lipids in the sperm plasma membrane (2), acrosome loss (3), lethal ROS production and apoptotic-like changes such as extended phospholipid scrambling and depolarisation of the inner mitochondrial membrane (4) and concomitant lipid peroxidation and DNA damage (5). Moreover, at the tail, these changes also result in ROS-induced tyrosine phosphorylation (which usually activates hyperactivated motility) despite the fact that the spermatozoa are immotile (6). (C) The end result is plasma membrane damage, the release of protein and vesicles (in blue) from the sperm membrane and cell deterioration. These physical and chemical stressors can be minimised by specific protocol adaptations mentioned in the text (see section ‘Sperm surface alterations during sperm handling’ and Fig. 3). In general, spermatozoa that survive handling appear to have similar surface properties to ejaculated spermatozoa. For further details and references, see section ‘Sperm surface alterations during sperm handling’.

formulated in the early 1970s. Optimisation of the process and tailoring for individual species were achieved through countless, continuing, empirical investigations (for species’ specific information, see bull (Vishwanath & Shannon 2000), ram (Salamon & Maxwell 2000), boar (Johnson *et al.* 2000, Grossfeld *et al.* 2008, Rath *et al.* 2009a) and mouse (Nakagata 2000)). The principles of cryobiology and the considerations made in the past and present to improve the process are valid for many species. Thus, cryopreservation protocols are fairly similar with slight tailoring of conditions to address the eccentricities of some species (e.g. high glycerol content required for koala spermatozoa (Zee *et al.* 2008)). Synthetic freezing diluents commonly contain tris or citrate to provide an isotonic environment and buffer pH, glucose or fructose as an energy source and non-penetrating and penetrating factors to protect spermatozoa from freeze-thaw damage, such as egg yolk and glycerol.

It is accepted that egg yolk minimises cold shock damage, but its mechanism of action is debated. Direct associations between the lipids of the egg yolk and the

sperm membrane have been reported (Pace & Graham 1974, Ollero *et al.* 1998, Ricker *et al.* 2006), and it is believed that these have a stabilising effect, minimising lateral phase separations involved in cold shock deterioration (Pettitt & Buhr 1998).

Glycerol and other penetrating cryoprotectants (e.g. dimethyl sulphoxide and polyvinyl pyrrolidone) are used to protect the cell interior and prevent intracellular ice formation. However, inclusion of such agents must be carefully managed to minimise potentially detrimental osmotic effects. Spermatozoa can then be frozen, and a rate of $-30\text{ }^{\circ}\text{C}/\text{min}$ has been shown optimal for boar spermatozoa through determination of the rates of water permeability by differential scanning calorimetry (Devireddy *et al.* 2004) and rapid thawing is recommended ($1200\text{--}1800\text{ }^{\circ}\text{C}/\text{min}$ (Hernandez *et al.* 2007b)).

In spite of considerable research, the cryopreservation process remains highly damaging, inflicting significant lethal and sub-lethal effects on spermatozoa (Parks & Graham 1992, Salamon & Maxwell 1995b, Bailey *et al.* 2000, Holt 2000, Bagchi *et al.* 2008). It is routinely and successfully applied only in certain species (e.g. the bull)

and the reasons for this are numerous and based on both biochemical and practical differences. For example, to overcome inefficiencies of sperm transport from freeze–thaw damage, spermatozoa must be deposited closer to the site of fertilisation, and this is easier to achieve in the cow, for instance, than in the ewe. In addition, some species show greater susceptibility to freeze–thaw damage (e.g. the pig).

Another confounding factor is the variation between animals in the ability of their spermatozoa to withstand freeze–thaw damage. In the boar, this is the major factor (70%) affecting cryosurvival (Roca *et al.* 2006), but this phenomenon is common in many species (bull (Chaveiro *et al.* 2006), mouse (Songsasen & Leibo 1997) and dog (Yu *et al.* 2002)) and influences whether donors can be used for cryopreservation. The reason for this significant male variation is not well understood but numerous causes, and potential markers, of freezing resistance have been investigated including regulation of cell volume (Chaveiro *et al.* 2006), genetic factors (Thurston *et al.* 2002), expression of housekeeping proteins (Casas *et al.* 2009, 2010) and seminal plasma proteins (Jobim *et al.* 2004, Asadpour *et al.* 2007).

Differences in freezing resistance also exist between, and within, ejaculates. In fact, in a given boar semen sample, a subpopulation of sperm cells has been identified that exhibit increased osmotic resistance and these cells would be expected to have inherently different membrane changes upon cryopreservation than the spermatozoa in other subpopulations (Petrunkina & Töpfer-Petersen 2000, Druart *et al.* 2009).

In the current review, we will only discuss alterations to the primary site of injury, the sperm membrane, and focus particular attention on how this damage may affect its protein and lipid components.

Freezing results in the concomitant coating and decoating of proteins of the sperm surface. Bull (Ollero *et al.* 1998) and boar (Huang *et al.* 2009) sperm surface proteins, collected before and after freeze–thawing, showed processing related differences using comparative SDS–PAGE analysis, but the effect of these protein alterations on sperm function remains unknown. The intensity of a fertility-associated protein (P25b; Parent *et al.* 1999) has also been shown to remain stable if bull spermatozoa were stored in liquid nitrogen for <5 days but longer storage periods (>28 days) reduce its abundance compared with that of fresh spermatozoa (Lessard *et al.* 2000). This result is interesting because it suggests that cryo-elution of the protein occurred in an environment where chemically and thermally driven reactions are minimised, and not during cooling or thawing where the majority of damage to the plasma membrane occurs.

Freeze–thawing also has distinct effects on the lipids of the sperm membrane (Buhr *et al.* 1994). Temperature fluctuations and cell dehydration induce changes in lateral-phase separation of lipids and thus a lateral

reordering of membrane components (Holt & North 1984, De Leeuw *et al.* 1990, Drobnis *et al.* 1993) and the loss of polyunsaturated fatty acids and cholesterol (Maldjian *et al.* 2005, Chakrabarty *et al.* 2007). This alters the permeability of the sperm surface to water, ions and cryoprotectants (Hagiwara *et al.* 2009, Oldenhof *et al.* 2010) and the final result is a weakening of the cell that reduces its ability to withstand future stress (see Fig. 2). However, at the same time, proteins and lipids of the cryoprotective diluent (e.g. milk, albumin or yolk) coat the membrane, providing partial protection from these deleterious effects of freezing (Ollero *et al.* 1998, Ricker *et al.* 2006), and prevent the binding of proteins that are detrimental for sperm survival (Bergeron *et al.* 2004).

The main advantage of cryopreservation is that it permits an indefinite hiatus of sperm development. However, spermatozoa are altered by this process and when revived by warming, they emerge in an advanced state characterised as a bypassing of the need for capacitation (Watson 1995, Maxwell & Watson 1996, Maxwell & Johnson 1997), for they possess the ability to undergo the acrosome reaction. This phenomenon is believed to follow a different pathway to that of physiological capacitation. Green & Watson (2001) found that lipid bilayer fluidity and tyrosine phosphorylation signalling pathways differed between boar spermatozoa that were cooled (5 °C) and re-warmed from those that were capacitated *in vitro*. Variations in protein tyrosine phosphorylation patterns between *in vitro* capacitated and cryopreserved spermatozoa have also been demonstrated in a number of species (bull (Cormier & Bailey 2003), boar (Bravo *et al.* 2005) and stallion (Thomas *et al.* 2006)).

It is speculated that a loss of cholesterol during freezing, and an increase in permeability of the sperm membrane to stimulating factors, allows frozen–thawed spermatozoa to bypass the physiological capacitation pathway. However, it is noteworthy that it has not been demonstrated that these signalling differences result in altered physiological capacitation outcomes (e.g. aggregation of rafts at the apical plasma membrane or docking of the apical plasma membrane with the outer acrosomal membrane; see section ‘Physiological alterations of the sperm surface’). Impairment of these processes probably relates to the poor fertility of frozen–thawed spermatozoa following artificial insemination. In fact, lateral phase separation of lipids in frozen spermatozoa is not reversibly restored upon thawing (De Leeuw *et al.* 1990), which may well impede the capacitation-specific lateral rearrangements of the sperm surface.

The cryopreservation process as a whole dramatically alters the composition of the sperm membrane and its extracellular matrix. In this respect, some surface changes are reversible while others are fatal (Watson 2000, Guthrie & Welch 2005). A similar effect is evident after spermatozoa are processed for sex-sorting.

Sex-sorting

New technologies have allowed the precise selection of subpopulations of spermatozoa with specific attributes. Selection of high-quality spermatozoa via flow cytometry (Paasch *et al.* 2007) magnetic sorting (Said *et al.* 2008) or microfluidic sorting (Wu *et al.* 2006) has proven useful for human ART (Lee *et al.* 2010) and it is even possible to sort viable sperm cells that are responsive to capacitation from those that are not (de Vries *et al.* 2003). Despite these possibilities, sorting is only routinely applied to separate X and Y chromosome-bearing spermatozoa for the production of offspring with pre-selected sex. This technique exploits differences in DNA content (Johnson *et al.* 1989) between the X and Y chromosomes, which can be detected by fluorescence-activated cell sorting after staining with a DNA binding dye (Hoechst 33342, a bisbenzimidazole-based membrane permeable probe). The technique has now been successfully applied to a wide range of species, produced innumerable live offspring after artificial insemination, and has been the subject of many reviews (Maxwell *et al.* 2004, Garner 2006, Bathgate 2008, Rath *et al.* 2009b, Vazquez *et al.* 2009). Despite its considerable advantages, industry adoption has been hampered by the additional expense and reduced capabilities of sex-sorted spermatozoa compared with their non-sorted counterparts (Rath *et al.* 2009b).

Sex-sorting exposes spermatozoa to multiple stressors, and while the individual contribution of each is hard to quantify, their combined effect causes advanced cell deterioration. Damage to spermatozoa during sex-sorting has been well documented and extensively reviewed (see Garner (2001), Bathgate (2008), Rath *et al.* (2009b) and Vazquez *et al.* (2009)). Thus, in this review, we will only describe these briefly and focus particular attention on how the sperm surface is affected.

The most limiting aspect of sex-sorting is the length of processing. The low sort rate ($\sim 20 \times 10^6$ spermatozoa of each sex per hour (Garner 2006)) requires the holding of spermatozoa in sub-optimal conditions for an extended period of time and is the main contributor to their increased cost. Technical improvements have increased sorting speed, but since each cell must be analysed individually, there are limitations on further improvement in this area. Thus, considerable man-hours have been invested into improving sorting conditions.

One of the most obvious differences between non-sorted and sorted sperm populations is their rate of dilution. During sorting, the sperm cells are highly diluted (5000- or 800-fold in the ram and boar respectively) and the media utilised are species specific and differ with the stage of processing (pre-sorting, sorting and post-sorting). These processing requirements expose spermatozoa to varying buffers, energy sources, protective coating factors (egg yolk and seminal plasma) and consequential fluctuations in osmolarity and pH

(Bathgate 2008). Variations in temperature also occur when spermatozoa are transferred from incubation with Hoechst (34 °C) to the thin tubing of a room temperature-conditioned flow cytometer (24 °C) and this sudden decrease in temperature can cause deterioration of capacitated cells (Flesch *et al.* 2001). The spermatozoa also face hydrodynamic pressure changes, orientating forces and are subjected to changes in electric charge. The consequences of these insults are largely unknown. Moreover, spermatozoa are exposed to significant mechanical insult when they are propelled through the machine and into a collection vessel at speed up to 90 km/h (Seidel & Garner 2002). Due to their highly dilute state, they are then re-concentrated to obtain an adequate insemination dose. This is commonly achieved through centrifugation, which may further stress the sperm membrane (Leahy *et al.* 2010a) and generate ROS (Agarwal *et al.* 1994, Shekarriz *et al.* 1995) and/or DNA damage (Twigg *et al.* 1998, Urrego *et al.* 2008). In the boar, concentration by sedimentation is a promising alternative and has been shown to increase the proportion of viable, motile and fertile spermatozoa after a 16–18 h storage (17 °C) period (Garcia *et al.* 2007).

These mechanical and chemical stressors alter the sperm surface (see Fig. 2). Spinaci *et al.* (2006) reported heat shock protein 70 (HSP70) redistributed from the equatorial sub-segment towards the equator of sperm cells after sorting; a relocation that reflects capacitation-like changes of the sperm membrane. Similarly, capacitation-like changes have been reported in numerous species after flow sorting (ram (Hollinshead *et al.* 2003), bull (Moce *et al.* 2006) and boar (Maxwell & Johnson 1997)). This is not particularly surprising as the processing steps for sorting are very similar to those previously described for *in vitro* modelling of capacitation. For example, spermatozoa are diluted and subjected to mechanical forces (sheath fluid pressure, expulsion from machine, centrifugation, etc.), which may promote the release of proteins from the sperm surface that are not discharged under physiological conditions (Nixon *et al.* 2006) and incubated with BSA, a known capacitating agent, during staining and post-sort collection. A combination of these factors is believed to result in the removal or alteration of binding sites on the sperm surface (Maxwell *et al.* 2007). Indeed, early proteomic studies on the surface of bull spermatozoa reported that sex-sorting caused the loss of two membrane proteins, 108 and 57 kDa, thought to originate from seminal plasma and the sperm membrane respectively (McNutt & Johnson 1996). Conversely, proteins of low molecular weight (<29 kDa), thought to be mainly of seminal plasma origin, were reported to remain adsorbed (McNutt & Johnson 1996). More recently, binder of sperm proteins (BSP) were reported on the membrane of non-viable ram spermatozoa gated out in the waste population during sorting, confirming

that seminal plasma proteins remain tightly associated with the sperm membrane during transit through the flow cytometer (Leahy *et al.* 2011).

Despite the aforementioned stressors, not all the effects of sex-sorting are negative. The orientating forces of the nozzle selects morphologically normal spermatozoa and counterstaining with non-toxic red food dye (FD&C40) penetrates the nuclear membrane of non-viable cells, quenching Hoechst 33342 fluorescence, and allowing these cells to be gated out to waste during flow sorting. This leaves a population of spermatozoa that exhibit some improved sperm parameters (e.g. membrane integrity and total motility) compared with their non-sorted counterparts (ram (de Graaf *et al.* 2006) and bull (Underwood *et al.* 2009c)). This was also noted in a proteomic investigation (Leahy *et al.* 2011), which showed a greater abundance of sperm membrane-associated protein 1 (SLLP1) in the waste sperm population compared with the final sex-sorted product. This protein is retained on the sperm membrane following acrosomal exocytosis, providing strong evidence that acrosome-reacted spermatozoa are gated out of the final sorted population.

These results show that sex-sorting is a discriminatory process that produces a sperm population that exhibits both superior and inferior traits compared with non-sorted spermatozoa, but which essentially have a limited lifespan. These changes are reflected in reduced fertility rates and higher incidence of pregnancy loss after artificial insemination (pig (Bathgate *et al.* 2008) and bull (Underwood *et al.* 2009a, 2009b)). However, in this regard, the ram appears to be the exception to the rule as sex-sorting did not reduce fertility of ram spermatozoa when it was used for intrauterine insemination (de Graaf *et al.* 2009).

Novel sperm surface manipulation methods

Stabilisation of the sperm surface with cholesterol and other lipids

Cholesterol efflux from the plasma membrane is one of the recognised responses of spermatozoa (see section 'Physiological alterations of the sperm surface') in a capacitation-supportive medium (Travis & Kopf 2002, Witte & Schafer-Somi 2007). While these modifications are thought to be essential for fertilisation (Parinaud *et al.* 2000), they also increase the fragility of the sperm surface, rendering it more sensitive to handling imposed deterioration (Van Gestel *et al.* 2005b). Similar to capacitated spermatozoa, frozen-thawed spermatozoa also show some signs of membrane lipid modification such as higher membrane fluidity, partial phospholipid scrambling (Thomas *et al.* 2006) and loss of polyunsaturated fatty acids and cholesterol (Maldjian *et al.* 2005, Chakrabarty *et al.* 2007).

It has long been known that specific vesicle additives can stabilise spermatozoa and protect them from freeze-thaw damage (Watson 1981, Holt & North 1988). The addition of certain lipid mixtures, including cholesterol (Moore *et al.* 2005), appeared to be the best way to prevent deterioration, and more recently, cholesterol-loaded cyclodextrins (CLC; molecules with specific affinity for free sterols (Shadan *et al.* 2004)) have been used (Moce *et al.* 2010, Serin *et al.* 2011). Such approaches led to increased cellular levels of cholesterol by monomeric transfer of these lipids from their carriers and spermatozoa pre-loaded with cholesterol are more stable during freeze-thawing than those that have not been pre-loaded (Moce & Graham 2006, Aksoy *et al.* 2010, Moraes *et al.* 2010, Oliveira *et al.* 2010). The responsiveness of the surviving cells to *in vitro* capacitating treatments was questioned but studies in the bull (Purdy & Graham 2004) and stallion (Spizziri *et al.* 2010) indicate that CLC-treated spermatozoa remain fertile.

Protection of the sperm surface and DNA by antioxidants

Several lines of evidence demonstrate that an active, well-balanced and controlled oxidant system regulates capacitation by generating cAMP and tyrosine phosphorylation (O'Flaherty *et al.* 2006, de Lamirande & O'Flaherty 2008, de Lamirande & Lamothe 2009). In line with this, ROS interact with cholesterol, a known mediator of capacitation, at the sperm surface. Brouwers *et al.* (2011) demonstrated that cholesterol can be oxidised during capacitation and that these oxidation products are preferentially adsorbed by albumin (the cholesterol depletory in IVF media). However, cholesterol oxidation and depletion was not observed in frozen-thawed spermatozoa, and this unique behaviour of cholesterol under different sperm conditions requires further elucidation.

However, uncontrolled ROS production leads to excessive lipid peroxidation, DNA damage and sperm deterioration (Aitken 1995, Aitken & Curry 2011). Therefore, there appears to be a window of opportunity for capacitation, as spermatozoa go from a non-responsive to a responsive state by mild and controlled ROS formation. If the responsive (capacitation) state is reached and ROS formation is not stopped, after a certain time period, the same process that initiated capacitation will now cause membrane and DNA deterioration (for review, see Aitken & Baker (2006) and Storey (2008)). What remains unclear is whether this is a sperm apoptosis phenomenon with specific cell death pathways (for model, see Aitken (2011)) or whether the ROS-imposed peroxidation just exceeds an irreversible limit of membrane dysfunction and chromatin damage. The latter cannot be excluded, as cholesterol oxidation and depletion, as well as DNA

deterioration, take place far away from the mid-piece, where mitochondrial induced apoptosis is to be expected (Brouwers *et al.* 2011). Moreover, as spermatozoa are translationally and transcriptionally silent, their chromatin has very specific features that may not be recognised by apoptosis-related DNA ligases of somatic cells.

Regardless of how spermatozoa deteriorate, sperm handling causes prolonged exposure of the cells to light (singlet oxygen formation) and oxygen that can create an oxidative environment and lead to detrimental peroxidative processes (Brouwers & Gadella 2003, Brouwers *et al.* 2005). Handling techniques, such as centrifugation (Agarwal *et al.* 1994, Shekarriz *et al.* 1995) and cryopreservation (Ball *et al.* 2001, Chatterjee & Gagnon 2001), may also reduce the presence and activity of naturally occurring antioxidants and cause spermatozoa to generate ROS (Bilodeau *et al.* 2000, Marti *et al.* 2008). Finally, it is well documented that freezing or sex-sorting increases the susceptibility of spermatozoa to ROS damage, even when compared with capacitated spermatozoa (Salamon & Maxwell 1995a, Brouwers & Gadella 2003, Neild *et al.* 2005, Leahy *et al.* 2010a).

For these reasons, antioxidant supplementation has been shown to increase the oxidative resistance of flow-sorted (Klinc & Rath 2007, Klinc *et al.* 2007, Leahy *et al.* 2010a), liquid-stored (Maxwell & Stojanov 1996, Aurich *et al.* 1997, Johnson *et al.* 2000, Aurich 2008) and frozen-thawed spermatozoa (Branco *et al.* 2010, Bucak *et al.* 2010, Malo *et al.* 2010, Kalthur *et al.* 2011). Such optimisation of processing techniques has resulted in increased fertility rates in numerous species, particularly with regard to liquid stored semen. However, if achieving the correct balance between oxidation and reduction were so simple, then antioxidants would be routinely included in all semen diluents. The reason this does not occur is that uncontrolled antioxidant treatment can also be detrimental, as cells generally function in a reduced state (termed the 'anti-oxidant paradox'; reviewed by Henkel (2011)). For instance, capacitation is reduction dependent and it is not clear whether excessive anti-oxidant protection during sperm handling renders the surviving cells responsive to a capacitation-inducing environment. It is probable that capacitation-specific oxysterol formation and depletion are hindered by anti-oxidants (see Fig. 3). This is also valid for the bicarbonate-dependent adenylate cyclase/cAMP/protein kinase A and tyrosine kinase signalling pathways and thus for the generation of hyperactivated sperm motility.

Stabilisation of the sperm surface with seminal plasma proteins

Due to the stabilising effect of seminal plasma on the sperm membrane, and its partial or complete removal during processing for freezing or sex-sorting, seminal

plasma has naturally been explored as an additive to mediate the detrimental effects of these technologies (see Fig. 3). The presence of seminal plasma during freezing is generally considered to protect the sperm membrane but the effect is more pronounced in certain species. In the ram (Graham 1994, Maxwell *et al.* 1999, Leahy *et al.* 2010c) and boar (Suzuki *et al.* 2002, Hernandez *et al.* 2007a, Vadnais & Roberts 2007, 2010, Garcia *et al.* 2010), inclusion of whole seminal plasma in the freezing regime results in the improvement of a whole host of functional parameters of spermatozoa upon thawing, including the ability to hold the sperm sample in a non-capacitated state.

Addition of seminal plasma as part of the sex-sorting protocol has met with similar success. Early on, it was shown that inclusion of ram or boar seminal plasma in a number of sorting diluents improved the viability and motility of the final product and reduced capacitation-like changes (Maxwell *et al.* 1996, 1998, Catt *et al.* 1997, Spinaci *et al.* 2006) induced by flow-sorting (Maxwell & Johnson 1997). While seminal plasma is now commonly added to the collection medium (Parrilla *et al.* 2005), or after concentration of sorted boar spermatozoa (Grossfeld *et al.* 2005), later studies in the ram were not as conclusive, reporting both beneficial (Leahy *et al.* 2009) and detrimental effects of seminal plasma on the function of sorted spermatozoa (de Graaf *et al.* 2007).

Such variations in the response of spermatozoa to seminal plasma are well documented (Katila & Kareskoski 2006, Maxwell *et al.* 2007, Muino-Blanco *et al.* 2008), as both the source and subsequent treatment of seminal plasma and spermatozoa influence the final outcome, and are thought to reflect differences in the relative abundance of beneficial and detrimental seminal factors. The major determinants include donor animal (e.g. good vs poor freezing boars (Hernandez *et al.* 2007a)), season (Leahy *et al.* 2010c), sperm fraction utilised (e.g. sperm rich (Saravia *et al.* 2009), as well as prior processing of seminal plasma (e.g. fractionation of seminal plasma (Ollero *et al.* 1997)) or sperm samples (e.g. washed vs non-washed (Perez-Pe *et al.* 2001)). Finally, protocol variations, such as point of addition of seminal plasma (e.g. pre-freeze vs post-freeze) and protein concentration (Leahy *et al.* 2009, 2010c), affect the response of spermatozoa to seminal plasma.

These variants contribute to the controversial effect of seminal plasma on field fertility. Supplementation of seminal plasma to frozen-thawed boar spermatozoa has been reported to improve farrowing rates (Okazaki *et al.* 2009) and litter size (Garcia *et al.* 2010). While other studies have found no effects on fertility (Abad *et al.* 2007a) or on the establishment of an oviductal sperm reservoir (Abad *et al.* 2007b). The situation is similar in the ram where cervical insemination of frozen-thawed sorted or non-sorted ram spermatozoa has been reported to improve or

Figure 3 Effect of surface stabilising methods on the fertilising ability of spermatozoa. (A) Methods to stabilise the spermatozoa during handling. Without such approaches, a greater proportion of cells will deteriorate during processing (membrane deterioration depicted here). These methods aim to keep the sperm surface in a stable, non-capacitated state (resembling that of ejaculated spermatozoa) during handling. To achieve this, physical and osmotic stressors must be minimised (see also Fig. 2B and C). At ejaculation, seminal plasma proteins (in orange) bind to the membrane and stabilise it but it is not known whether they replace decapacitation factors removed from the sperm surface during handling, or if their association physically inhibits the removal process (1). The application of high hydrostatic pressure (HHP) may result in firmer attachment of seminal plasma factors to the sperm surface (2), providing additional stability to the sperm cells, or result in higher adaptation rates to a changing *in vitro* environment (e.g. temperature, pH, osmolytes and pressure). Cholesterol oxidation can be inhibited by the addition of anti-oxidants and/or albumin (in purple) and cholesterol efflux by inclusion of cyclodextrin (in red) or vesicles (in white) that are preloaded with saturated amounts of cholesterol (in black) or other lipids (3). (B) Methods to remove these stabilising factors in order to achieve a functionally capacitated spermatozoon capable of fertilising the oocyte. It is debatable whether subsequent destabilisation of processed spermatozoa will allow them to enter a true capacitated state (4). IVF treatments that are currently employed (5) include bicarbonate (stimulates adenylate cyclase/cAMP/protein kinase A and tyrosine kinase signalling pathways) heparin (enhances stripping of the sperm surface) and fatty acid-free albumin (depletes cholesterol). Novel stimulation methods include cholesterol-accepting vesicles (in white) or cholesterol-free cyclodextrin (in red) or the use of certain pro-oxidants (6) (Rodríguez & Beconi 2009, Rodríguez *et al.* 2011). For further details and references, see section 'Novel sperm surface manipulation methods'.

have no effect on field fertility (Maxwell *et al.* 1999, El-Hajj Ghaoui *et al.* 2007, O'Meara *et al.* 2007, Leahy *et al.* 2010b).

This variability could be reduced if the exact components responsible for the beneficial or detrimental effects of seminal plasma were identified. Separation and characterisation of bull seminal plasma proteins have greatly expanded knowledge on the signalling pathways of capacitation and the protective action of extender components (for review, see Manjunath *et al.* (2007)). The main molecules of functional dominance in bull seminal plasma are BSP proteins of the fibronectin type II

family, which bind tightly to choline phospholipids on the sperm membrane upon ejaculation (Manjunath *et al.* 2009). This initially stabilises the sperm membrane but extended association causes efflux of cholesterol in a dose- and time-dependent manner (Therien *et al.* 1998, 1999). As BSP proteins can also bind high-density lipoprotein and heparin-like glycosaminoglycans (Gwathmey *et al.* 2003, 2006) – known capacitation factors in follicular and oviductal fluids – *in vivo*, they may act as regulators of capacitation (Lane *et al.* 1999). However, their presence for extended periods *in vitro* is not beneficial to sperm survival. Fortunately, the

common use of egg yolk and skim milk in cryoprotective diluents appears to have unintentionally masked this detrimental effect, as low-density lipoproteins and casein micelles present in these diluents preferentially bind BSP proteins, reducing the number available to bind to the sperm membrane (Manjunath *et al.* 2002).

In the boar, the effect of heparin-binding proteins is not as well documented and has been reported to have both positive and negative effects on sperm function, particularly in relation to the inhibition or stimulation of capacitation. Vadnais & Roberts (2010) reported that a heparin-binding fraction of boar seminal plasma, consisting of three spermadhesins (AQN-3, AQN-1, AWN) and the BSP protein, pB1, inhibited *in vitro* capacitation and cooling-induced capacitation-like changes (Vadnais & Roberts 2010). This agrees with early structural studies, showing that non-aggregated forms of AWN-1 and AQN-3 are able to bind directly to membrane lipids (Dostalova *et al.* 1995) and are partly released from the sperm surface upon capacitation (Sanz *et al.* 1993, Dostalova *et al.* 1994, Calvete *et al.* 1997), suggesting they act as decapacitation factors. However, AQN-3 has also been shown to be involved in the binding of capacitated boar spermatozoa to the zona pellucida (van Gestel *et al.* 2007), indicating that release of seminal plasma factors from the sperm surface is not a straightforward process. Moreover, the heparin-binding BSP protein, pB1, has also been shown to stimulate capacitation of epididymal boar spermatozoa by inducing cholesterol and phospholipid efflux from the sperm membrane (Lusignan *et al.* 2007), which has been extensively documented with bull BSP proteins. Incubation of highly diluted boar spermatozoa (extended to a level that mimicked sex-sorting: $0.3\text{--}1 \times 10^6$ sperm/ml) with heparin-binding proteins was detrimental to *in vitro* sperm function (Centurion *et al.* 2003), causing a time- and dose-dependent decrease in the motility, viability and mitochondrial integrity of spermatozoa. Non-heparin-binding proteins showed the opposite effect (Caballero *et al.* 2006) and the protective effect was largely afforded by the PSP-I/PSP-II heterodimer. Subsequent investigation narrowed down the protective effect to the peptidic fragment of the PSP-II subunit (Garcia *et al.* 2006), suggesting that a simple sperm preserver could potentially be commercially synthesised containing only this fragment (Caballero *et al.* 2008). Immunolocalisation studies have revealed that the PSP-I/PSP-II heterodimer is mainly located on the acrosomal region of boar spermatozoa and that this association holds the cell in a time-limited non-capacitated state by maintaining low intracellular calcium levels (Caballero *et al.* 2009). After long-term incubation, the heterodimer redistributes to the post-acrosomal region. Interestingly, heparin-binding proteins show the opposite pattern, and this redistribution has also been related to capacitation status (Dapino *et al.* 2009).

These somewhat contradictory results highlight the importance of prior treatment of spermatozoa on the subsequent effect of seminal plasma protein supplementation. The aforementioned studies investigated epididymal, ejaculated and highly diluted boar spermatozoa. As it is thought that pro-capacitation factors only have effects on the sperm surface after appropriate removal of other coating factors, it is probable that the differing effects reported for boar heparin-binding proteins are due to differences in the extracellular matrix of these sperm types. However, these processing differences do not explain the interesting variation between species when supplemented with BSP proteins.

As outlined above, seminal plasma proteins are well known to protect ram spermatozoa from cold shock (cooled or frozen in the absence of cryoprotectants) or during routine freezing (for review, see Maxwell *et al.* (2007) and Muino-Blanco *et al.* (2008)). The main protective components were reported to be a 14 kDa BSP protein, which was shown to prevent (Barrios *et al.* 2005) or reverse (Barrios *et al.* 2000) cold shock damage. The reason why these proteins do not appear to destabilise the ram sperm membrane, as shown in the bull, is not clear but may be due to differences in stimulation of capacitation. Colas *et al.* (2008) showed that ram spermatozoa are relatively insensitive to cholesterol-depleting reagents (e.g. albumin) that provoke tyrosine phosphorylation and capacitation in other species.

These multiple influences explain why, despite having known about the decapacitation effect of seminal plasma (Chang 1957) soon after discovery of the process itself (Austin 1952), we have failed to reliably exploit this natural phenomenon to protect spermatozoa during processing. Nevertheless, dissection of the effects of specific proteins has provided novel insights into the capacitation pathway and the function of previously unknown cryoprotective diluents (e.g. milk and egg yolk) and has also produced promising protective factors (e.g. PSP-II) for inclusion in well-defined sperm handling protocols.

The use of stress to improve stress tolerance

It is well recognised that *in vitro* manipulations have a detrimental impact on sperm quality, and new insights in this area have been reviewed in the current manuscript. However, this long-held dogma is challenged by a new evidence demonstrating that exposure to carefully controlled levels of stress can be beneficial to sperm survival. The aim of this novel manipulation method is to trigger the gametes' survival mechanisms by subjecting them to sub-lethal levels of stress (pre-stress), which then improves their resistance to subsequent challenges (e.g. storage, sex-sorting, embryo culture, etc.). The technique has been performed on various cell types (spermatozoa, oocytes, embryos and stem cells) using numerous

stressors (e.g. hydrostatic pressure, osmotic, heat and oxidation). The preferred method is high hydrostatic pressure (HHP), as it can be applied with the aid of a programmable device in an instantaneous, uniform and consistent manner (Pribenszky *et al.* 2010). Optimisation of pressure, temperature and exposure time has produced improvements in the quality and fertility of fresh, chilled and frozen-thawed bull and boar spermatozoa (reviewed by Callesen (2010), Pribenszky *et al.* (2010) and Pribenszky & Vajta (2011)). However, the mechanism by which HHP treatment confers increased resistance to stress is not known. It has been suggested that it may result from the production of chaperone proteins (Pribenszky & Vajta 2011), but no differences between HHP-treated and non-treated boar spermatozoa have been observed (Huang *et al.* 2009) and this explanation is controversial as the biosynthetic capabilities of spermatozoa are limited.

HHP is known to alter cell morphology, membrane fluidity, protein conformation and protein interactions (McCarthy & Grigera 2006). These effects are considered reversible at the levels applied during HHP treatment but it is possible that these changes alter the permeability of the sperm membrane to penetrating cryoprotectants (e.g. glycerol) and cause a quickening of diffusion processes during freezing, which would reduce the length of time the cells are exposed to an adverse osmotic environment. Alternatively, HHP treatment may result in firmer attachment of extracellular material to the sperm surface (e.g. egg yolk and seminal plasma proteins) providing additional stability to the sperm cell. In fact, spermadhesins (AWN) have been reported to be of greater abundance on the membrane of HHP-treated boar spermatozoa compared to those without HHP treatment (Huang *et al.* 2009). Such a finding would have important implications for the concurrent use of HHP treatment with other surface-stabilising components discussed in the current review, and its mechanism of action requires further attention.

General conclusion

Modern sperm surface stabilising strategies (see section 'Novel sperm surface manipulation methods') can partly prevent or reverse the detrimental effects of sperm handling (see section 'Sperm surface alterations during sperm handling'). Further developments in this area would benefit from clarification of the differences between physiological sperm capacitation (see section 'Physiological alterations of the sperm surface') and handling-induced capacitation-like changes. It has already been shown that protein tyrosine phosphorylation patterns may differ (Green & Watson 2001), and as we gain further insight into the capacitation process, it is becoming unlikely that other intricate stimulation mechanisms such as cholesterol depletion, ROS-induced specific signalling of spermatozoa and removal of seminal plasma proteins from the

sperm surface occur in a controlled manner during 'capacitation' induced by sperm handling as they do during physiological capacitation.

It also remains to be seen whether these stabilising components render spermatozoa unresponsive to subsequent incubation in a capacitation-supportive environment. Strategies to stimulate capacitation, through the depletion of cholesterol from the sperm membrane, or the triggering of capacitation-specific sperm signalling pathways, are emerging and have been shown to increase the success of IVF in rodents (Takeo *et al.* 2008, Seita *et al.* 2009). However, the hypothesis that oxidants (especially peroxynitrite) could prepare spermatozoa for fertilisation has not been tested, despite their ability to initiate capacitation (Rodriguez & Beconi 2009, Rodriguez *et al.* 2011). Such studies may shed light on achieving an optimal balance between anti-oxidant inclusion, to prevent sperm damage during handling and pro-oxidant concentration to trigger capacitation when required.

The recent observations and hypotheses described in this review provide further insight into the dogma of sperm capacitation and handling-induced sperm damage. Overall, the studies show the following: i) in order to survive handling, spermatozoa need to be stabilised at their surface, ii) physiological capacitation is a very specific surface destabilisation event involving very subtle and intricate processes and iii) it is possible that we override such processes during *in vitro* handling of spermatozoa. Careful balancing of strategies to stabilise the sperm surface during handling to prevent premature deterioration, and destabilisation of the sperm surface in the correct time and place, are crucial factors in obtaining the desired ART outcome.

Declaration of interest

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

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