

Review: Sperm–oocyte interactions and their implications for bull fertility, with emphasis on the ubiquitin–proteasome system

P. Sutovsky[†]

Division of Animal Sciences, Department of Obstetrics, Gynecology & Women's Health, University of Missouri, Columbia, MO 65211-5300, USA

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Fertilization is an intricate cascade of events that irreversibly alter the participating male and female gamete and ultimately lead to the union of paternal and maternal genomes in the zygote. Fertilization starts with sperm capacitation within the oviductal sperm reservoir, followed by gamete recognition, sperm–zona pellucida interactions and sperm–oolemma adhesion and fusion, followed by sperm incorporation, oocyte activation, pronuclear development and embryo cleavage. At fertilization, bull spermatozoon loses its acrosome and plasma membrane components and contributes chromosomes, centriole, perinuclear theca proteins and regulatory RNAs to the zygote. While also incorporated in oocyte cytoplasm, structures of the sperm tail, including mitochondrial sheath, axoneme, fibrous sheath and outer dense fibers are degraded and recycled. The ability of some of these sperm contributed components to give rise to functional zygotic structures and properly induce embryonic development may vary between bulls, bearing on their reproductive performance, and on the fitness, health, fertility and production traits of their offspring. Proper functioning, recycling and remodeling of gamete structures at fertilization is aided by the ubiquitin–proteasome system (UPS), the universal substrate-specific protein recycling pathway present in bovine and other mammalian oocytes and spermatozoa. This review is focused on the aspects of UPS relevant to bovine fertilization and bull fertility.

Keywords: fertilization, artificial insemination, sperm capacitation, acrosome, zygote

Implications

Fertilization is an intricate cascade of events that irreversibly alter the participating male and female gamete and ultimately lead to the union of paternal and maternal genomes in the zygote. Proper functioning, recycling and remodeling of gamete structures at fertilization is aided by the ubiquitin–proteasome system (UPS), the universal substrate-specific protein recycling pathway present in bovine and other mammalian oocytes and spermatozoa. Research focused on the UPS-regulated aspects of the fertilization process has a potential to impact the management of bull fertility, the optimization of artificial insemination, genomic selection for production and fertility-related traits, and the improvement of sperm viability after sexing and cryopreservation in cattle.

Introduction: prelude to sperm–oocyte interactions

However, for the semen to exert its effect on the eggs, it must enter their little bodies, as it seems unlikely that it could animate them by the mere contact with their skin (Spallanzani, 1780).

Quoted above, Italian pioneer of reproductive research and father of fertilization biology Lazzaro Spallanzani (1729–99), known for conducting the first recorded experiments with artificial insemination in frogs and canines, captured the essence of the fertilization process long before his successors were able to observe the actual union of a spermatozoon and an oocyte and decipher the molecular machineries that guide it. To paraphrase the words of another luminary of fertilization research, C.R. Moore, fertilization is a precisely orchestrated cascade of irreversible events that starts long before the male and female gametes meet. This intricate wedding dance starts with the mature gametes reaching the site of fertilization within the female oviduct. Spermatozoa have to bypass multiple hurdles crossing from the site of deposition through the female cervix (in vaginal depositors), uterus and uterotubal junction, to finally reach the site of oviductal sperm reservoir and bind, transiently, to its apical epithelia surfaces (Suarez, 2015). In bulls, sperm transport is believed to take between 4 and 16 h. The sperm–oviductal epithelium binding is mediated by acrosomal surface ligands and the fucose-containing glycans of the plasma membrane annexins, expressed by the oviductal epithelial cells. The sperm release upon capacitation requires proteolytic cleavage and shedding of the acrosomal

[†] E-mail: sutovskyp@missouri.edu

binder of sperm proteins, particularly binder of sperm protein 1 (BSP1) (Suarez, 2015).

Upon ovulation, signals from the ovary, oocyte–cumulus complex and its accompanying follicular fluid may be responsible for the capacitation and hyperactivation of spermatozoa bound to the oviductal sperm reservoir. Capacitation endows the reservoir-bound spermatozoa with fertilizing ability and helps them detach from the reservoir epithelium. As in other species, bull sperm capacitation encompasses cholesterol efflux, calcium influx, pH increase, remodeling of plasma membrane, and increase in tyrosine phosphorylation of sperm head and sperm tail proteins (Parrish, 2013). Such structural and molecular remodeling brings about hyperactivation, manifested by an increased amplitude and frequency of flagellar movement. Chemo-attractants issued from the oocyte and its surrounding cumulus cells are likely responsible for gamete recognition in mammals. Although the exact identity of the attractants involved in bovine gamete recognition is not known, progesterone from cumulus cells has been shown to fulfill such a role in other species (Oren-Benaroya *et al.*, 2008).

Ubiquitin–proteasome system and fertilization

Ubiquitin–proteasome system (Figure 1) is likely involved in all steps of the reproductive process, including gametogenesis, fertilization, zygotic and preimplantation embryonic development, implantation, and beyond (Sutovsky, 2003). Protein ubiquitination is a stable, covalent post-translational

modification by which the substrate proteins can be targeted for degradation by 26S proteasome, lysosome or autophagosome (Cohen-Kaplan *et al.*, 2016). Alternatively, protein ubiquitination, which is reversible by deubiquitinases, can serve a regulatory function, for example, through receptor endocytosis and histone code modification. Ubiquitination is a substrate-specific process that requires an energy donor (ATP) and a set of specific activator, carrier and ligase enzymes; it starts with the activation of single ubiquitin molecules by the ubiquitin-activating enzyme E1 (HUGO acronym UBA1). The activated ubiquitin is passed onto one of several carrier enzymes (E2 or UBE2) and ligated to an internal lysine residue of a substrate protein by substrate-specific ubiquitin ligases (E3 enzymes or UBE3), of which there is a great variety. Additional ubiquitin molecules are linked to the substrate-bound monoubiquitin by E3 enzymes, sometimes assisted by chain elongation factors (E4 enzymes). Substrate proteins linked to multi-ubiquitin chains of four or more ubiquitin molecules are degraded by the 26S proteasome, a multi-subunit holoenzyme composed of a 20S proteolytic core capped with a 19S proteasomal regulatory complex responsible for the recognition and removal of the multi-ubiquitin chain, and for the priming of the substrate protein before its transport to the 20S core.

While the proteasomes have been studied in spermatozoa of a number of mammalian species, as well as in other vertebrate and invertebrate animals (Sutovsky, 2011), little is known about the role of UPS in the early events of ungulate fertilization. Involvement of the sperm-borne proteasomes in

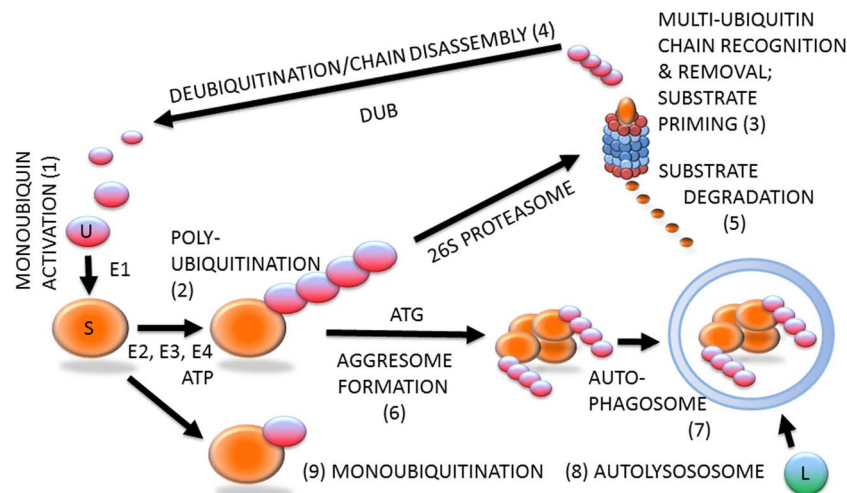


Figure 1 (Colour online) Protein ubiquitination and degradation. Unconjugated monoubiquitin (U; 1) binds covalently to its substrate (S) protein, catalyzed by ubiquitin-activating and -conjugating enzymes (E1, E2, E3) and fueled by ATP, to form a multi-ubiquitin chain (2), which is recognized by the 26S proteasome (3). The multi-ubiquitin chain is removed by the 19S proteasomal regulatory complex to be disassembled by deubiquitinating enzymes (DUB) and re-enters the pool of monoubiquitin available for conjugation (4). The substrate is degraded in the 20S proteasomal core (5). Proteasomal degradation has been implicated in sperm–zona pellucida binding, acrosomal exocytosis and sperm–zona penetration. Proteasomal proteolysis also regulates cell cycle progression, and pronuclear development and apposition in the zygote. During autophagy, which has been implicated in the regulation of early embryo development and post-fertilization sperm mitophagy, polyubiquitinated substrate molecules are aggregated by autophagy-associated proteins (ATG) to form an aggresome (6). Aggresomes are recognized and engulfed by the autophagophore that encloses them to form an autophagosome (7), which upon fusion with a lysosome (L) becomes an autolysosome (8) capable of degrading the entire aggresome, or an ubiquitinated organelle such as sperm mitochondrion. The ubiquitin–proteasome system-regulated post-fertilization sperm mitophagy mediates clonal, maternal inheritance of mammalian mitochondrial genes. Monoubiquitination (9) is reversible and serves regulatory purposes relevant to gametogenesis, fertilization and early development, such as histone modification to establish the epigenetic histone code, or plasma membrane receptor internalization to change cell responsiveness to specific external stimuli/ligands.

sperm capacitation has been described in human and porcine spermatozoa (reviewed by Kerns *et al.*, 2016). A recent study details the role of proteasomal degradation of the A-kinase anchoring fibrous sheath protein AKAP3 (A-kinase anchoring protein 3) in bull sperm capacitation, which was correlated with capacitation and acrosomal exocytosis ability that varied among individual bulls (Hillman *et al.*, 2013). More is known about the involvement of UPS in later events of fertilization, as will be discussed next.

Sperm–zona pellucida interactions

Bull and other mammals' spermatozoa bind to oocyte zona pellucida (ZP) by the apical ridge of their acrosome, composed of the acrosomal matrix sandwiched between the inner and outer acrosomal membranes (IAM and OAM, respectively), with the IAM laid atop subacrosomal perinuclear theca (PT) (Oko and Sutovsky, 2009). Bovine ZP is composed of three heavily glycosylated proteins, ZPA (homolog of human ZP2), ZPB (homolog of human ZP4/ZP3 β) and ZPC (homolog of human ZP3/ZP3 α), with the ZPC/ZP3 and ZPB/ZP4 being the predicted primary receptors for sperm binding and induction of acrosomal exocytosis (Yonezawa, 2014). In this scenario, ZPA/ZP2 is proposed to be responsible for secondary/sustained sperm–zona binding (Gadella, 2010).

During fertilization, the spermatozoa primed by acrosomal membrane remodeling during capacitation bind to ZP surface, undergo acrosomal exocytosis enacting the loss of OAM and acrosomal matrix in all parts of the acrosome except the equatorial segment, and penetrate the ZP with only the IAM left intact after exocytosis (Gerton, 2002). The OAM and acrosomal matrix do not merely disperse from zona surface as previously thought; they remain associated with it in the form of acrosomal shroud. In addition to enabling sperm–zona penetration, acrosomal exocytosis prepares spermatozoa for fusion with the oolemma, by exposing oolemma-binding proteins on the sperm head equatorial segment (Cuasnicu *et al.*, 2016). Based on imaging of live mouse spermatozoa with green fluorescent acrosomes, it was proposed that acrosomal exocytosis is initiated before sperm contact with the oocyte, that is, during sperm migration along the female oviduct (La Spina *et al.*, 2016). It is not clear whether such concept applies to bovine fertilization. Furthermore, one has to be cautious about interpreting such observations as the true acrosomal exocytosis, as opposed to the remodeling of acrosomal membranes induced by sperm capacitation.

The signaling cascade of acrosomal exocytosis, induced by the binding of sperm head ligands to ZPC, activates signaling through phospholipases, and both the tyrosine and serine/threonine protein kinases which leads to the influx of external calcium, depolymerization of the actin microfilaments scaffolding the acrosome, and the activation of membrane fusion pathways leading to the fusion and hybridizing vesiculation of plasma membrane and OAM, resulting in the formation of acrosomal shroud (Belmonte *et al.*, 2016; Romarowski *et al.*, 2016).

Bull spermatozoa display all three major proteasomal core enzymatic activities (Pizarro *et al.*, 2004). Proteasomes are localized in all three layers of the mammalian sperm acrosome, including OAM, matrix and IAM (Sutovsky *et al.*, 2004; Zimmerman *et al.*, 2011). Acrosomal proteasomes may serve a dual purpose during sperm–ZP interactions, first as the mediators of acrosomal exocytosis and subsequently as proteases contributing to localized degradation of the zona matrix that results in the formation of the fertilization slit (Zimmerman *et al.*, 2011; Miles *et al.*, 2013). Bull spermatozoa capacitated in the presence of proteasomal inhibitor epoxomicin had reduced ability to undergo acrosomal exocytosis induced *in vitro* by progesterone and to fertilize oocytes *in vitro* (Sanchez *et al.*, 2011). Addition of proteasomal inhibitor MG132 into *in vitro* fertilization (IVF) medium significantly reduced the rate of bovine fertilization (Rawe *et al.*, 2008). Solubilized ZP protein degradation by bull sperm proteasomes has not been described, to our knowledge, but the degradation of solubilized ZP proteins by sperm proteasomes has been shown in humans (Saldivar-Hernandez *et al.*, 2015) and Japanese quail (Sasanami *et al.*, 2012). In agreement with their proposed role in sperm–zona interactions, a subpopulation of proteasomes are exposed on the bull sperm surface and prominently represented in the sperm surface proteome (Byrne *et al.*, 2012), as has also been described in other mammals. It is thus possible that a third function could be assigned to them: that of secondary or alternative receptor for ZP. In agreement the proteasomes of human and porcine spermatozoa are part of a large multimeric zona-binding complex that also contains chaperonins, zona-binding receptors, acrosin and its binding protein, and seminal plasma proteins (Redgrove *et al.*, 2011; Kongmanas *et al.*, 2014). In summary, UPS is likely involved in multiple events during sperm interactions with oocyte ZP.

Gamete adhesion and fusion, and sperm incorporation

Commonly referred to as gamete fusion, the process by which the fertilizing spermatozoon contacts and passes through the oocyte plasma membrane, the oolemma, is likely more complex than originally thought. It is thus prudent to distinguish between its adhesion and fusion phase (reviewed in Sutovsky, 2009). Moreover, important is to note that gamete adhesion and fusion is enabled by sperm capacitation and acrosomal exocytosis during which proteins involved in the sperm–oolemma interactions become exposed on the sperm head equatorial segment (e.g. equatorin/MN-9 (Manandhar and Toshimori, 2001), CD9 (Ito *et al.*, 2010) and IZUMO1 (Satouh *et al.*, 2012)).

Early studies implicated sperm disintegrins and oolemma integrins in sperm–oolemma adhesion, though none such receptors have fusogenic properties. Gene knock-out studies demonstrated that these molecules are either non-essential or compensable in mouse fertilization (Primakoff and Myles, 2007). Alternatively proposed sperm receptors on the oolemma, tetraspanins CD9 and CD81 were knocked out, resulting in reduced fertility of single gene knock-out and

complete infertility of double knock-out (Benammar *et al.*, 2016). The most convincing evidence for gamete adhesion receptors came with the discovery of the sperm-specific immunoglobulin family cell adhesion protein IZUMO (Inoue *et al.*, 2005) and its oocyte-binding partner, the folate-binding receptor JUNO (Bianchi *et al.*, 2014). Mice null in either of these proteins are infertile. Both IZUMO (Fukuda *et al.*, 2016) and JUNO (Zhao *et al.*, 2017) are present in bovine gametes, but there is not yet a direct proof of their involvement in bovine gamete adhesion. A plausible model for bovine/mammalian sperm–oolemma interaction is thus the tetraspanin web on the oolemma, in which said tetraspanins are the organizers of various receptors for sperm equatorial segment ligands, including integrins, JUNO and the downstream signaling proteins that relay the adhesion signals to oocyte cortical cytoskeleton (Sutovsky, 2009; Wright and Bianchi, 2015). Accordingly, microfilaments in the bovine oocyte microvilli (Sutovsky *et al.*, 1996) and cortex are required for physical incorporation of the sperm head and tail in the ooplasm and probably for signaling in response to the initial adhesion between sperm and oocyte plasma membranes, preceding their fusion, as exemplified by gamete adhesion induced signaling via oocyte proline-rich tyrosine kinase (PYK2) in zebra fish (Sharma and Kinsey, 2012). Consequently, microfilament disrupting drug cytochalasin B prevents sperm incorporation without interfering with sperm–oolemma fusion or with the release of sperm PT proteins that trigger oocyte activation (Sutovsky *et al.*, 1996; Sutovsky *et al.*, 1997; Sutovsky *et al.*, 2003).

Cortical actin cytoskeleton appears to be regulated in part by UPS. Interference with ubiquitin C-terminal hydrolase (UCHL1), enriched in the mammalian oocyte cortex can thus alter polyspermy rate *in vitro* (Yi *et al.*, 2007; Mtango *et al.*, 2011b). The expression of *Uchl1* gene in bovine zygotes was altered by heat stress (Sakatani *et al.*, 2015), wherein the UCHL1 inhibition impaired migration and exocytosis of cortical granules, the major line of defense against polyspermic fertilization (Susor *et al.*, 2010). A related deubiquitinase, UCHL3, is associated with oocyte meiotic spindle and may regulate oocyte maturation (Mtango *et al.*, 2014 and 2011a). Altogether, it is likely that the UPS mainly regulates the later events of sperm–oocyte adhesion and fusion; in particular, sperm incorporation and activation of the anti-polyspermy defense.

Oocyte activation

While adhesion and fusion between oocyte and sperm plasma membranes initially occurs within the sperm head equatorial segment, it quickly extends distally to post-acrosomal sheath (Sutovsky *et al.*, 2003). The current paradigm of oocyte activation assumes that a soluble sperm factor(s) released from post-acrosomal PT triggers calcium release from oocyte endoplasmic reticulum that triggers a signaling cascade guiding the completion of oocyte meiosis, mobilization of anti-polyspermy defense, zygotic/pronuclear development and ultimately embryo cleavage (Okó *et al.*, 2017). Initial studies of this process in mammals were conducted in rodent and

bovine IVF systems. It was demonstrated that freeze-thaw sperm extracts activate hamster oocytes (Swann, 1990). The removal and solubilization of sperm PT was shown to occur during bovine IVF even when sperm incorporation was prevented by treatment with actin depolymerizing drug cytochalasin B (Sutovsky *et al.*, 1997).

While the dissolution of PT can be hindered by protease inhibitors (Perry *et al.*, 2000), it is not clear whether oocyte proteasomes are directly involved. However, UPS clearly regulates zygotic cell cycle by mediating the oocyte spindle exit from metaphase II through degradation of EMI1 (synonym F-box protein 5/FBOX5) protein that maintains the activity of oocyte cytostatic factor and by degrading the cyclin component of the M-phase promoting factor to assure successful metaphase–anaphase transition (Glotzer *et al.*, 1991; Tung *et al.*, 2005; Karabinova *et al.*, 2011).

Stepwise extraction of PT from mouse sperm heads progressively eliminated their ability to trigger oocyte activation after intracytoplasmic sperm injection, and the activity was shown to be associated with specific fractions of PT that had differential extraction resistance (Kimura *et al.*, 1998; Perry *et al.*, 2000). Eventually, the currently favored sperm-borne oocyte factor (SOAF) candidate, phospholipase C zeta 1 (PLCZ1) was identified *in silico* and shown to trigger oocyte calcium oscillations when a recombinant protein or its messenger RNA (mRNA) were injected in metaphase II oocytes (Saunders *et al.*, 2002). Persistent concerns about localization of PLCZ outside of post-acrosomal sheath (PAS) and its promiscuity with regard to oocyte-activating sperm cytosol fractions prompted a search for alternative or synergistic sperm SOAF components, chief among them male germline-specific WW-domain-binding WBP2NL (ww-domain binding protein 2 N-terminal like) (alias post-acrosomal WW-domain binding protein (PAWP)) and its somatic cousin WBP2 (Wu *et al.*, 2007b; Okó *et al.*, 2017). As in other mammals, PAWP is prominent in bull sperm PT where it is transported from the cytoplasmic lobe along the caudal manchette in the elongating spermatid phase of spermiogenesis, coinciding with the spermatids' acquisition of oocyte-activating ability (Wu *et al.*, 2007a). In the mouse, targeting neither the *Plcz1* (Hachem *et al.*, 2017) nor the *Wbp2nl* (Satouh *et al.*, 2015) gene appears to eliminate male fertility. Intriguingly, increased expression of *Wbp2nl* gene is observed in the testis of *Plcz1* null mice (Hachem *et al.*, 2017), suggesting compensation in the knock-out mouse and possible synergy between the two gene products in wild type animals.

With regard to bull fertility, individual sperm levels of WBP2NL vary between sires and within individual ejaculates. Excessive amounts of WBP2NL were found in macrocephalic spermatozoa with abnormally large sperm head and PAS, while certain morphologically deviant spermatozoa, particularly the ones with abnormally shaped sperm heads and those positive for ubiquitin, in this case applied as a sperm quality biomarker (Sutovsky *et al.*, 2015), lacked WBP2NL altogether (Kennedy *et al.*, 2014). High abundance of *Plcz1* mRNA in spermatozoa was also associated with fertility of Holstein bulls (Kasimanickam *et al.*, 2012), a breed in which *Plcz1* gene

polymorphism also appears to be a contributing factor (Pan *et al.*, 2013). In conclusion, while the identity of the sperm-borne oocyte-activating factor is still being researched, there appears to be a consensus that it is a partially soluble protein (or several proteins) in the sperm head PT.

Zygotic centrosome assembly and pronuclear apposition

Somatic cell centrosome is a cellular microtubule organizing center composed of two perpendicularly oriented, barrel-shaped (pinwheel-like on cross-section) centrioles and pericentriolar material (PCM) with the ability to nucleate tubulins to polymerize into microtubules. In bovine gametes, the centrosome is reduced in a complementary fashion, with oocytes retaining PCM but devoid of centrioles, and spermatozoa carrying a single, duplication-competent centriole (or no centrioles, as in rodents) and little, if any PCM (Sathananthan *et al.*, 1997; Manandhar *et al.*, 2005). As in other non-rodent Eutherian mammals, bull sperm centrosome is reduced to a single centriole with little or no PCM during spermiogenesis/spermatid elongation in the testis. This centriole is housed within the complex structure of the sperm tail connecting piece and thus termed the proximal centriole (Sutovsky *et al.*, 1996; Simerly *et al.*, 1999). The distal centriole is degraded during spermiogenesis, and only some remnants of centrosomal material may be found within the distal centriolar vault (Manandhar *et al.*, 2005). Failure of proper centrosome remodeling during spermiogenesis has been associated with the tail stump defect in infertile bulls (Blom and Birch-Andersen, 1980).

At fertilization, soon after sperm head and sperm tail mid-piece incorporation, the striated columns caging the centriole break up, a disassembly process required for the formation of zygotic centrosome and leading up to the organization of microtubule-made sperm aster responsible for the apposition of paternal and maternal pronuclei (Sutovsky *et al.*, 1996; Sutovsky and Schatten, 1997; Sutovsky, 2010) (Figure 2). The gamma-tubulin (TUBG) containing, microtubule-nucleating PCM is recruited from bovine oocyte cytoplasm by the liberated sperm centriole (Shin and Kim, 2003). The ability of bull spermatozoa to form sperm aster after IVF has been correlated with the developmental potential of bovine IVF embryos in artificial insemination (AI) bulls (Navara *et al.*, 1996; Hara *et al.*, 2015; Hochi, 2016). Inspired by those findings, bovine oocytes have been injected with human spermatozoa as a way of testing for human male infertility due to sperm centrosomal insufficiency (Nakamura *et al.*, 2001). It was noted that in both human and bovine zygote, proteasomal activity is required for the formation of sperm aster, possibly because proteasomes are present in the sperm tail connecting piece and necessary for its disassembly that liberates the sperm centriole and makes it competent to recruit centrosomal proteins from ooplasm (Rawe *et al.*, 2008). Altogether, it is well documented that bull spermatozoa contribute crucial components of the zygotic centrosome at fertilization, and such a mode of centrosomal inheritance is similar to primates and other ungulates, but different from rodents.

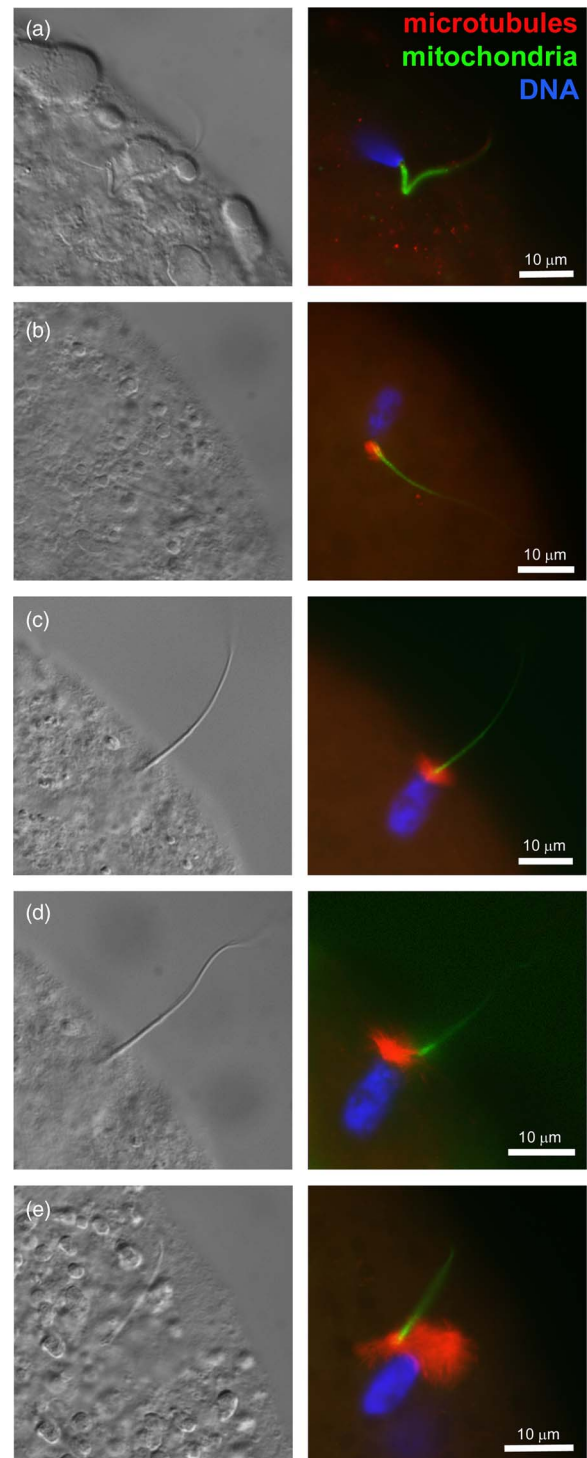


Figure 2 (Colour online) Progression of sperm incorporation, sperm nucleus decondensation and sperm aster formation during bovine fertilization *in vitro*. In all panels, sperm aster microtubules were labeled with monoclonal anti-tubulin antibody E7 (red), sperm mitochondria were pre-labeled with vital stain MitoTracker Green FM (green) before IVF and DNA in the sperm nucleus (blue) was counterstained with 2-(4-amidinophenyl)-1H-indole-6-carboxamide. Left columns show parfocal bright-field images acquired with differential interference contrast optics (DIC). The samples were examined and photographed under a Nikon Eclipse 800 epifluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) with Cool Snap camera (Roper Scientific, Tucson, AZ, USA) and MetaMorph software (Universal Imaging Corp., Downingtown, PA, USA). Images were edited and contrast balanced by Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA).

Pronuclear development

Before sperm–oolemma fusion, bull sperm head found within oocyte perivitelline space is composed of a nucleus covered with a reduced nuclear envelope and PT all around, and IAM in the acrosomal region and plasma membrane over the equatorial segment and post-acrosomal region. With the exception of a small contingent of somatic and sperm-specific histones in the nucleus and PT (Aul and Oko, 2002; Tovich and Oko, 2003) sperm DNA is packaged with protamines, mainly protamine 1 (PRM1) (Balhorn, 2007). Proper sperm protamination correlates with bull fertility in AI service (Dogan *et al.*, 2015).

The sperm nucleus is demembrated in a stepwise fashion at sperm–oolemma fusion and entry in the ooplasm, when the sperm plasma membrane comingles with oolemma, PT, first in the post-acrosomal and then in the subacrosomal region spreads across the ooplasm, and the sperm nuclear envelope, devoid of nuclear pore complexes and nuclear lamina, dissolves (Sutovsky *et al.*, 1997; Sutovsky *et al.*, 1998). Next, protamines, inserted during sperm nucleus compaction at spermiogenesis, are removed and replaced with oocyte-specific histones, which themselves will later be replaced with somatic cell type histones (Gao *et al.*, 2004). At the time of protamine–histone

exchange reversal, the nuclear envelope is reconstituted by membrane vesicle fusion and nuclear lamina formation on both maternal and paternal chromatin, and nuclear pores are reinserted, possibly from a premade stock of ooplasmic annulate lamellae (Sutovsky *et al.*, 1998).

As the maternal and paternal pronuclei grow, maternally stored nucleolar proteins re-enter the nuclear compartment to form nucleolus precursor bodies that will be remodeled into active ribosomal RNA producing nucleoli at the time of major zygotic genome activation (four to eight cells in bovine) (Maddox-Hyttel *et al.*, 2007; Ogushi *et al.*, 2008). The zygote-driven remodeling of bovine sperm nucleus is facilitated by multiple ooplasmic factors, including the disulfide bond reducing peptide glutathione, histones, lamins, nucleoporins and nucleoplasmin (Sutovsky and Schatten, 1997; Burns *et al.*, 2003). Such an extensive restructuring is aided by oocyte proteasomes that accumulate in both pronuclei from the onset of pronuclear development (Figure 3). In addition to pronuclear accumulation, proteasomes are abundant in mammalian zygote cytoplasm, as shown in the zygotes of a transgenic pig expressing proteasomes tagged with green fluorescent protein (Miles *et al.*, 2013).

Proteasomal inhibitors alter male pronuclear chromatin (Huo *et al.*, 2004; Rawe *et al.*, 2008) possibly interfering with protamine removal and/or oocyte–somatic histone swap. The

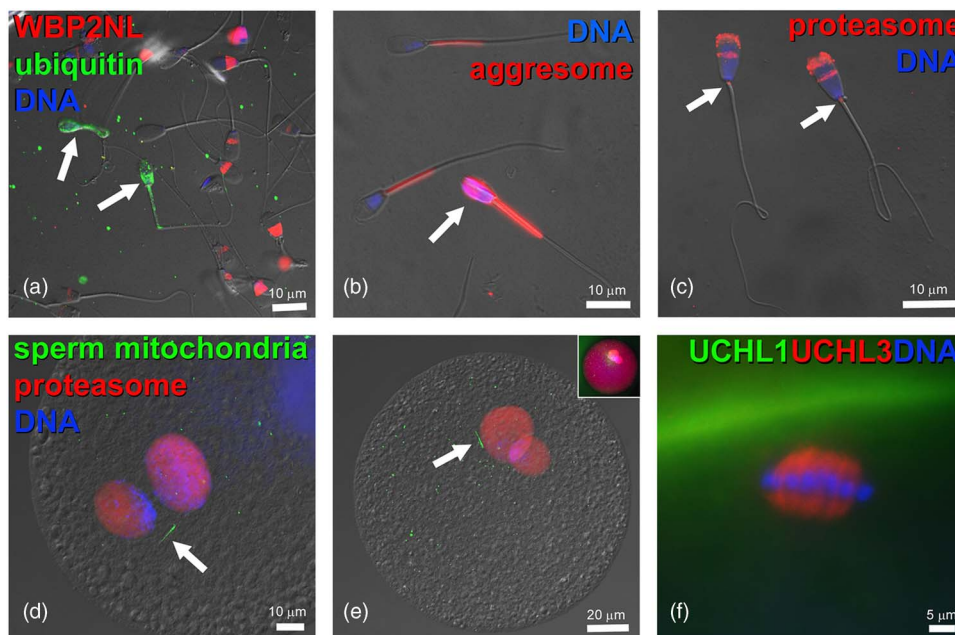


Figure 3 (Colour online) Components of ubiquitin–proteasome system in bovine gametes and embryos. (a) Bull spermatozoa (arrows) with abnormal phenotypes are ubiquitinated on their surface (green) and lack the WBP2NL/PAWP protein (red) present at varied levels in the post-acrosomal sheaths of surrounding morphologically normal spermatozoa. (b) Aggresome-binding ProteoStat probe (red) binds exclusively to mitochondrial sheaths of normal spermatozoa but detects aggresomes in the deformed sperm head of an abnormal spermatozoon (arrow). (c) Proteasomes (red) detected in bull sperm head acrosome and sperm tail connecting piece (arrows) by polyclonal antibody recognizing the 20S proteasomal core subunit PSMB10 (PW8150, Enzo Lifesciences, Ann Arbor, MI, USA). (d,e) Proteasomes (red) in the apposing (d), and apposed (e) maternal and paternal pronuclei of bovine zygotes, detected by a polyclonal antibody recognizing multiple 20S proteasomal core subunits (PW8155, Enzo Lifesciences). Inset in (e) shows the same zygote from which the differential interference contrast layer has been subtracted and red fluorescence brightened to reveal cytoplasmic proteasome labeling. Bovine oocytes were fertilized by spermatozoa pre-labeled with MitoTracker Green FM (green) to detect sperm tail mitochondrial sheaths (arrows). (f) Detection of deubiquitinases ubiquitin C-terminal hydrolase L1 (UCHL1) (green) and UCHL3 (red) in the bovine oocyte cortex and meiotic spindle, respectively. DNA in all panels was counterstained with 2-(4-amidinophenyl)-1H-indole-6-carboxamide. The samples were examined and photographed under a Nikon Eclipse 800 epifluorescence microscope with Cool Snap camera and MetaMorph software. Images were edited and contrast balanced by Adobe Photoshop CS5.

ensemble of epigenetic histone marks, the zygotic histone code, which is reset at the onset of embryo development, is also influenced by UPS (Nevoral and Sutovsky, 2017), possibly through UPS regulation of sirtuin 1 (SIRT1), a histone deacetylase (Adamkova *et al.*, 2017). The involvement of UPS extends beyond the zygote. Genes encoding UPS components such as proteasomal subunits, ubiquitin-conjugating enzymes and ubiquitin ligases likely regulate later events of bovine preimplantation development, including the major zygotic genome activation and blastocyst differentiation (Massicotte *et al.*, 2006; Adjaye *et al.*, 2007). It can be concluded that the UPS regulates multiple facets of early zygotic development in bovines and other mammals.

Mitochondrial inheritance and the fate of sperm tail accessory structures

At fertilization, bull spermatozoa carry into ooplasm several dozen mitochondria organized into a helical structure of the mitochondrial sheath in the sperm tail midpiece. Early studies demonstrated that bull sperm mitochondria are sought out and targeted for degradation by the fertilized oocyte, typically lasting only up to 4-cell stage (Sutovsky *et al.*, 1996). Such targeted mitophagy, not observed in interspecies zygotes of domestic cattle oocytes and gaur (*Bos gaurus*) spermatozoa (Sutovsky *et al.*, 1996) may be developmentally advantageous due to the high potential for sperm mitochondrial DNA (mtDNA) damage, mutation and deletion during spermatogenesis and fertilization (reviewed in Song *et al.*, 2014). Failure of this mechanism in cattle, causing heteroplasmy by leakage of paternal mtDNA, could affect offspring health and production traits influenced by mitochondrial fitness, such as milk protein content and meat quality (Gibson *et al.*, 1997).

Bovine post-fertilization mitophagy depends on the progression of the zygotic cell cycle, and it is not a coincidence that both processes are co-regulated by UPS. Consequently, sperm mitochondria become ubiquitinated inside the oocyte (Sutovsky *et al.*, 1999) and targeted for degradation by proteasomal proteolysis and lysosomal/autophagic machinery (Sutovsky *et al.*, 2000). Recent studies in domestic pig zygotes indicate that the central role in sperm mitophagy is played by the ubiquitin-binding autophagy receptor SQSTM1 and the proteasome-serving ubiquitinated protein dislocase valosin containing protein (VCP) (Song *et al.*, 2016). The participation of VCP explains why proteasomal inhibitors block sperm mitophagy, but proteasomes seldom associate directly with the sperm mitochondrial sheath inside the zygote. Some mitochondrial membrane and matrix proteins, such as prohibitin (Thompson *et al.*, 2003), are already tagged with ubiquitin in sperm mitochondria in bull spermatozoa, and the properties of the heavily disulfide bond cross-linked, ubiquitinated sperm mitochondrial sheath are similar to those of an aggresome, a ubiquitinated protein aggregate subject to autophagy in somatic cells (Thompson *et al.*, 2003; Kennedy *et al.*, 2014).

Attention has also been given to sperm tail accessory structures including the fibrous sheath covering the principal piece

and the axonemal outer dense fibers running the whole length of the sperm tail except the connecting piece, where they are transformed into striated columns. While the fibrous sheath appears to dissolve very quickly within the first few hours of bull sperm incorporation, the outer dense fibers and microtubules may persist past one cell stage (Sutovsky *et al.*, 1996). Given the minute size of bovine sperm tail, its resident proteins are not likely to be the source of nutrition for the embryo, as has been proposed in *Drosophila*, a species endowed with an enormous sperm tail. However, the densely amalgamated tail accessory structures could harbor paternal RNAs that affect embryo development, as will be discussed below.

Research on sperm ubiquitin has been extended beyond sperm mitochondrion ubiquitination and led to the discovery of sperm surface ubiquitination in defective bull spermatozoa (Sutovsky *et al.*, 2001; Baska *et al.*, 2008), and later on to identification of aggresomes outside bull sperm mitochondrial sheath (Kennedy *et al.*, 2014). Sperm surface ubiquitination has been validated as a sperm quality biomarker in bulls and other species (Sutovsky *et al.*, 2015) and correlated with sperm acrosome (Odhiambo *et al.*, 2011) and DNA integrity (Sutovsky *et al.*, 2002), expression of fertility-related sperm proteins (platelet activating factor receptor) (Sutovsky *et al.*, 2007) and PAWP/WBP2NL, which also correlated with sperm aggresome content in AI bulls (Kennedy *et al.*, 2014). Identification of sperm surface ligands unique to defective spermatozoa enabled the development of nanopurification for the improvement of bull AI semen (Odhiambo *et al.*, 2014). At present, there is already enough evidence to conclude that the clonal maternal inheritance of bovine mitochondrial genes is regulated by UPS through proteasomal and autophagosomal protein/organelle degradation.

Regulatory molecules in bull spermatozoa that affect early embryo development

Mammalian spermatozoa carry their own transcriptome (Ostermeier *et al.*, 2004), which is likely reflective of genome activity before germ cell meiosis and particularly early after meiosis, when round spermatids remain transcriptionally active until the process of histone–protamine exchange is initiated. Approximately 3500 species of mRNA alone are found in human spermatozoa (Ostermeier *et al.*, 2002), among which the genes within UPS appear to be dysregulated in teratospermic infertile men (Platts *et al.*, 2007). Recent sequencing effort revealed transcripts of over 13 000 genes in bull spermatozoa (Selvaraju *et al.*, 2017). Beside mRNA, non-coding RNAs such as micro-RNA (miR), long Piwi-interacting RNAs and small nucleolar RNAs could be carried by bull spermatozoa and influence embryo development and inheritance (Hossain *et al.*, 2012). The miR-196b, targeting *homeobox* genes, appears to be the most abundant miR in bull spermatozoa (Selvaraju *et al.*, 2017). The miR content may vary between a high- and low-motility spermatozoa (Capra *et al.*, 2017), as well as between bulls of varied fertility (Govindaraju *et al.*, 2012; Fagerlind *et al.*, 2015).

Protamine *Prm1* is among the most abundant bull sperm mRNAs (Card *et al.*, 2013), probably reflecting its abundance in spermatids, wherein its product is required for the proper packaging of sperm DNA. Sperm levels of several other transcripts, including ubiquitin-conjugating enzyme gene *Ube2e3* correlate with bull AI fertility (Parthipan *et al.*, 2017). This and other UPS-related sperm-borne mRNAs could be translated by the zygote, for example, to assist the post-fertilization clean-up of maternally stored proteins.

Some studies suggest the lone presence of spermatozoa and seminal plasma can stimulate specific transcriptional response in the female reproductive system that may be favorable for the establishment of pregnancy. Seminal plasma may influence gene expression in uterine epithelia directly and indirectly regulate embryo development through uterine secretion of embryotrophic growth factors (Bromfield *et al.*, 2014). Such findings warrant further inquiry into the transcriptome of bovine seminal plasma, from which some RNA containing protein complexes could be adsorbed on the bull sperm surface and carried past cervix during mating, or be introduced directly in uterus with extended semen during AI.

Mouse studies provide additional insight into potential roles of sperm RNAs in zygotic development and inheritance. Some of the sperm mRNA contributed to zygote could be translated to regulate early development and even to promote the RNA-mediated non-Mendelian inheritance, as suggested for the mouse sperm transmitted mutated *Kit* allele dominating the mRNA produced from wild type allele (Rassoulzadegan *et al.*, 2006). Another example, miR-34c is present in spermatozoa but absent from oocytes and appears to be required for embryo cleavage (Liu *et al.*, 2011). Pronuclear injection of miR-24 caused paramutation in the mouse zygote coinciding with heritable histone modification associated with promoter region of transcription factor *Sox9* gene (Grandjean *et al.*, 2009). Pertinent to the regulation of zygotic development by UPS, injection of eight different sperm derived miR in mouse zygotes caused degradation of maternal, oocyte stored mRNA that, among other effects on gene expression, affected the genes encoding aforementioned, UPS-regulated SIRT1 protein and ubiquitin ligase UBE3A, both involved in histone code modification (Rodgers *et al.*, 2016). Similarly, sperm-borne transfer RNA fragments, acquired during epididymal passage seem to repress expression of specific genes in the murine embryo and may mediate epigenetic influences between father and offspring (Sharma *et al.*, 2017). Altogether, new evidence indicates that RNAs with both the protein coding and regulatory functions are present in bull spermatozoa and contributed to the zygote at fertilization.

Effects of paternal genome on conception rate and offspring fertility

Gene polymorphisms and epigenetic marks transmitted to offspring by the father can directly influence offspring fertility and have an effect on the development of their own

offspring. Efforts are under way to identify genes in bulls with extreme high *v.* low fertility that bear on sperm phenotype and sperm production traits (Taylor *et al.*, 2018). There are already some examples of gene polymorphisms that affect bull sperm phenotype. Genes encoding for sperm head proteins, integrin β 5 (ITGB5) and collagen type I α 2 (COL1A1), have been associated with bull fertility (Feugang *et al.*, 2009). Other polymorphisms associated with bull sperm phenotypes include genes encoding for sperm surface CD9, which may influence sperm motility (Kumar *et al.*, 2015) and TEX11 (testis expressed 11) associated with scrotal circumference (de Camargo *et al.*, 2015). Holstein bull fertility may be influenced by single-nucleotide polymorphisms in the miR target sites of gene encoding spermatid transitional protein 1 (TNP1) (Zhang *et al.*, 2015) and by polymorphism-driven alternative splicing of the sperm flagella 2 (SPEF2) gene (Guo *et al.*, 2013). The copy number in spermatogenesis associated multi-copy genes such as preferentially expressed antigen in melanoma may also result in varied bull fertility (Yue *et al.*, 2013). More attention should be directed to epigenetic marks on sperm-borne genome and chromatin that are generally thought to be erased in the zygote and re-established in the early embryo. Both sperm histone H3 modifications (Kutchy *et al.*, 2017) and sperm DNA methylation (Kropp *et al.*, 2017) have been associated with bull fertility. Finally, genes that are transcribed from paternal allele during early development and may support developmental competence and pregnancy establishment, or guard against pregnancy loss will be examined for potentially detrimental polymorphisms (Cochran *et al.*, 2013). Ongoing genomic studies will likely identify a number of gene mutations that may contribute to fertility or convey subfertility to bulls used in AI service.

Summary

Gamete interactions during bovine fertilization represent an intricate mesh of remodeling, recycling and signaling that results in transformation of gamete-specific structures into zygotic components. Thus, the sperm acrosome is remodeled during capacitation causing the exposure of zona-binding ligands on the OAM, and is partially lost to acrosomal exocytosis induced by the oocyte ZP. The sperm head PT dissolves in ooplasm to release oocyte-activating factors, and the denuded sperm nucleus is turned into a zygotic paternal pronucleus. The reduced sperm centrosome contributes the formation of zygotic centrosomes and initiates nucleation of microtubule sperm aster necessary for maternal and paternal pronucleus apposition. Various sperm-borne RNAs may regulate early embryo development and even impose epigenetic marks. Sperm contributed paternal genome affects developmental competence of embryos and ultimately imposes heritable traits on offspring.

While some sperm accessory structures are simply recycled after fertilization as they become obsolete, the fate of sperm mitochondria is of particular interest as these have to be eliminated to promote clonal, maternal inheritance of

mitochondrial genes and avoid heteroplasmy. Similarly, the regulatory influence of sperm-borne RNAs on zygotic and embryonic development warrants further investigation. Altogether, the ability of some sperm contributed structures and molecules to develop into the functional zygotic components and properly induce embryonic development may vary between bulls, bearing on their reproductive performance, and on the fitness, health, fertility and production traits of their offspring.

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Declaration of interest

There is no conflicts of interest to declare.

Ethics statement

This is a review article; consequently, there are no applicable ethics/compliance committee approvals to be listed.

Software and data repository resources

There are no deposited data or models associated with this article.

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