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New aspects of sperm physiology and sperm oocyte interactions

F.J. Peña[1]

Laboratory of Equine Reproduction and Spermatology, Veterinary Teaching Hospital,
University of Extremadura, Cáceres, Spain.

Abstract

Recent aspects of sperm biology that readily impact on reproductive medicine and biotechnology are briefly overviewed in this paper. The new concept of reactive oxygen species (ROS) homeostasis, and its implications on sperm functions, the heterogeneous nature of the ejaculate and the epigenetic information contained will be overviewed. Additionally, the concept of sperm senescence will be discussed as some aspects of sperm oocyte interaction.

Keywords: CASA, ROS, sperm, subpopulations.

Introduction

In recent years the knowledge of sperm biology has increased exponentially, and new findings in sperm biology are impacting reproductive medicine and technology in animals and humans. In this paper, aspects of sperm biology that have been object of research in the last years in the laboratory of the author will be discussed.

The spermatozoa a redox regulated cell

Reactive oxygen species are byproducts of various metabolic processes, and now are recognized as important regulators of many cellular functions (Stowe and Camara, 2009). The mitochondrion is considered as the major source of reactive oxygen species in most cells. Superoxide ($O_2\bullet^-$) can be generated at different points within the electron transport chain, by univalent reduction of oxygen and spontaneously or enzymatically dismutates to H_2O_2. Most superoxide is converted to H_2O_2 by superoxide dismutases inside and outside of the mitochondrial matrix, and, superoxide in low and controlled amounts exerts important regulatory cellular functions. Excess of H_2O_2 can combine with Fe^{2+} to form reactive ferryl species. In the presence of nitric oxide (NO•), O2•- forms the reactant peroxynitrite (ONOO•), and ONOOH induced nitrosylation of proteins, DNA, and lipids can modify their structure and function (Stowe and Camara, 2009). Numerous studies indicate that ROS are important regulators of sperm function (de Lamirande and Gagnon, 1993, 2002, 2003; Zini *et al*., 1995; de Lamirande *et al*., 1997, de Lamirande and Lamothe, 2009). An important aspect, sometimes neglected, is the type, origin and main role of the different ROS that can be formed during sperm metabolism. Superoxide is short lived ($t_{1/2}$ 1ms) and cell impermeant, while H_2O_2 is more stable and cell permeant. Nitric oxide (NO•) is synthesized through the conversion of l-arginine to l-citruline by nitric oxide synthase (NOS). These enzymes are present in the stallion spermatozoa, possibly as sperm specific isoforms (Ortega Ferrusola *et al*., 2009a). Moreover recent evidence suggests that stallion sperm mitochondria produce significant amounts of NO (Ortega Ferrusola *et al*., 2009a). Nitric oxide has a relatively long half life (1 s) and is more reactive than $O_2\bullet^-$. These compounds are considered, when produced in a controlled manner, as signaling molecules involved in a variety of sperm functions. Other molecules such as the hydroxyl radical (•OH) the peroxynitrite anion ($ONOO^-$) and lipid peroxides are considered more toxic to the spermatozoa and with less regulatory functions. Functions believed to be redox regulated in spermatozoa include activated and hyperactivated motility, chemotaxis, capacitation and the acrosome reaction. Controlled ROS production occurs during capacitation in spermatozoa (Agarwal *et al*., 2014), this controlled production triggers signaling pathways initiated by an increase in cyclic adenosine 3'-5' monophosphate cAMP. Increased cAMP activates protein Kinase A (PKA) and the subsequent phosphorylation of extracellular regulated kinase like proteins and finally tyrosine phosphorylation of proteins in the fibrous sheath of the spermatozoa, leading to sperm hyperactivation. Acrosome reaction and sperm oocyte fusion also depend of ROS activated cellular pathways.

Sperm not only DNA

The sperm genome has to be intact to participate in embryo development. It is, however, susceptible to oxidative DNA damage, so it is important to determine whether it remains intact when semen is manipulated and cryopreserved. In addition, semen also contains a series of small regulatory non-coding RNA (ncRNA) that contain 19 to 22 nucleotides. These microRNA (miRNA) are found in both the seminal plasma and spermatozoa. The miRNA are key of gene expression. They act epigenetically and play an important posttranscriptional modifiers role in the acquisition and maintenance of male fertility. They are

[1]Corresponding author: fjuanpvega@ unex.es
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abundant in bull sperm and show differential expression in relation to fertility levels of different sires. They are delivered to the oocyte at fertilization and modulate the first cleavage divisions (Kleene *et al*., 2010).

Mammalian ejaculate is composed of different sperm subpopulations

Growing consensus exists within the scientific community regarding the existence of sperm subpopulations within the mammalian ejaculate (Abaigar *et al*., 1999). Rather than an homogenous population in which all the spermatozoa shows the same behaviour, the mammalian ejaculate is an heterogeneous group of different sperm subpopulations showing different responses to physiological or biotechnological stimuli, motility patterns and even different morphometric characteristics. Over the years the ejaculate has been considered and analyzed as an entity, and the spermatozoa considered as a single sperm subpopulation. In recent years, the complex nature of the mammalian ejaculate is being unveiled more and more (Abaigar *et al*., 2001; Nunez-Martinez *et al*., 2007). In many species semen is ejaculated in fractions, where spermatozoa are embedded in the secretions of the male accessory glands, in variable numbers, with variable amounts of fluid and, probably proteins, in each fraction (Rodriguez-Martinez *et al*., 2009). This heterogeneity in the composition of seminal plasma may affect greatly sperm function, as has been demonstrated *in vitro*. However once the ejaculate is deposited in the female genital tract, the spermatozoa losses contact with the seminal plasma, and are exposed to different environments within the female reproductive tract. Using the boar species as model, and in experiments performing heterospermic inseminations (Satake *et al*., 2006) the role of the presence of sperm subpopulations in sperm selection has been disclosed. When spermatozoa from two or more boars are mixed and females inseminated the resulting litters are skewed in favour of one male. Bicarbonate responsiveness varies among sperm subpopulations between males, and specific oviductal proteins modulate this response. This mechanism selects sperm subpopulations to reach the oocytes for fertilization. All these scientific evidences provide an explanation of why the overall correlation with fertility is usually low when laboratory test of sperm function that ignore the sperm subpopulation structure are performed.

Kinematic subpopulations

Sperm motility is measured to provide information about the likely fertility of an individual an/or to assess the viability of a semen sample after liquid storage or cryopreservation In these situations motility has to be considered in three ways, first the percentage of cells showing movement, secondly the characteristics of the movement itself and thirdly the speed of the movement. Although sperm motility evaluation is normally one of the first steps of the spermiogramme (Rodriguez-Martinez, 2003), the amount of predictive information given, even using computerized systems, have been scarce and even disappointing. In spite of this, recent evidences suggest that the pitfall in the predictive value of motility relays in the use of the information given and not in the intrinsic value of the motility itself (Holt *et al*., 2007). One of the reasons for the lack of power of conventional parameters is the inherent heterogeneity of mammalian semen; it is one of the most variable of all biological fluids. Sperm motility is the most widely used indicator of sperm quality, however this parameter is dependent of a large number of factors and usually its estimation is largely subjective. In addition sperm motility is evaluated *in vitro*, and what is seen *in vitro* may not reflect at all the behaviour of the spermatozoa during the fertilization process. The fact is that in mammals the significance of sperm motility is a very complex phenomenon, since the spermatozoa are exposed to very different environments, with capability to modify cell signaling pathways, both in the male and female genital tract. In addition, most of the sperm transport process is mediated by the female genital tract, and not by the intrinsic motility of the spermatozoa. During sperm transport, spermatozoa are selected. At coitus billions of spermatozoa are deposited in the female genital tract, but only a few thousands (those of better quality) reach the uterotubal junction. Depending of the species, the spermatozoa may find two main barriers through its journey to reach the oocyte, the cervical barrier (humans, ruminants, canine) and the uterotubal junction in all species. It is clear that only a minority of the spermatozoa present in the ejaculate is retained in the female reproductive tract. In humans it has been estimated that only less than 1% of the spermatozoa are retained in the female reproductive tract and that this supports the notion that only a minority of sperm enters the cervical mucus and ascends higher in the female reproductive tract (Suarez and Pacey, 2006). The uterotubal junction acts as a selective barrier, allowing only the "best spermatozoa" reaching the sperm reservoir (Liu *et al.*, 1991). In this regard, high sperm velocities appear to be a critical factor to colonize the oviduct (Liu *et al*., 2010). The fact is that when sperm motility is evaluated using traditional approaches, a number of important factors are ignored: a) that not all the spermatozoa in the semen sample will reach the oviduct; b) that sperm motility will be displayed in different manner in response to different environments within the female genital tract; c) that sperm velocities are the key factors to colonize the oviduct, and d) that the sample is a mix of different sperm subpopulations with different ability to response to signals arising in the female genital tract. However the analysis of the sperm subpopulation structure, using sperm velocities as

variable to disclose the heterogeneity of the ejaculate has proven to be more informative to asses the quality of a given semen sample.

Morphometric subpopulations

Most studies on sperm subpopulations have been performed using kinematic parameters, but some research has been done using computerized morphometry to disclose sperm subpopulations. In the laboratories of the authors, the sperm morphometric subpopulation structure has been studied using the canine, equine and porcine species as models. Also the sperm subpopulation structure of wild ruminants has been described. Studies in boars, using sequentially principal component analysis (PCA), clustering, and discriminant analyses have been developed to identify sperm morphometric subpopulations in well-defined portions of the fresh boar ejaculate. In a recent study using the boar as a model (Saravia *et al*., 2007) within morphologically normal spermatozoa, different sperm subpopulations were disclosed based in head size and other morphological parameters, such as the regularity of sperm head shape.

Spermatozoa are present in the ejaculate in different physiological status

The spermatozoa are eliminated at ejaculation embedded in the secretions of the male accessory glands. In species such as pig, horses and dogs, the ejaculate is eliminated in different jets, accompanied of different subsets of secretions from the accessory glands. Whether or not this modifies sperm physiology is still to be fully determined, but the fact is that not all the spermatozoa within a given ejaculate are in the same physiological status. Studies in pigs demonstrate that the sperm present in the first 10 ml of the sperm rich fraction have different motility patterns, capacitation status, and even different size. Also these spermatozoa are the first to colonize the sperm reservoir at the oviduct. Studies in horses demonstrate that a significant subset of spermatozoa present in a given ejaculate is activated to experience an apoptotic process shortly after ejaculation (Ortega Ferrusola *et al*., 2008). These spermatozoa are unlikely to be able to fertilize an oocyte. So, a physiological explanation for these phenomena is warranted. The fate of most of the spermatozoa from a given ejaculate is to be removed from the female genital tract, either due to semen reflux after breeding, either being phagocytosed within the female uterus. Only a few thousand spermatozoa colonize the sperm reservoir at the utero- tubal junction. This means that the loose of spermatozoa by reflux and phagocytosis is a part of a process of sperm selection. It is believed that the heterogeneous nature of the ejaculate is involved in this sperm selection. In fact, although to be demonstrated in all the species, spermatozoa depicting PS externalization are preferentially phagocytosed in humans. Spermatozoa depicting PS externalization can be considered as prematurely activated, probably acting as a barrier against the female macrophages, thus protecting other spermatozoa. In the porcine species the proportion of spermatozoa with PS externalization varies between ejaculate fractions (Peña *et al*., 2003). In stallions, fresh sperm present a high percentage of spermatozoa showing caspase activity, both pro-caspases and active caspases 3, 7 and 9 have been detected in fresh samples (Ortega Ferrusola *et al*., 2008). From all these findings is clear that the spermatozoa present in a given ejaculate are in different physiological states at ejaculation, and this heterogeneity may be related to sperm selection within the female reproductive tract.

Born to dye; only one last. Do mitochondria control the lifespan of ejaculated spermatozoa?

Although sperm death after ejaculation is due to ATP depletion, other forms of sperm demise are described. Both “apoptotic like events” (Aitken *et al*., 2012a, b; Gallardo Bolanos *et al*., 2014), and an “autophagy like” mechanism may also be involved in sperm death after ejaculation (Gallardo Bolanos *et al*., 2012; Bolanos *et al*., 2014). More interestingly, different subpopulations of spermatozoa are in more advanced stages of senescence and may die at different intervals after ejaculation (Auger *et al*., 1993; Barroso *et al*., 2006; Gallon *et al*., 2006). Many spermatozoa with apoptotic changes appear in the ejaculate, and apoptotic changes including phosphatidylserine (PS) translocation (Peña *et al*., 2003; Brum *et al*., 2008; Martin *et al*., 2004), increase in membrane permeability (Ortega Ferrusola *et al*., 2009a), low mitochondrial membrane potential (da Silva *et al*., 2011; Aitken *et al*., 2012a; Garcia *et al*., 2012), activated caspases (Caselles *et al*., 2014; Gallardo Bolanos *et al*., 2014) and DNA fragmentation (Smith *et al*., 2013; Gillan *et al*., 2005) have been characterized in the ejaculate. Although the significance of these changes is still under debate, some points of consensus are being achieved. Perhaps, the most important aspect is that all spermatozoa are programmed to die, and that only one sperm reaches immortality through fertilization (Aitken and Koppers, 2011; Aitken *et al*., 2012a). Importantly many sperm biotechnologies accelerate this pathway to sperm death (Ortega-Ferrusola *et al*., 2008; Balao da Silva *et al*., 2013; Peña *et al*., 2011; Petyim *et al*., 2014). This form of sperm death appears to be dependent on the activation of an intrinsic apoptotic cascade originated in the mitochondria after unbalanced mitochondrial ROS generation (Aitken and Curry, 2011; Koppers *et al*., 2008). This later may occur after exhaustion of intracellular antioxidant defenses, particularly intracellular glutathione. Although this mechanism has been primarily described in humans, evidences suggest

that a similar scenario occurs in stallions, mainly during processes of conservation of sperm (Ortega-Ferrusola *et al*., 2008, 2009b). The maintenance of sperm viability depends on the phosphorylation status of specific pro-survival proteins. One of this, Akt is activated through phosphorylation at threonine-308 or serine-473 (Alessi *et al*., 1997; Yu *et al*., 2005). After phosphorylation, Akt functions through phosphorylation and inhibition of Bad (serine-136) or caspase-9 (Cardone *et al*., 1998). Bad is a proapoptotic member of the Bcl family that promotes cell death by dimerization with Bcl-2 or Bcl-X_L (Yang *et al*., 1995). Bad phosphorylation at four different serine residues (serine-112, -136, -155, or -170) has been characterized as inactivating Bad (Datta *et al*., 1997; Lizcano *et al*., 2000; Dramsi *et al*., 2002; Danial *et al*., 2003). Provided that sperm Akt is phosphorylated at Ser473 and/or Thr308 sperm motility and integrity are maintained; with de-phosphorylation of Akt, caspases are activated and motility is rapidly lost (Gallardo Bolanos *et al*., 2014), a similar mechanism occur in human sperm (Koppers *et al*., 2011). It appears that de-phosphorylation of Akt depends of different factors such as ATP depletion, unbalanced ROS and removal of pro-survival factors (Pujianto *et al*., 2010). On the contrary, dead receptor mediated sperm death may also occur. Some evidences indicate the sperm death can be triggered through the activation of toll like receptors by bacteria (Das *et al*., 2011; Fujita *et al*., 2011), ROS released by death sperm (Roca *et al*., 2013), and TNFα or other pro death factors released by other spermatozoa activating extrinsic apoptotic pathways (Macias Garcia *et al*., 2012; Mendoza *et al*., 2013).

Fertilization: the miracle of life

For fertilization to occur in mammals, ejaculated spermatozoa must reach the egg, that after ovulation has move from the ovary to the fallopian tube. Very few of the ejaculated spermatozoa, less than 1 million in humans reach the fallopian tube, moreover the spermatozoa undergo capacitation before being able to penetrate the cumulus layer, bind to the sperm receptor of the egg coat, and undergo the acrosome reaction that allows sperm penetration through the egg coat and fusion with the egg. The percentage of capacitated spermatozoa is low (around 10% in humans), so the number of spermatozoa that can reach and fertilize the egg is small. Due to this slow number of capacitated sperm, mechanisms of sperm guidance are crucial for successful fertilization to occur. Two active mechanisms of sperm guidance have been described in mammals, chemotaxis and thermotaxis (Eisenbach and Giojalas, 2006). Only the small fraction of capacitated sperm is chemotactically active. The spermatozoa use both active swimming and passive drag by female genital tract muscular contraction to reach the storage site at the oviduct. Once here, a small percentage of spermatozoa undergoes capacitation; capacitated sperm reach the egg guided by a combination of chemotaxis, termothaxis and, perhaps oviductal contractions. Chemoattractants are present in the oviductal fluid and are also secreted by the egg and surrounding cumulus cells. Initially capacitated spermatozoa at the storage site, use an ovulation-dependent temperature gradient between this site and the fertilization site (Eisenbach and Giojalas, 2006). As spermatozoa approach the fertilization site they probably sense a chemoatractant gradient that guides them to the egg. Confirmed chemoatractants for mammalian spermatozoa are atrial natriuretic peptide, bourgeonal, lyral, small peptides from the follicular fluid (1.3 to 13 kDa), progesterone and rantes (Eisenbach and Giojalas, 2006).

The fertilization site in the oviduct has been shown to be 1-2ºC warmer that the storage site for spermatozoa in rabbits and pigs, this change is due more to a temperature decrease in the storage site than increase at the fertilization site (Eisenbach and Giojalas, 2006).

Fertilization triggers a complex cellular programthat transforms two highly specialized meiotic germ cells, the oocyte and the sperm, into a totipotent embryo. Sperm cells initially bind to the zona pellucida of the egg made of just a few glycoproteins, zona pellucida binding protein 1 (ZP1) to ZP4 in humans (Clift and Schuh, 2013). Early studies identified ZP3 as a potential primary sperm receptor, however now is considered that ZP3 alone is not sufficient for sperm binding. It is possible that ZP proteins together adopt a three dimensional structure that presents a binding site for sperm. This binding site is lost after fertilization preventing polyspermy. The protease responsible for ZP2 cleavage has been identified, and is termed ovastacin. ZP proteins are modified with oligosaccharides at Asp (N-linked) and/or Thr (O-linked resiues). Most of them terminate with the sialyl Lewis X tetrasaccharide motif, that plays a major role in sperm-egg binding (Clift and Schuh, 2013). Upon binding to the egg the sperm experiences the acrosome reaction with release of hydrolytic and proteolytic enzymes that opens a way for the fusion of the spermatozoa with the plasma membrane of the egg. The fusion of the sperm with the egg triggers the completion of the second meiotic division and the transition to mitosis. The sperm induces rise in free Ca2+ in the egg, in mammals a series of Ca2+ oscillations triggers a temporally ordered sequence of events, including the release of cortical granules, completion of the second meiotic division, translation of maternal mRNAs, and ultimately the transition of meiosis to mitosis. Increase of Ca2+ is triggered by phospholipase Cζ (PLCζ) that is introduced in the egg by the sperm. PLCζstimulates the production of inositol 1-4-5 trisphosphate, which binds to receptors in the ER causing the release of Ca2+, and triggering the exit from meiosis. Eggs arrest in metaphase of the second of the second meiotic division,

while they await for fertilization. This arrest is mediated by the activity of cytostatic factor (CSF). An essential mediator of CSF activity is EMI2 (early mitotic inhibitor 2). EMI2 maintains metaphase II arrest by inhibiting the anaphase-promoting complex, which targets cyclin B and the separase inhibitor securin for degradation (Clift and Schuh, 2013). The increase in intracellular Ca^{2+} levels upon fertilization activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which phosphorylates EMI2. EMI2 is further phosphorylated by Polo-like kinase 1 (PLK1) and subsequently targeted for degradation by the SCF (SKP2-cullin 1-F-box protein) ubiquitin ligase. This leads to the activation of the APC/C, destruction of cyclin B and securin, elimination of half of the sister chromatids into the second polar body and the formation of the female pronucleus (Clift and Schuh, 2013). Almost immediately after fertilization, the increase in intracellular Ca^{2+} levels triggers exit from meiosis and the formation of a female pronucleus, while the sperm genome undergoes decompaction. Protamines are rapidly removed from the sperm pronucleus, and the DNA is re-wrapped around nucleosomes that contain the histone H3 variant H3.3, which is replaced with canonical histone H3 during DNA replication. Although now equivalently structured in nucleosomes, the two pronuclei retain some parent-specific histone methylation patterns, particularly at pericentromeric heterochromatin regions that are equilibrated gradually during the first embryonic divisions. For the zygote to acquire totipotency, the parental genomes must also undergo extensive epigenetic reprogramming, which involves global DNA demethylation. The sperm genome is highly methylated compared with that of the egg. Within hours of fertilization, however, the sperm pronucleus undergoes rapid active demethylation before DNA replication.

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