**Article Addendum**

**Communication between female tract and sperm**

Saying NO• when you mean yes

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Signaling through [Ca\(^{2+}\)] is central to regulation of sperm activity and is likely to be the mechanism that transduces signals from the female reproductive tract to regulate sperm motility. In a recent paper we showed that exposure of sperm to nitric oxide mobilizes stored Ca\(^{2+}\) in human sperm, an effect that occurs through nitrosylation of protein thiols. Not only did we find that NO• production by cells of the human female tract would be sufficient to elicit this effect, but progesterone, which is also present in the female tract and is synthesized by the oocyte vestments, acted synergistically with NO• to mobilize Ca\(^{2+}\) and enhance flagellar beating. Here we argue that a Ca\(^{2+}\) store at the junction of the sperm head and flagellum is subject to regulation by both progesterone and NO• and that ryanodine receptors at the store may be the point at which coincidence detection and synergistic interaction occurs.

The sole purpose of sperm is to deliver the male genome to the oocyte. In internal fertilizers, such as mammals, large numbers of sperm are ejaculated and deposited in the female tract (∼100,000,000 cells in the human). Successful fertilization leading to healthy offspring requires that a least one but not excess numbers of sperm encounter the egg in the oviduct and that they are prepared to fertilize. Freshly ejaculated sperm cannot immediately fertilize. Competence to do so is gained through a ‘suite’ of biochemical and ‘structural’ modifications termed capacitation, that occurs during residence in the female reproductive tract and can also be induced (under appropriate conditions) in vitro.

Both sperm transport to the oocyte and capacitation are closely regulated by the female reproductive tract. In humans fewer than 100 sperm reach the site of fertilization in the oviduct (Fig. 1A), a ‘success’ rate of ∼10\(^{-6}\) and of these only a fraction may be competent to fertilize. Success in this endeavor not only requires high levels of motility, but is also crucially dependent on the ability of the sperm to detect and respond appropriately to a series of cues provided by the cells of the female tract, the oocyte itself and the cumulus cells that surround it. In response to these cues the sperm may bind to the epithelium in the isthmal region of the oviduct (Fig. 1A) to await ovulation, detach at the appropriate time, direct their swimming toward the oocyte (chemotaxis), alter their swimming style from progressive motility to ‘whiplash’ or hyperactivated motility (required to penetrate the vestments that surround the oocyte) and undergo acrosomal exocytosis of the acrosome (a single vesicle in the sperm head) upon reaching the surface of the glycoprotein coat (zona pellucida) that surrounds the oocyte (Fig. 1B). Due to the highly condensed nature of the sperm nucleus and the absence of ribosomes (differentiating sperm shed almost all their cytoplasm and mature cells have no endoplasmic reticulum) sperm are generally considered to lack translational or transcriptional activity (though this is disputed). Regulation of sperm activity must, therefore, be achieved through post-translational modification of the activity of proteins ‘inherited’ from the differentiating germ cell. It is well established that phosphorylation of sperm proteins is pivotal to the process of capacitation and various serine/threonine and tyrosine kinases, regulated by cAMP, Ca\(^{2+}\) and other second messenger cascades are crucial in regulation of the sperm activity and competence to fertilize the egg.

The identity of the signaling molecules that regulate the activity of sperm is only beginning to be understood. Surface receptors on the epithelial cells lining the tract bind sperm and regulate their progress and contact with the surface of the zona pellucida activates processes leading to acrosomal exocytosis (Fig. 1B). However, sperm must also detect and respond to soluble messengers that the tract and cumulus-oocyte complex secrete into the luminal fluid. Of these, the most studied is the steroid progesterone. Low concentrations of progesterone derived from the circulation are present throughout the female tract, but a particularly potent source for the sperm is the cumulus oophorus (Fig. 1B), a mass of granulosa cells which surround the oocyte and continue to synthesize progesterone after ovulation. Exposure of human sperm to nM-μM doses of...
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Progesterone causes Ca\textsuperscript{2+} influx at the plasma membrane (probably by activating a surface, non-genomic receptor) and also mobilizes Ca\textsuperscript{2+} stored in an organelle (or organelles) situated at the sperm neck (junction of head and tail; Fig. 1C). This causes [Ca\textsuperscript{2+}]\textsubscript{i} transients and oscillations in this region of the sperm.\textsuperscript{10}

Progesterone-induced release of stored Ca\textsuperscript{2+} requires Ca\textsuperscript{2+} influx at the plasma membrane, is modified by pharmacological agents that act on ryanodine receptors (RyRs) and appears to be a form of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR). The mobilization of Ca\textsuperscript{2+} stored at the sperm neck causes a marked increase in flagellar bend angle,
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particularly in the proximal flagellum adjacent to the neck.\textsuperscript{10,11} The well-known hyperactivating effect of progesterone on human sperm probably reflects this Ca\textsuperscript{2+} store mobilization, a conclusion consistent with the work of Suarez and colleagues on the role of the sperm neck Ca\textsuperscript{2+} store in hyperactivation of mouse and bovine sperm.\textsuperscript{12,13} At sub-nanomolar doses progesterone causes chemotaxis\textsuperscript{14} and there is now evidence that this is the primary or even the only chemoattractant released from the cells of the cumulus oophorus.\textsuperscript{15,16} The identity and mode of action of other diffusible regulators of sperm activity, secreted by the female tract, is an area of considerable interest.

More than 20 years ago the free radical gas nitric oxide (NO\textsuperscript{*}) was shown to act as an intercellular messenger.\textsuperscript{17} NO\textsuperscript{*} is synthesized by nitric oxide synthase (NOS) either as a ‘puff’ of NO\textsuperscript{*} in response to a cellular signal such as a [Ca\textsuperscript{2+}] elevation or as a more prolonged signal generated by an unregulated (inducible) NOS. NO\textsuperscript{*} diffuses freely from its point of synthesis but its effects are localized because of its high reactivity and consequent very short lifetime.\textsuperscript{18} The classical mode of action of NO\textsuperscript{*} in the ‘target’ cell is activation of soluble guanylate cyclase (sGC), leading to downstream actions of cGMP such as kinase activation and gating of ion channels\textsuperscript{19} but more recently other modes of action have been discovered including S-nitrosylation of exposed protein thiols,\textsuperscript{20} which acts as a functional switch similar to protein phosphorylation. Reports that NOS is present in both the walls of the female tract and the cells of the cumulus-oocyte complex\textsuperscript{21-23} suggest that NO may be one of the messengers that act on sperm as they approach the egg. We have confirmed that fresh human oviduct and cumulus express NOS and actively synthesize NO\textsuperscript{*}.\textsuperscript{1} Experiments with nitric oxide donors (molecules that, degrade spontaneously in solution to liberate NO\textsuperscript{*}) have provided evidence that NO\textsuperscript{*} has a number of effects on sperm function, those on motility being of particular interest in the context of sperm ascending the tract. Some results are inconsistent but the consensus ‘take home message’ seems to be that at high concentrations NO\textsuperscript{*} is toxic, suppressing sperm motility and blocking fertilization, whereas at lower concentrations NO\textsuperscript{*} enhances motility and affects the ‘pattern’ of the sperm’s activity, possibly even acting as a chemoatractant.\textsuperscript{24-26} [Ca\textsuperscript{2+}]\textsubscript{i} is a crucial regulator of sperm motility (see above), being pivotal both to hyperactivation and to chemotaxis.\textsuperscript{27} Thus Ca\textsuperscript{2+} signaling in the sperm is likely to be the ‘tool’ by which NO\textsuperscript{*} regulates the activity of sperm in the female tract.

We treated human sperm with nitric oxide donors and observed a rapid rise in [Ca\textsuperscript{2+}]\textsubscript{i}, that stabilized at a significantly higher concentration within 5–10 min, often with superimposed [Ca\textsuperscript{2+}]\textsubscript{i} spikes or oscillations.\textsuperscript{1} NO\textsuperscript{*} was also effective in Ca\textsuperscript{2+} free medium and localization of the Ca\textsuperscript{2+}-signal indicated that, like progesterone, NO\textsuperscript{*} mobilized Ca\textsuperscript{2+} stored in region of the sperm neck. We discovered that NO\textsuperscript{*} was not acting through activation of sGC and generation of cGMP but through an effect that was reversed by thiol reducing agents and mimicked by S-nitroso-glutathione (GSNO), a protein S-nitrosylating agent. Use of the biotin switch method confirmed that, under these conditions, protein S-nitrosylation occurred with kinetics indistinguishable from the effects of NO\textsuperscript{*} and GSNO on [Ca\textsuperscript{2+}]\textsubscript{i}. Co-incubation of sperm with explants from the human oviduct caused levels of S-nitrosylation comparable with those induced by NO\textsuperscript{*} donors and GSNO. Both protein S-nitrosylation and Ca\textsuperscript{2+}- mobilisation reversed rapidly upon washing off the NO\textsuperscript{*} donor. Significantly, application of Angeli’s salt, a donor of HNO (nitrooxy, which also affects protein function by modification of exposed thiols and is more reactive than NO\textsuperscript{*}\textsuperscript{28}) had similar effects (Fig. 1D).

These findings suggest that NO\textsuperscript{*} mobilizes (or at least sensitizes) the Ca\textsuperscript{2+} store in the neck region of human sperm (Fig. 1C) by a mechanism that is not the ‘classical’ regulation of sGC leading to responses induced by cGMP or PKG, but instead involves a direct effect on ‘target’ proteins by S-nitrosylation, presumably at one or more key residues.\textsuperscript{20} Modulation of protein function enhances Ca\textsuperscript{2+} leak from the store (leading to a [Ca\textsuperscript{2+}]\textsubscript{i} plateau) and periodic store emptying occurs (leading to Ca\textsuperscript{2+} spikes and oscillations). This interpretation poses two important questions:

1. What is the target (or targets) for S-nitrosylation that regulates mobilization of Ca\textsuperscript{2+} stored at the sperm neck?

2. What is the potential significance of this effect in vivo?

With regard to the target protein, nitrosylation of exposed thiols has been shown to change the activity of many proteins. We studied the S-nitrosoproteome of human sperm exposed to NO donors and to GSNO under conditions equivalent to those that induce mobilization of stored Ca\textsuperscript{2+}.\textsuperscript{29} S-nitrosylated proteins were bionitylated (using the biotin switch method) and purified, followed by MS/MS of tryptic peptides for identification. The list of proteins that we detected included a number of interest including heat shock proteins, A kinase anchoring proteins and glycolytic enzymes. However, of particular interest with regard to effects of S-nitrosylation on Ca\textsuperscript{2+} signalling in sperm was detection of ryanodine receptor (RYR)2. RYRs are intracellular Ca\textsuperscript{2+} channels that underlie Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR; responsible for Ca\textsuperscript{2+} waves and Ca\textsuperscript{2+} oscillations) in many cell types. These channels are subject to S-nitrosylation in the presence of NO\textsuperscript{*}, leading to increased open probability and Ca\textsuperscript{2+} mobilization, an effect which is rapidly reversed by thiol reducing agents,\textsuperscript{30} paralleling our observations on [Ca\textsuperscript{2+}]\textsubscript{i}. SNO, which mobilizes stored Ca\textsuperscript{2+} in human sperm (Fig. 1D) is also a highly potent activator of these channels through reaction with exposed thiols.\textsuperscript{31} The presence of RyRs in sperm has been difficult to prove and is still controversial\textsuperscript{10,32-34} probably because these channels are present at very low abundance. RyRs have such high conductance that in a mature sperm (a cell with a minimal cytoplasmic volume) the presence of a single channel could be sufficient to generate a significant elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. However, evidence is accumulating: pharmacological manipulation of RyRs modulates progesterone-induced Ca\textsuperscript{2+} oscillations derived from the sperm neck store\textsuperscript{10} and also affects Ca\textsuperscript{2+} mobilization by NO\textsuperscript{*} (Machado-Oliveira G, unpublished). Furthermore, immunostaining of human sperm with RyR1 or RyR2 antibodies localizes to the sperm neck region (Leftièvre L, unpublished) where the NO\textsuperscript{*}-sensitive store is situated.

The biological significance of NO-induced Ca\textsuperscript{2+} mobilization in human sperm is not yet clear, but a key point here may be the convergence of the actions of progesterone and NO\textsuperscript{*} on the Ca\textsuperscript{2+} store at the sperm neck (at the RyR2). Experiments on interaction of these two stimuli showed a strong synergistic effect.\textsuperscript{1} Pre-treatment with NO\textsuperscript{*} greatly enhanced Ca\textsuperscript{2+}-mobilisation induced by progesterone and caused the effects of progesterone on flagellar beating to be prolonged. Furthermore, pre-treatment of sperm with threshold doses of progesterone (100 pM, no detectable effect on [Ca\textsuperscript{2+}], in many cells) could greatly enhance the response to NO\textsuperscript{*}, though this
effect was not always observed. Since both progesterone and NO• are synthesized by the cells of the cumulus, the Ca²⁺ store at the sperm neck (through the RyR) can act as a coincidence detector in sperm approaching the oocyte. Simultaneous Ca²⁺ influx (caused by progesterone) and S-nitrosylation of key residues can act synergistically to cause Ca²⁺ mobilization in the sperm neck and transition of flagellar activity to a form required for penetration of the egg vestments (Fig. 1E). Since the effects of S-nitrosylation on sperm [Ca²⁺]i are rapidly reversed, other sources of NO and progesterone lower in the female tract may also be important, perhaps contributing to regulation of motility during penetration of mucus in the cervix or release of bound cells from the isthmic surface (Fig. 1A). Coincidence detection is known to be crucial in controlling the ‘switching’ of cellular activities (e.g., the role of the NMDA receptor in synaptic plasticity). Coincidence detection at the Ca²⁺ store in the sperm neck, involving nitrosylation of key protein thiols, may play a key role in regulating transition in the motility of mammalian sperm. Where sperm are concerned, NO• means yes!

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