Chapter 8

Nitric Oxide: Key Features in Spermatozoa

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http://dx.doi.org/10.5772/67551

Abstract

Several *in vitro* studies have pointed to the importance of nitric oxide (NO) in the female and male reproductive system in mammals. Its functions vary from preventing oocyte aging, improving the integrity of the microtubular spindle apparatus in aged oocytes, modulating the contraction of the oviduct, to regulating sperm physiology by affecting the motility, inducing chemotaxis in spermatozoa, regulating tyrosine phosphorylation, enhancing the sperm-zona pellucida binding ability, and modulating the acrosomal reaction. In spermatozoa, NO exerts its functions in different ways, which involve key elements such as the soluble isoform of guanylate cyclase, cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), adenylate cyclase, and the extracellular signal-regulated kinase (ERK) pathway. Furthermore, NO leads to the S-nitrosylation of several sperm proteins, among them a substantial group associated with energy generation and cell movement, but also with signal transduction, suggesting a role for S-nitrosylation in sperm motility and in modulating the sperm function, respectively. In this chapter, an overview of how NO modulates the sperm physiology is presented, based on the knowledge acquired to this day.

Keywords: nitric oxide, nitric oxide synthase, S-nitrosylation, spermatozoa, fertilization

1. Introduction

NO is a small hydrophobic molecule which can easily diffuse through biological membranes [1]. *In vivo*, it is synthesized during the conversion of L-arginine to L-citrulline by nitric oxide synthase, with the help of co-factors such as the reduced form of nicotinamide adenine dinucleotide phosphate, flavin mononucleotide, flavin adenine dinucleotide, and tetrahydrobiopterin [2].

The nitric oxide synthase (NOS) may be found in three different isoforms. Two of them, the endothelial and the neuronal NOS (eNOS, nNOS), require calcium/calmodulin to be activated and are responsible for the continuous basal release of NO. The third isoform, known as inducible NOS (iNOS), is calcium independent [3, 4]. Since NOS activity depends on the availability of its substrate and its co-factors, all these elements jointly determine the cellular rates of NO synthesis [5].

Substantial evidence indicates that NO is a crucial biological messenger involved in a wide variety of physiological and pathological processes in different systems in mammals, including the vascular, nervous, and reproductive system [1, 6].

2. NOS/NO duo in the reproductive system

The NOS/NO duo regulates key functions in both the female and male reproductive systems [6].

All three isoforms of NOS have been identified in the oviduct [7, 8], oocytes, and cumulus and corona cells [9, 10] of several species [7, 11, 12]. The expression of NOS isoforms differs during the estrous cycle in the follicles as well as in the oviduct [13]. Tao *et al*. [10] showed that the immunoreactivity of eNOS in early antral follicles was restricted to the oocyte and increased from small and medium to large follicle-enclosed oocytes. In contrast, no immunoreactivity for iNOS was found in primordial, early antral follicle, or the cumulus-oocyte complexes aspirated from small and medium follicles.

In the oviduct, the endogenous basal release of NO regulates its contraction and the ciliary beating of the ciliated epithelial cells and induces chemotaxis in human spermatozoa via activation of the nitric oxide/soluble isoform of guanylate cyclase/cyclic guanosine monophosphate pathway (NO/sGC/cGMP) [14–16]. NO forms a vital component of the oocyte microenvironment and has been positively implicated in meiotic resumption [17], in preventing oocyte aging and improving the integrity of the microtubular spindle apparatus in aged oocytes [18]. It may also contribute as an anti-platelet agent during implantation [19].

As far as the male gamete is concerned, research was first concentrated on determining the effects of NO-releasing compounds on sperm motility and viability. Low concentrations of sodium nitroprusside (SNP), an NO-releasing compound, stimulated sperm hyperactivation in mouse, fish, and hamster [20–22] and were beneficial to the maintenance of post-thaw human sperm motility [23]. On the other hand, high concentrations of NO-releasing compounds decreased sperm motility [20, 24–26].

Numerous studies have also been conducted to determine the presence and localization of NOS in sperm from several species (**Table 1**). For example, Herrero *et al*. [27] located nNOS in the head of freshly ejaculated human spermatozoa, with a more concentrated fluorescent staining toward the equatorial region. O'Bryan *et al*. [28] described the pattern of eNOS expression in human spermatozoa, finding that morphologically normal spermatozoa exhibited post-acrosomal and equatorial eNOS immunostaining. Interestingly, though, abnormally shaped sperm cells exhibited aberrant staining, especially in the midpiece and/or head region, which correlated negatively with the percentage of motile sperm.

Species	Authors	Techniques	Identified isoforms
Human	Herrero *et al*. [27]	Immunofluorescence	nNOS
	O'Bryan *et al*. [28]	Immunocytochemistry	eNOS
Mouse	Herrero *et al*. [29]	Kinetic assays measuring the conversion of L-arginine to L-citrulline	nNOS, iNOS, eNOS
		Western blot	
Bull	Meiser and Schulz [30]	Modified griess reaction [92]	nNOS, eNOS
		Western blot	
		Immunofluorescence	
Boar	Hou [31]	NO assay kit with the Griess reagent	nNOS, iNOS, eNOS
	Aquila *et al*. [32]	Western blot	
Stallion	Ortega Ferrusola *et al*. [33]	Flow cytometry	nNOS, eNOS
		Western blot	
Cat	Liman and Alan [34]	Histochemistry	nNOS, iNOS, eNOS
		Immunohistochemistry	
		Western blot	

Table 1. Summary of *in vitro* studies and the techniques used to identify NOS isoforms in different species.

NOSs were revealed in mature mouse spermatozoa by means of biochemical techniques and Western blot. Herrero *et al*. [29] showed that mouse spermatozoa can synthesize L-citrulline, depending on the concentration of L-arginine present in the incubation medium while different concentrations of N(G)-nitro-L-arginine methyl ester (L-NAME) inhibit the formation of the amino acid. Furthermore, when sperm protein extracts were incubated under denaturing and nonreducing conditions and then subjected to immunoblotting assay, a protein fraction of 140 kDa was recognized by the three anti-NOS antibodies.

Bull spermatozoa were examined for the presence of constitutive NOS [30]. NO generation seemed to be enhanced by L-arginine and abolished by the NOS-inhibitor, L-NAME. In addition, Meiser and Schulz [30] verified the presence of NOS in bull sperm cells by immunohistochemistry, which was confirmed by Western blot. Confocal laser microscopy localized nNOS-related immunofluorescence at the acrosome cap and the main part of the flagellum. The same technique also identified eNOS staining spread over the spermatozoan head. Moreover, when these findings were confirmed by Western blot, immunoreactive bands at 161 kDa (nNOS) and 133 kDa (eNOS) were identified.

Hou *et al*. [31] investigated whether boar sperm can generate NO, finding that porcine spermatozoa synthesized low levels of NO under noncapacitating conditions, but that the NO concentration almost doubled when sperms were capacitated. Furthermore, NO production

was significantly inhibited when capacitated sperms were treated with L-NAME. In another study [32], Western blot analysis was performed to identify NOS enzymes in boar sperm samples. The immunoblots showed three distinct bands: ~160, ~130, and ~135 kDa, corresponding to nNOS, iNOS, and eNOS, respectively.

NO production was evaluated in stallion spermatozoa before and after freezing/thawing [33] by means of flow cytometry, after loading the sperm suspension with an NO detection probe. NO synthesis was positively correlated with sperm motility after thawing and, interestingly, the presence of egg yolk in the semen extender radically reduced the amount of NO produced. The authors further investigated in fresh and frozen/thawed stallion sperm the presence of NOS enzymes by Western blot, using anti-nNOS, anti-eNOS, and anti-universal NOS antibodies. Two bands of approximately 83 kDa and 96 kDa were labeled by the antibodies anti-nNOS and anti-eNOS, respectively. Moreover, the other antibody, which recognized an epitope present in all the NOS isoforms described so far, showed two similar bands of 84 and 92 kDa.

Recently, Liman and Alan [34] investigated the localization of NOS isoforms in spermatozoa within the intratesticular and excurrent duct systems of adult domestic cats. Overall, the spermatozoa head did not exhibit immunoreactivity. On the other hand, immunoreactivity for all three isoforms was observed in the flagellum, in the proximal cytoplasmic droplets of spermatozoa (located in the neck region) within the lumen of the intratesticular and efferent ducts, in the epididymal duct of the caput epididymis, and in the distal cytoplasmic droplets of spermatozoa (located at the mid-principal piece junction of the tail) within the lumen of corpus and cauda epididymis and the vas deferens.

3. Role of NO on sperm functionality

Several *in vitro* studies were conducted in order to determine the effects that NO has on sperm physiology (**Figure 1**). It has been shown that NO affects sperm motility [28, 35, 36], acts as chemoattractant [16, 37], regulates the tyrosine phosphorylation of different sperm proteins [38, 39], enhances the sperm-zona pellucida binding ability [40], and modulates the acrosomal reaction [41, 42].

In detail, NO seems to play an important role in the maintenance of sperm motility at physiological levels. A study [36] showed that the basal release of NO by spermatozoa from normozoospermic samples tended to be greater than that from asthenozoospermic samples, suggesting a physiological and beneficial role for endogenous NO in the preservation of sperm motility. These observations agree with a previous report that normozoospermic spermatozoa express more NOS and generate more nitrite than asthenozoospermic spermatozoa [35]. On the other hand, as previously mentioned, it has been shown that spermatozoa with an abnormal morphology show aberrant staining for eNOS, which was negatively correlated with the motility [28]. A detrimental effect on motility has also been reported by Rosselli *et al*. [24] and Weinberg *et al*. [25] when millimolar concentrations of exogenous NO donors were added to sperm samples.

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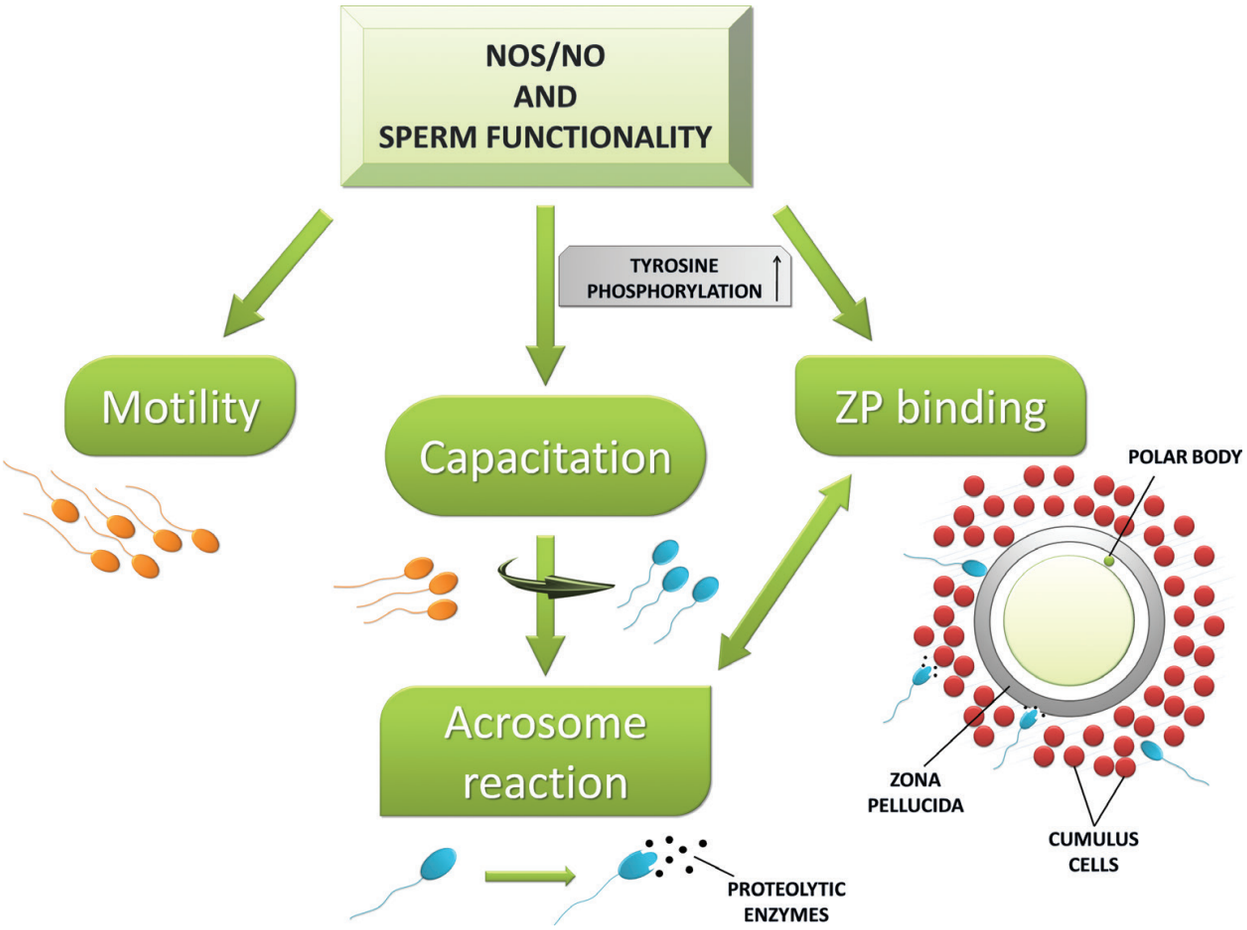

Figure 1. Some aspects of the sperm physiology modulated by the NOS/NO system. At physiological levels, endogenous NO has a beneficial role in maintaining sperm motility, enhances tyrosine phosphorylation, which, in turn, promotes the capacitation process. NO also increases the sperm-zona pellucida binding ability and leads to a rise in the percentage of reacted spermatozoa, especially in the presence of follicular fluid or protein-enriched extracts of follicular fluid.

It has been suggested that, upon approaching and entering the cumulus oophorus, both NO and progesterone, which are synthesized by the cumulus cells [9, 10, 43–45], provide a synergistic stimulus to mobilize stored calcium in the sperm neck/midpiece [46]. As a consequence, they can modulate flagellar activity and contribute to the hyperactivation that is vital for penetration of the oocyte vestments [47].

Interestingly, it has also been suggested that NO may exert a chemoattractant effect on spermatozoa. In fact, the percentage of mouse sperm migrating toward the medium containing an NO donor increased significantly [37]. Similar results were obtained when human spermatozoa were exposed to an NO donor [16]. In the latter case, the signal transduction pathway was also studied. It was proposed that NO exerts its chemoattractant effect through the activation of the NO/sGC/cGMP pathway, since the use of an NO scavenger and/or an sGC and cGMP-dependent protein kinase inhibitor reverted the NO donor-induced migration of sperm.

Since tyrosine phosphorylation in different sperm proteins is associated with the capacitation process [48], this aspect was investigated in order to further define the involvement of

NO in capacitation. Herrero *et al*. [38] observed an increase in tyrosine phosphorylation when human sperm capacitation was accelerated by an NO-releasing compound. On the other hand, when sperm capacitation was inhibited by L-NAME, there was an attenuation in the tyrosine phosphorylation of sperm proteins. In addition, Thundathil *et al*. [39] reported that L-NAME prevented, and a NO donor promoted, the increase in threonine, glutamine, and tyrosine phosphorylation in human spermatozoa. Furthermore, the addition of L-arginine reversed the inhibitory effect of L-NAME on the capacitation and the associated increase in phosphorylation.

The correlation between NO and sperm-zona pellucida binding ability was investigated by Sengoku *et al*. [40], who reported that when treated with low concentrations of a NO donor, the number of spermatozoa which binds to the hemizona is higher than in sperm treated with a higher concentration. Additionally, a NO quencher lowered the enhancement of sperm binding by the NO donor.

NO also seems to modulate the acrosome reaction. The percentage of acrosome loss induced by human follicular fluid or by calcium ionophore was studied when human spermatozoa were capacitated in the presence/absence of NO-releasing compounds or NOS inhibitors [38]. NO donors induced sperm cells to respond faster to human follicular fluid, whereas NOS inhibitors decreased the percentage of acrosome reaction. Similar results were obtained by Revelli *et al*. [41], who showed that different NO-releasing compounds were able to increase the percentage of reacted spermatozoa in the presence of protein-enriched extracts of human follicular fluid. Also, hemoglobin, a NO scavenger, inhibited the follicular fluid–induced acrosomal reaction. In an in-depth analysis of the signaling pathway of the nitric oxide–induced acrosome reaction in human spermatozoa [42], the authors suggested that the acrosome reaction-inducing effect of exogenous NO on capacitated human spermatozoa is accomplished via the NO/sGC/cGMP pathway, which leads to the activation of cGMP-dependent protein kinase (PKG). In fact, both the intracellular cGMP levels and the percentage of reacted spermatozoa were significantly increased after incubation with SNP. Furthermore, the SNP-induced acrosome reaction was significantly reduced in the presence of sGC inhibitors, a reduction that was reversed by the addition of a cell-permeating cGMP analogue to the incubation medium. Finally, PKG inhibition reduced the SNP-induced acrosome reaction.

4. NOS-activating molecules

As previously stated, NOS activity depends on the availability of its substrate and co-factors [5]. However, the scientific literature does not include many studies on the molecules present in the female reproductive tract which may activate, in one way or another, NOS enzymes in spermatozoa.

Starting from follicular fluid samples, Revelli *et al*. [41] obtained a protein-enriched follicular fluid solution (PFF), which was then used to study its effects on NOS activity, citrulline synthesis, and acrosome reaction in human sperm. Interestingly, this study showed for the first time that the endogenous NOS activity of human sperm may be increased by PFF. Moreover,

the authors demonstrated that PFF-mediated induction of sperm NOS activity leads to acrosome reaction in the same cells, thereby, establishing a link between follicle-derived substances, the activation of NO synthesis in sperm and biological responses.

Furthermore, the increase in NO synthesis mediated by PFF was not associated with a rise in the expression of NOS catalytic units, which is not surprising since specialized cells possess very poor, if any, transcriptional activity [41]. The authors hypothesized that PFF first determines the transient enzyme activation of sperm NOS, which is subsequently strengthened by a more stable modification of the enzyme.

However, more studies should be performed in order to identify the NOS-activating molecule(s) in the follicular fluid.

5. NO pathway in spermatozoa

In spermatozoa, NO acts via three main pathways (**Figure 2**) [13]. First, NO is able to activate sGC, leading to a rise in the intracellular levels of cGMP [49]. The latter activates the cyclic nucleotide-gated channels (CNG) localized in the flagellum of mammalian spermatozoa [50, 51]. These channels seem to play an important role in the sperm motility control, by allowing the entry of Ca^{2+} ions to the cytoplasm during the capacitation process of mammal sperm [50]. Their activation is one of the first events that occur during capacitation in the mouse spermatozoa [52]. cGMP also activates PKG [53, 54], which is involved in the serine/threonine phosphorylation of proteins that promote sperm capacitation and the acrosome reaction [55, 56]. Furthermore, since cGMP and cAMP compete for the catalytic sites of phosphodiesterases [57, 58], an increase in the intracytoplasmic cGMP concentration may inhibit cAMP degradation via cyclic nucleotide phosphodiesterase type 3 [59], thus increasing cAMP intracellular levels and activating protein kinase A (PKA). The latter leads to an increase in protein tyrosine phosphorylation [60].

Second, NO is directly involved in tyrosine phosphorylation by modulating the cAMP/PKA and the extracellular signal-regulated kinase (ERK) pathways. The cAMP/PKA pathway can be influenced by NO via activation of sGC (as described above), but it can also be regulated directly. In fact, S-nitrosylation of adenylate cyclase (AC) has been suggested as a possible mechanism of action of NO [61]. Low levels of NO may activate AC, consequently increasing the cAMP concentration and activating PKA [62]. However, high levels of NO can inhibit AC [61]. As far as the ERK pathway is concerned, NO reacts with the cysteine residues of the RAS protein, inducing its activation [35]. In turn, RAS triggers the RAF, MEK, and ERK1/2 complex, necessary for tyrosine phosphorylation [63].

Third, NO regulates the post-translational protein modification in spermatozoa via S-nitrosylation [64], a process similar to phosphorylation and acetylation [65, 66]. S-nitrosylation consists of the covalent incorporation of NO into thiol groups (-SH) to form S-nitrosothiols (S-NO), a modification that is selective and reversible [13].

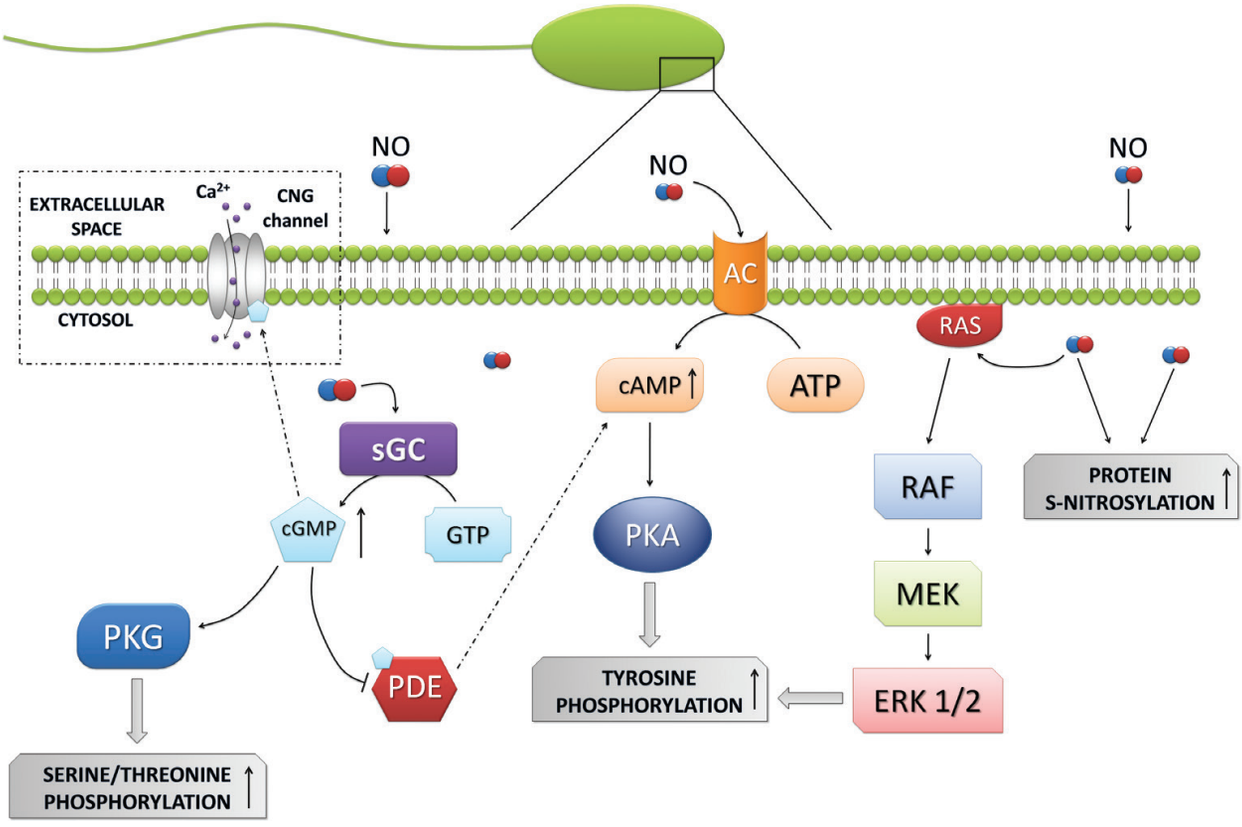

Figure 2. Representation of the main pathways through which NO acts in spermatozoa. NO leads to an increase in the intracellular levels of cyclic guanosine monophosphate (cGMP) by activating the soluble isoform of guanylate cyclase (sGC). The cGMP can activate the cyclic nucleotide-gated channels (CNG) localized in the flagellum of mammalian spermatozoa, which regulate the influx of Ca^{2+} ions to the cytoplasm during the capacitation process and also activates the cGMP-dependent protein kinase (PKG), leading to the serine/threonine phosphorylation of different proteins. It can also inhibit cyclic adenosine monophosphate (cAMP) degradation via cyclic nucleotide phosphodiesterase (PDE), which leads to the activation of cAMP-dependent protein kinase A (PKA) and tyrosine phosphorylation. Furthermore, NO is involved in the tyrosine phosphorylation process in a direct manner, by activating adenylate cyclase (AC) and the extracellular signal-regulated kinase (ERK) pathway. Finally, NO determines post-translational protein modification in spermatozoa via S-nitrosylation.

6. Function of S-nitrosoproteins in spermatozoa

An extensive study by Lefièvre *et al*. [64] described a large number of proteins present in the sperm of normozoospermic men, which can be subjected to S-nitrosylation in the presence of NO donors. Although the function of some nitrosylated proteins remains to be discovered, a considerable group of them are known to be metabolic proteins and proteins associated with energy generation and cell movement, suggesting a role for S-nitrosylation in sperm motility. This agrees with a previous proteomic analysis [67], in which the most abundant group was also involved in energy production.

Other considerable groups of proteins were those involved in signal transduction, which agrees with a role for S-nitrosylation in modulating the sperm function [64]. Interestingly, since sperms are generally assumed to be transcriptionally inactive, a small percentage of the

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S-nitrosylated proteins identified by Lefièvre *et al*. [64] was related to transcription. Previous proteomic studies in sperm also observed the presence of proteins involved in transcription [67, 68]. However, when comparing the human sperm S-nitrosoproteome with proteins identified during a proteomic study of sperm-oocyte interaction, only three proteins were found in common, suggesting that S-nitrosylation is not a regulatory mechanism employed during fertilization [64, 69].

It is known that the mobilization of Ca^{2+} stored in the sperm neck/midpiece is necessary for the hyperactivation process [46]. The Ca^{2+} store in the neck of the sperm coincides with the region occupied by the redundant nuclear envelope (RNE) [70] and in order to mobilize Ca^{2+} from this site, ryanodine receptors (RyRs), which are intracellular Ca^{2+}-release channels involved in regulation of cytosolic calcium levels [71], need to be activated. These proteins contain a large number of thiol groups and are thus prone to S-nitrosylation by NO [64, 72, 73]. S-nitrosylation can potentiate the opening of RyRs [74–79], probably through the generation of the membrane permanent product S-nitrosocysteine [80]. It has been shown that an increase in Ca^{2+} induced by NO is accompanied by an increase in S-nitrosylation levels of endogenous RyRs [81, 82] while these Ca^{2+} channels may be inhibited under strongly nitrosylating conditions or at high doses of NO (**Figure 3**) [76, 79, 82]. Furthermore, progesterone acts synergistically with NO to mobilize Ca^{2+} in the sperm neck/midpiece by activation of RyRs [47], contributing to the hyperactivation process.

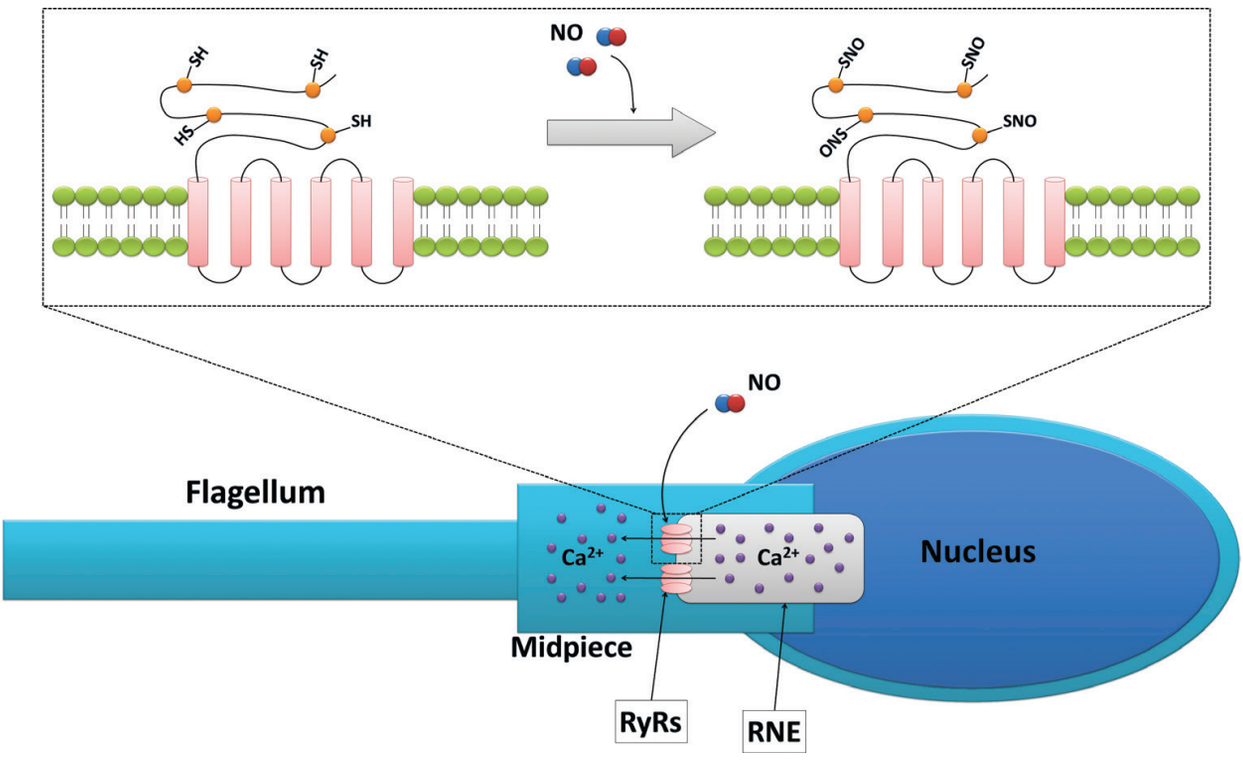

Figure 3. S-nitrosylation process. NO acts on the thiol groups (-SH) of the cysteines in proteins to form S-nitrosothiols (S-NO). At the sperm neck/midpiece, the S-nitrosylation occurs in ryanodine receptors (RyRs) allowing the release of calcium from the redundant nuclear envelope (RNE), which is required for sperm hyperactivation. Adapted and modified from López-Úbeda and Matás [13].

Other examples of proteins which can undergo S-nitrosylation in sperm and have a known biological significance are the A-kinase anchoring proteins (AKAPs) [64]. Both AKAP3 and AKAP4 are present in the fibrous sheath of the sperm flagellum, control PKA activity and undergo phosphorylation during the capacitation process [83–85]. AKAP complexes also modulate the motility of sperm. In fact, phosphodiesterase inhibitors were seen to significantly increase sperm motility [86], whereas PKA-anchoring inhibitor peptides arrested sperm motility [87]. Since the effects of NO on sperm motility are well established, the S-nitrosylation of AKAPs would be an interesting subject for additional studies.

A number of heat shock proteins (HSPs) may also be targets of S-nitrosylation in sperm [64], and some of them have been reported to act as important modulators of sperm capacitation. For instance, Asquith *et al*. [88] reported that heat shock protein 1 and endoplasmin undergo tyrosine phosphorylation during mouse sperm capacitation, whereas Nixon *et al*. [89] suggested that they form part of a zona pellucida complex, allowing successful sperm-egg interaction in the same species. Heat shock 70 kDa protein 8 and heat shock protein 90α also undergo tyrosine phosphorylation during human sperm capacitation [83], but whether they function in a zona receptor complex is still unknown [64]. Furthermore, HspA2 has been shown to be a marker of sperm maturity [90], its expression in infertile men with idiopathic oligoteratozoospermia being lower than in normozoospermic men [91].

7. Concluding remarks

In recent years, our knowledge of the involvement of the NOS/NO system in mammalian fertilization has grown, and there is clear evidence that NO acts as a significant modulator of the male and female gamete. However, many aspects regarding the NOS/NO duo, such as the presence of NOS-activating molecule(s) in the fertilization site or how the biological function of the S-nitrosylated proteins changes, remain to be discovered. Shedding light on these mechanisms will increase our understanding of the etiopathology of subfertility/infertility problems and how such problems can be overcome.

Acknowledgements

This work was supported by H2020 MSC-ITN-EJD 675526 REP-BIOTECH, the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (FEDER), Grants AGL2015–66341-R.

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