

## REVIEW

# New insights into male (in)fertility: the importance of NO

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Infertility is a global problem that is on the rise, especially during the last decade. Currently, infertility affects approximately 10–15% of the population worldwide. The frequency and origin of different forms of infertility varies. It has been shown that reactive oxygen and nitrogen species (ROS and RNS) are involved in the aetiology of infertility, especially male infertility. Various strategies have been designed to remove or decrease the production of ROS and RNS in spermatozoa, in particular during *in vitro* fertilization. However, in recent years it has been shown that spermatozoa naturally produce a variety of ROS/RNS, including superoxide anion radical ( $O_2^{\cdot-}$ ), hydrogen peroxide and NO. These reactive species, in particular NO, are essential in regulating sperm capacitation and the acrosome reaction, two processes that need to be acquired by sperm in order to achieve fertilization potential. In addition, it has recently been shown that mitochondrial function is positively correlated with human sperm fertilization potential and quality and that NO and NO precursors increase sperm motility by increasing energy production in mitochondria. We will review the new link between sperm NO-driven redox regulation and infertility herein. A special emphasis will be placed on the potential implementation of new redox-active substances that modulate the content of NO in spermatozoa to increase fertility and promote conception.

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### Abbreviations

AR, acrosome reaction; EDRF, endothelial-derived relaxing factor; eNOS, endothelial NOS; ETC, electron transport chain; iNOS, inducible NOS; IVF, *in vitro* fertilization; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; MMP, mitochondrial membrane potential; mtNOS, mitochondrial NOS; NG, nitroglycerine; nNOS, neuronal NOS;  $O_2^{\cdot-}$ , superoxide anion radical; RNS, reactive nitrogen species; ROS, reactive oxygen species; sGC, soluble GC; SNP, sodium nitroprusside; SOD, superoxide dismutase; ZP, zona pellucida

### Introduction

Infertility is one of the most serious medical problems worldwide. The prevalence of infertility is on the rise, especially

during the last decade. Globally, one of six to seven couples worldwide currently has some difficulty with conception (Sharma *et al.*, 2013). Infertility is usually defined as the inability to conceive after 1 year of regular unprotected

intercourse, but in couples in which the female partner is >35 years of age, infertility is diagnosed after an inability to conceive for 6 months (Cooper *et al.*, 2010). Although the frequency and origin of infertility varies, it has been established that nearly 40% of the issues involved with infertility are attributable to a male factor, another 40% due to a female factor, and 20% result from combined male and female factors (Sharlip *et al.*, 2002).

Over the past decade, significant advances have occurred in the diagnosis and treatment of reproductive disorders. However, some of causative factors underlying infertility can only be successfully overcome with the use of assisted reproductive technologies. Various techniques, such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection, intrauterine insemination using a partner or donor's semen, frozen embryo replacement and egg donation, have been developed to treat infertility. Since 1978, when the first IVF baby was born, the techniques used in IVF have advanced dramatically; indeed, the current procedure success rate based on clinical pregnancies is 25–50%, clearly showing that advances in general and reproductive medicine occur simultaneously. However, modern lifestyle patterns, such as social and environmental factors, account for part of the increase in the prevalence of infertility. For these reasons, one of the most important biomedical directions today is the development of new therapeutic approaches for improvement in fertilization, both under natural and assisted reproduction methods.

Extensive research in infertility has confirmed that excessive production of reactive oxygen and nitrogen species (ROS and RNS), that is, oxidative stress, is involved in the aetiology of infertility, especially male infertility (MacLeod, 1943; Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992; Sukcharoen *et al.*, 1996; Agarwal and Allamaneni, 2004). All of these reports have associated ROS/RNS, generated both exogenously and endogenously, with several aspects of male infertility, such as decreased sperm motility, abnormal morphology and decreased sperm–egg penetration. The mechanism of action of ROS and RNS involves lipid peroxidation of the sperm plasma membrane, which is highly susceptible to oxidative damage due to large amounts of polyunsaturated fatty acids, by impairing membrane fluidity and mobility (Alvarez and Storey, 1995; Baker *et al.*, 1996). In addition, ROS and RNS can damage sperm axoneme, and impair mitochondrial function, DNA, RNA and proteins (de Lamirande and Gagnon, 1992; Agarwal and Prabakaran, 2005).

Thus, for many years, ROS and RNS have been considered as harmful and various strategies were designed to remove or decrease their production, especially during IVF, when sperm is exposed to higher concentrations of oxygen than *in vivo*. Some of the strategies used include supplementation of culture media with antioxidants (vitamin E and disulphide-reducing agents), removal of ROS-producing cells (mostly leukocytes and damaged spermatozoa) by density gradient for sperm preparation, and incubation under low O<sub>2</sub> tension and illumination (de Lamirande and Gagnon, 1992; Aitken *et al.*, 1996; Irvine, 1996; Parinaud *et al.*, 1997). Nevertheless, in contrast to the involvement of ROS and RNS in male infertility, supplemental intake of antioxidants in humans has been shown to improve reproductive functions only in select cases (Dawson *et al.*, 1992; Kessopoulou *et al.*, 1995).

Moreover, some of the studies have reported no changes in semen parameters or male fertility after prolonged oral administration of antioxidants (Hughes *et al.*, 1998; Rolf *et al.*, 1999). These data have given rise to a concern and debate regarding the effectiveness of oral administration of antioxidants as therapy for male infertility.

The basis for a 'failure' of antioxidant therapy in the treatment of male infertility is that ROS and RNS in small, controlled, physiological concentrations are needed for normal sperm functioning. Thus, ROS/RNS must be maintained at appropriate levels to ensure a physiological function. Specifically, it has been shown that as a product of normal metabolism in germ cells – spermatozoa and oocytes – small amounts of ROS and RNS were produced, and these reactive species, when produced in controlled, physiological concentrations, function as secondary messengers that regulate crucial reproductive processes, especially in sperm cells (de Lamirande *et al.*, 1997; Leclerc *et al.*, 1997). Specifically, it has been shown that NO and superoxide anion radical (O<sub>2</sub><sup>•−</sup>) are essential in regulating sperm capacitation and acrosome reaction (AR), two processes required for sperm to acquire fertilization potential (de Lamirande *et al.*, 1997; Leclerc *et al.*, 1997). It has also been shown that ROS and RNS stimulate sperm-hyperactivated motility (Suzuki *et al.*, 1997) and binding to the zona pellucida (ZP) (Aitken *et al.*, 1989), emphasizing that essential sperm fertilization events are redox sensitive.

It is important to note that the major production site and target for ROS and RNS are the mitochondria. Consequently, by production of ROS/RNS and subsequent activation of various redox-dependent signalling pathways, mitochondria are involved in the regulation of sperm functions. In addition, it has been shown that through ATP production, mitochondria regulate spermatogenesis, differentiation, the AR, oocyte fusion and fertilization (Agarwal *et al.*, 2008). Furthermore, through Ca<sup>2+</sup> signalling, mitochondria regulate sperm hyperactivation, flagellar activity during events leading up to fertilization and energy metabolism (Publicover *et al.*, 2008). The importance of mitochondria in sperm functioning was strengthened with recent evidence showing that mitochondrial function is positively correlated with human sperm fertilization potential and quality (Kasai *et al.*, 2002; Marchetti *et al.*, 2004; Gallon *et al.*, 2006; Otasevic *et al.*, 2013). In contrast, mitochondria are the main site for ROS and RNS targeting. In agreement with this observation, multiple regulatory effects of NO on mitochondria functioning have become evident during the last decade (Nisoli *et al.*, 2003; Petrović *et al.*, 2005; 2008; 2010a,b; Vucetic *et al.*, 2011). In our recent work, we have shown that NO achieves beneficial effects on sperm function through improvement of functional status of sperm mitochondria (Otasevic *et al.*, 2013). For these reasons, involvement of NO on sperm mitochondria and sperm function warrants more consideration, and is discussed in more detail in this review.

The current review has addressed infertility from the aspect of redox regulation. We focused on male (in)fertility and NO-mediated redox regulation of crucial sperm-fertilizing events. A special emphasis was placed on the potential implementation of new redox-active substances that through modulation of the content of NO in spermatozoa could increase male fertility and promote conception.

## NO as a signalling molecule: a historical overview

NO represents a simple diatomic gas that functions as a cell signalling molecule in mammalian cells, controlling fundamental functions in the cardiovascular system, brain, host defence, immunity, reproductive system, gastrointestinal system and secretory tissues (Alderton *et al.*, 2001; Govers and Oess, 2004). The first description of NO was linked to the discovery of nitroglycerine (NG) in 1847 by the Italian scientist Sobrero (Marsh and Marsh, 2000). A few years later, NG was used to treat headaches, while since 1870 it has been used in the treatment of angina pectoris. A century after, it was shown that NG activates soluble GC (sGC), as well as increasing the production of cGMP. The largest contribution to the discovery of the effects of NO was the discovery of endothelial-derived relaxing factor (EDRF) by Furchgott and Zawadzki (1980). Seven years later, Ignarro *et al.* (1987a,b) showed that EDRF is actually NO, and a year later Moncada's group (Palmer *et al.*, 1988) reported that mammalian cells endogenously synthesize NO from L-arginine. The importance of this discovery was demonstrated by the naming of NO as the 'molecule of the year' by the editor of *Science* in 1992. In 1998, the scientists who were involved in the discovery of NO and defined the role of NO in the cardiovascular system were awarded the Nobel Prize in Medicine.

## NO synthesis

NO is synthesized in virtually all mammalian cells via L-arginine oxidation by a family of NOS isoforms (Moncada *et al.*, 1989). Three distinct NOS isoforms have been identified thus far (see Alexander *et al.*, 2013), as follows: neuronal (nNOS), which was originally described in neuronal tissue; endothelial (eNOS), which was originally described in endothelial cells; and inducible NOS (iNOS), which was originally identified in macrophages (Moncada *et al.*, 1991; Alderton *et al.*, 2001). All mammalian NOS isoforms characterized are haem-containing proteins that are dimeric in native conditions with a monomeric molecular mass of 126–160 kDa. NOS isoforms display different affinities for calmodulin, whereas nNOS and eNOS are Ca<sup>2+</sup>-calmodulin dependent, and iNOS is nearly Ca<sup>2+</sup>-calmodulin independent. Another dissimilarity between the NOS isoforms is related to the level of NO production because iNOS has been shown to produce higher NO levels (μM–mM) and to remain active for a longer period of time compared with nNOS and eNOS (NO concentrations from nM–μM) (Moncada *et al.*, 1991; Alderton *et al.*, 2001; Buzadzic *et al.*, 2006; Otasevic *et al.*, 2011). All NOS isoforms may be found attached to or within the mitochondria, in which case NO synthase is referred to as the fourth isoform mitochondrial NOS (mtNOS) (Kobzik *et al.*, 1995; Bates *et al.*, 1996; Ghafourifar and Richter, 1999). However, the existence of mtNOS is controversial and the full characterization of mtNOS is still in progress.

## Mechanisms by which NO acts

In general, NO has been shown to exhibit its effects through two different signalling pathways as follows: cGMP-dependent signalling and cGMP-independent signalling (classical and non-classical signalling respectively) (Martínez-Ruiz *et al.*, 2011; Otasevic *et al.*, 2011). Classical signalling involves activation of sGC, generation of cGMP, and the

subsequent activation of specific cGMP-dependent enzymes (protein kinases, channels and phosphodiesterases). The sensitivity of sGC to NO is within the nanomolar range (Martínez-Ruiz *et al.*, 2011). Non-classical signalling occurs through covalent post-translational modification of target proteins, that is, S-nitrosylation, S-glutathionylation and tyrosine nitration (Mieyal *et al.*, 2008; Souza *et al.*, 2008; Lima *et al.*, 2010). This non-classical signalling is governed by NO concentrations higher than needed for activation of sGC. S-nitrosylation of the proteins and thiols is mediated not only by NO, but also by other NO species, metal-NO complexes, peroxynitrite or nitrite (Otasevic *et al.*, 2011). Almost all classes of proteins, including receptors, enzymes and transcriptional factors, are targets of S-nitrosylation. In contrast, protein modifications by tyrosine nitration, which are mainly mediated by peroxynitrite, potentially result in alteration, loss or gain of function, but reversibility has not been demonstrated, and hence a clear assignment to redox regulation cannot be made.

It is important to highlight specifically NO acting in a cGMP-independent manner, in which NO interacts with other haemoproteins, such as cytochrome *c* oxidase (Cleeter *et al.*, 1994). This cGMP-independent pathway is a less classic but equally important pathway for NO, which might have important implications for cell respiration and metabolism (Brookes *et al.*, 2002; Erusalimsky and Moncada, 2007; Vucetic *et al.*, 2011).

## NOS isoforms in spermatozoa

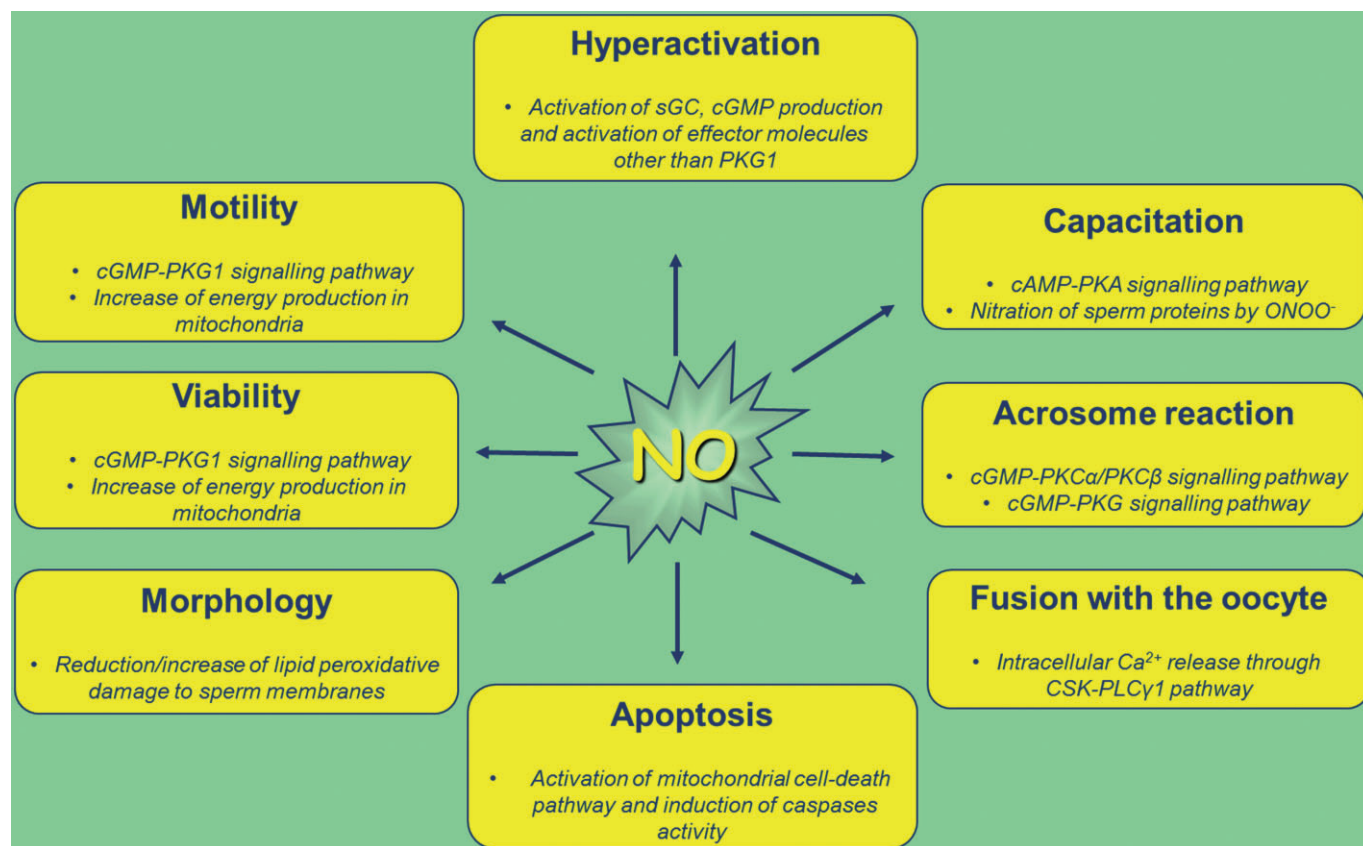
Human spermatozoa express all three isoforms of NOS, which are located in the head and/or flagellum regions of the sperm (Herrero *et al.*, 2003; de Lamirande and Lamothe, 2009; de Lamirande *et al.*, 2009). It has been shown that under capacitating conditions, the activity of NOSs increases in a time-dependent manner (de Lamirande and Lamothe, 2009; de Lamirande *et al.*, 2009). Furthermore, the presence and activity of sperm NOSs has been recently shown to depend on the maturity of male germ cells (Roessner *et al.*, 2010). The correlation between NOS activity and sperm maturity clearly underlies the physiological role played by NO in spermatozoa. It is often very difficult to define a function regulated exclusively by one specific NOS isoform. However, Table 1 summarizes the reported specific function, so far, that each NOS isoform plays in the regulation of events important for sperm functionality.

Specifically, NO is known to be involved in the regulation of sperm motility, viability, hyperactivation, capacitation, AR and fusion with the oocyte (Figure 1) (Herrero *et al.*, 1999; 2003; de Lamirande and Lamothe, 2009; de Lamirande *et al.*, 2009; Kothari *et al.*, 2010; Doshi *et al.*, 2012).

## Effects of NO on sperm-fertilizing effects

### Sperm motility, morphology and viability

Sperm motility is a crucial parameter of semen quality (Wang *et al.*, 2012). Accordingly, a primary cause of male infertility is decreased sperm motility, and men with poorly motile or immotile sperm are usually sterile (Turner, 2006). The very first report that showed a regulatory role of NO on sperm motility came from Hellstrom *et al.* (1994), who



**Figure 1**

NO regulates sperm functions. At physiological levels, NO and RNS regulate essential sperm-fertilizing events. At supra-physiological levels NO achieves detrimental effects on above shown sperm function. PLCγ1, phosphoinositide-specific phospholipase γ1.

**Table 1**

Specific function of NOS isoforms in the regulation of events important for sperm functionality

Isoform	Localization (Lee and Cheng, 2008)	Function
eNOS	Germ cells, Sertoli cells, Leydig cells, myofibroblasts, endothelial cells in blood vessels, head and midpiece of the spermatozoon	Control of tight junction in the testis, germ cell apoptosis, sperm motility and follicular fluid-induced acrosome reaction, spermatogenesis, regulating penile erection (Burnett <i>et al.</i> , 1996; Zini <i>et al.</i> , 1996; 1998;1999; O'Bryan <i>et al.</i> , 1998; Revelli <i>et al.</i> , 1999; Lee and Cheng, 2008)
nNOS	Sertoli cells, acrosome, Leydig cells, myofibroblasts, endothelial cells in blood vessels, head and midpiece of the spermatozoon	Sperm motility, regulating penile erection (de Lamirande <i>et al.</i> , 1984; Burnett <i>et al.</i> , 1996; Schaad <i>et al.</i> , 1996; Donnelly <i>et al.</i> , 1997)
iNOS	Leydig cells, Sertoli cells, germ cells, head and midpiece of the spermatozoon	Control of tight junction in the testis, germ cell apoptosis, fertilization and different aspects of sperm–egg interaction during fertilization, germ cell apoptosis and sperm number (Lue <i>et al.</i> , 2003; Yang <i>et al.</i> , 2005; Vera <i>et al.</i> , 2006; Lee and Cheng, 2008)
TnNOS	Leydig cells	Steroidogenesis (Wang <i>et al.</i> , 2002)

TnNOS, testis-specific nNOS.



determined the effects of NO-releasing compounds on sperm motility and viability. They showed (Hellstrom *et al.*, 1994) that the NO releaser sodium nitroprusside (SNP) in low concentrations (25–100 nM) is beneficial to the maintenance of post-thaw human sperm motion and viability. In contrast, several reports have shown a decrease in progressive sperm motility in the presence of high concentrations of pure NO (25–125  $\mu$ M) and SNP (0.25–2.5 mM), or S-nitroso-N-acetylpenicillamine (12–600  $\mu$ M) and 3-morpholiniosydnonimine (100–125  $\mu$ M) (Herrero *et al.*, 1994; Rosselli *et al.*, 1995; Weinberg *et al.*, 1995; Nobunaga *et al.*, 1996). It is clear that, as in other systems, NO exerts a bimodal action on sperm motility; specifically, low concentrations of NO increase sperm motility, while high concentrations of NO reduce the motility of spermatozoa (Weinberg *et al.*, 1995; Herrero and Gagnon, 2001; Miraglia *et al.*, 2011; Doshi *et al.*, 2012). Consistent with this finding, high NO levels have been reported in the semen of infertile men with decreased sperm motility (Rosselli *et al.*, 1995; Perera *et al.*, 1996; Balercia *et al.*, 2004). Moreover, it has been shown that production of nitrite by asthenozoospermic men is lower than that in normozoospermic men, indicating that a decrease in endogenous NO influences sperm motility, and consequently sperm-fertilizing potential (Lewis *et al.*, 1996; Bolanos *et al.*, 2008). Accordingly, the addition of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) to the sperm-incubating medium significantly reduces human sperm motility (Donnelly *et al.*, 1997; Yeoman *et al.*, 1998).

Miraglia *et al.* (2011) reported that NO influences sperm motility via the activation of sGC, the subsequent synthesis of cGMP and the activation of cGMP-dependent protein kinases. In addition, our recent data suggest that NO increases the motility of spermatozoa by increasing energy production originating in the mitochondrial compartment (Otasevic *et al.*, 2013). Accordingly, high levels of NO in spermatozoa detected in infertile men are linked to a decrease in sperm metabolism (Doshi *et al.*, 2012).

Moreover, NO contributes to normal sperm morphology, which has been shown to facilitate the accurate prediction of fertility status and pregnancy outcomes during IVF (Revelli *et al.*, 2001). Nevertheless, similar to sperm motility, it has been shown that in high concentrations NO induces atypical sperm morphology (Wu *et al.*, 2004). Sperm viability is also affected by NO. Specifically, researchers have reported that viability of sperm in the presence of 10<sup>−4</sup> M SNP is significantly lower than sperm without this treatment (Doshi *et al.*, 2012).

### Sperm capacitation

Motile and morphologically mature human spermatozoa are not fertile immediately after ejaculation, but rather achieve fertilizing ability during transition in the female genital tract. Sperm first undergoes a complex and timely series of biochemical and physiological changes, which is termed capacitation (Chang, 1951; Austin, 1952). In fact, capacitation is the final maturation process that ensures that only fertile spermatozoa are able to reach, bind to and penetrate the oocyte (Baker and Aitken, 2004). Membrane and metabolic changes that occur during capacitation confer upon the spermatozoa an ability to gain hyperactive motility, interact with the oocyte ZP, undergo an AR and initiate oocyte plasma membrane fusion (Baker and Aitken, 2004).

The involvement of NO in capacitation was first demonstrated in human spermatozoa incubated with low concentrations of NO-releasing compounds (Zini *et al.*, 1995). Specifically, it has been shown that NO donors (with different potencies) accelerate capacitation, while NOS inhibitors significantly decrease this process (Herrero *et al.*, 1999). These data clearly demonstrate that endogenous NO is necessary for spermatozoa to display full fertilizing ability. Furthermore, it was subsequently shown that this NO-dependent redox control appears crucial for capacitation triggered by different agents, such as albumin, progesterone and fetal cord serum ultrafiltrate, which modulate the same actions independently of the inducer (O'Flaherty *et al.*, 2006; de Lamirande and O'Flaherty, 2008).

At the molecular level, capacitation involves a cascade of events (an efflux of cholesterol, fluxes of bicarbonate, and a rise in cAMP, Ca<sup>2+</sup> and intracellular pH) that is initiated by ROS and RNS produced by spermatozoa (de Lamirande *et al.*, 1997; 2009; Herrero *et al.*, 1999; 2003; O'Flaherty *et al.*, 2006; de Lamirande and O'Flaherty, 2008; de Lamirande and Lamothe, 2009). Specifically, it has been shown that NO and O<sub>2</sub><sup>−</sup> are generated in spermatozoa from the beginning of capacitation and control the increase in cAMP and most of the known serine/threonine phosphorylation of PKA substrates, tyrosine phosphorylation of fibrous sheath proteins as well as tyrosine nitration of proteins. Moreover, it has been shown that NO is needed over the course of capacitation and more than one NOS is probably involved in this process (de Lamirande and Lamothe, 2009).

### Hyperactivation

Hyperactivation coincides with the onset of capacitation and represents a subcategory of capacitation. In non-hyperactive spermatozoa, low-amplitude flagellar movements are associated with slow, linear movements of the cell. In contrast, hyperactive spermatozoa exhibit high amplitude asymmetric flagellar movements and non-linear motility. In the female genital tract, hyperactivated spermatozoa exhibit less stagnation in the oviductal epithelium and the surrounding viscous mucus and a propulsive force to penetrate the cells of the oocyte (Miraglia *et al.*, 2007; Kothari *et al.*, 2010). Results supporting the role of NO in the initiation of hyperactivation are well accepted. Similar to the NO effects on 'regular' sperm motility, it has been shown that NO concentrations < 1  $\mu$ M increase sperm hyperactivation, while NO in concentrations > 1  $\mu$ M decreases sperm-hyperactivated motility (Lewis *et al.*, 1996; Miraglia *et al.*, 2011; Otasevic *et al.*, 2013).

### AR

When hyperactivated motility has drowned the capacitated spermatozoa to the ovum, sperm binds to the ZP and begins to bypass this barrier by the exocytotic release of proteolytic enzymes in a process referred to as the AR. The AR encompasses the release of proteolytic enzymes from the acrosome cap of the spermatozoa that create a pore in the extracellular matrix of the ZP and allows spermatozoa to bind appropriately, penetrate the barrier and fuse with the oocyte. At the molecular level, the AR shares a significant overlap with molecular events of capacitation, such as phosphorylation of similar tyrosine residues in proteins, an influx of Ca<sup>2+</sup>, activities of AC, cAMP and PKA (de Lamirande and

O'Flaherty, 2008). Similar to capacitation, reactive species, including NO, stimulate the AR by activating AC that triggers downstream target molecules to initiate the exocytotic event (Zini *et al.*, 1995).

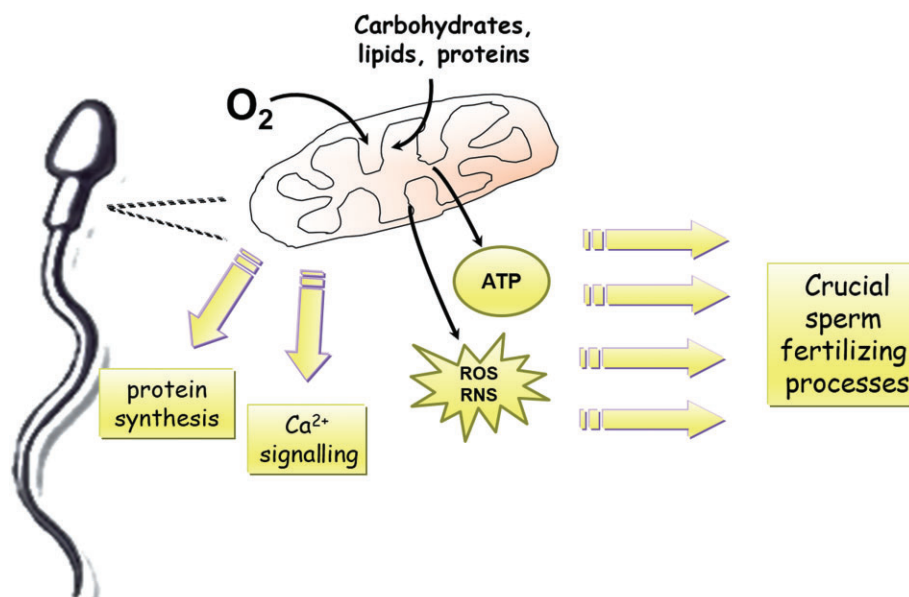
The fact that NO participates in ZP binding, as well as in the AR has been well characterized (Herrero and Gagnon, 2001). Treatment of human spermatozoa with low concentrations of SNP in the capacitation medium increases the number of spermatozoa bound to hemizona (Sengoku *et al.*, 1998), and increases sperm affinity for the ZP. In contrast, it has been demonstrated that addition of various concentrations of L-NAME to capacitated spermatozoa do not affect ZP binding, but significantly inhibit the fusion effect (Francavilla *et al.*, 2000). Similarly, NO-releasing compounds induce the AR in human spermatozoa in a concentration-dependent manner, whereas haemoglobin, as a NO scavenger, prevents the AR in spermatozoa capacitated by human follicular fluid (Revelli *et al.*, 1999).

### NO and sperm mitochondria

Mitochondria are essential in the regulation of a variety of cellular activities, including ATP synthesis, intracellular calcium homeostasis and apoptosis (Duchen, 2000). In sperm cells, mitochondria supply sperm with energy for several purposes (Figure 2), and thus play important roles in spermatogenesis, differentiation, motility, AR, oocyte fusion and fertilization (Agarwal *et al.*, 2008). In addition to the generation of ATP to supply the above sperm fertilization processes, other functions of mitochondria in sperm, such as  $\text{Ca}^{2+}$  signalling and protein synthesis, have been documented (Gur and Breitbart, 2006; Publicover *et al.*, 2008) (Figure 2). Several studies have confirmed that mitochondrial functionality

positively correlates with human sperm fertilizing potential and quality (Kasai *et al.*, 2002; Marchetti *et al.*, 2004; Gallon *et al.*, 2006; Otasevic *et al.*, 2013). The major determinants of sperm mitochondrial functionality are the activity and expression of components of the electron transport chain (ETC) (Ruiz-Pesini *et al.*, 1998; Hüttemann *et al.*, 2003) as well as mitochondrial membrane potential (MMP) (Marchetti *et al.*, 2004; Gallon *et al.*, 2006; Otasevic *et al.*, 2013). Accordingly, any disruption of mitochondrial ETC components or MMP is associated with decreased sperm motility, reduced sperm fertilization potential and male sterility (Marchetti *et al.*, 2002; 2004; Rossato, 2008; Otasevic *et al.*, 2013).

NO has been shown to affect the mitochondria on several levels, as follows: regulates mitochondrial respiration; biogenesis, remodelling and increasing  $\text{O}_2$  and substrate supply to the mitochondria (Nisoli *et al.*, 2003; Petrović *et al.*, 2005; 2008; 2010a,b; Vasiljevic *et al.*, 2010; Vucetic *et al.*, 2011). Current data show that NO can adversely influence the mitochondrial function of sperm cells depending on the NO level. A growing amount of data have shown that higher levels of NO ( $\mu\text{M}$ ) inhibit respiration in sperm mitochondria (Rosselli *et al.*, 1995; Weinberg *et al.*, 1995), while iNOS-derived high (mM) NO levels have been shown to induce mitochondrial hyperpolarization, cytochrome *c* release and sperm cell death (Mishra and Shaha, 2005). Low concentrations of NO (nM) increase sperm motility and this has been shown to be strongly associated with energy production in mitochondria (Hellstrom *et al.*, 1994). Our very recent work revealed that NO improved the functional status of sperm mitochondria and sperm quality (Otasevic *et al.*, 2013). The functional state of sperm mitochondria, as indicated by the percentage of positive MitoTracker<sup>®</sup>Green FM (Molecular Probes, Inc.,



**Figure 2**

Importance of mitochondria in regulation of sperm functions. Mitochondria supply spermatozoa with energy (ATP) for vital sperm-fertilizing processes. In addition, through production of ROS and RNS and subsequent activation of various redox-dependent signalling pathways, mitochondria are involved in the regulation of sperm functions. Other roles of mitochondria in spermatozoa, such as  $\text{Ca}^{2+}$  signalling and protein synthesis, have also been presented.

Eugene, OR, USA) sperm cells and the expression of components of ETC, was significantly higher in sperm samples with higher NO concentration. These data were positively correlated with sperm motility, a major indicator of semen quality, and clearly showed that NO improved the fertilizing potential of spermatozoa.

### Therapeutic applications

To date, the majority of trials oriented to NO-dependent improvement of sperm function have been performed with a precursor of NO synthesis, L-arginine (Scibona *et al.*, 1994; Aydin *et al.*, 1995; Patel *et al.*, 1998; Stanislavov *et al.*, 2009). In addition to L-arginine *per se*, the products of its metabolism play significant roles in the regulation of fundamental cellular processes and physiological functions (Vasilijevic *et al.*, 2007; Stancic *et al.*, 2012). However, many of the positive effects of L-arginine in male fertility disorders (improvement in the sperm count and motility in oligospermic and asthenospermic patients) have been proven to be mediated through increased biosynthesis of NO (Scibona *et al.*, 1994; Aydin *et al.*, 1995; Srivastava *et al.*, 2000). Stanislavov *et al.* (2009) reported that supplementation of subfertile men with a combination of pycnogenol and L-arginine aspartate in a double-blind, placebo-controlled, crossover study significantly increased sperm volume, concentration, motility and % normal morphology through the antioxidative effect of pycnogenol, induction of eNOS and consequent endogenous production of NO in spermatozoa.

However, important progress in L-arginine research suggests that this amino acid and its metabolites play a more complex role in the regulation of whole body energy homeostasis, and thus dietary supplementation of L-arginine must be taken with vigilance. When supplemented in appropriate physiological doses, the duration and intake frequency of L-arginine shows multiple beneficial effects; however, with supplementation in high, supra-physiological doses (Luiking

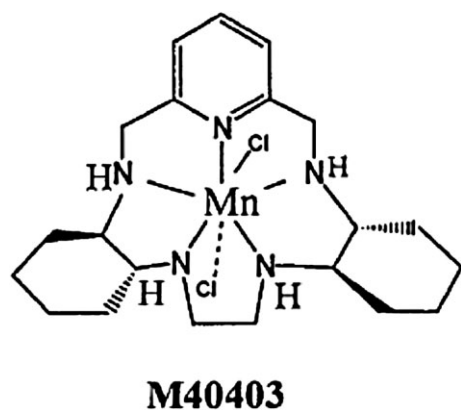
*et al.*, 1998; Grimble, 2007) and over a long-term period (Park, 1993; Tome *et al.*, 1999), L-arginine can exert some side effects (nausea, gastrointestinal discomfort, diarrhoea, and changes in electrolytes in the blood, including potassium). In addition, there are a few pathophysiological states in which L-arginine must be applied with caution, such as myocardial infarction (Schulman *et al.*, 2006), asthma (Takano *et al.*, 1998), viral infections (Tankersley, 1964), cancer (Yeatman *et al.*, 1991; Grossie, 1996), and renal and liver diseases (Tome *et al.*, 1999; Appleton, 2002). Bearing in mind all of these findings and the fact that infertility currently affects one in seven couples worldwide (NB, many experts in the field predict this could double over the next decade), it is clear that novel strategies to treat sterility must be developed (Table 2).

Accordingly, our recent data shed light on the development of new pharmacological strategies to treat male infertility using novel, safe and effective redox-active substances (Otasevic *et al.*, 2013). Due to the important implications of the redox status in male factor infertility, we examined how changes in the sperm redox milieu affect sperm functionality. To modulate the sperm cell redox state, sperm incubation medium was supplemented with the superoxide dismutase (SOD) mimic, M40403, which is representative of the Mn II pentaazamacrocyclic class of complexes (Figure 3). This is a potent low molecular mass SOD mimic with marked protective effects in inflammation, stroke, atherosclerosis and hypertension (Salvemini *et al.*, 2002; Vallance and Leiper, 2002). The main advantage of M40403 is high selectivity for O<sub>2</sub><sup>-</sup>, lack of peroxidase/catalase activity and lack of reactivity with NO. Our results have shown that M40403 restores the population of sperm with functionally active mitochondria, thus stimulating sperm mitochondrial activity and subsequent sperm quality. The SOD mimic positively affected another component of the functional status of sperm mitochondria, that is, increased mRNA expression of the nucleus

**Table 2**

List of redox-active substances in clinical trials or commercially available that ameliorate sperm-fertilizing capacity by (at least partially) modulating the NO-signalling pathway

Compound	Effect	
L-arginine	Increases sperm volume, concentration, motility and morphology by increasing eNOS activity	Scibona <i>et al.</i> , 1994; Aydin <i>et al.</i> , 1995; Srivastava <i>et al.</i> , 2000
Superoxide dismutase mimic, M40403	Improves the functional status of sperm mitochondria, and thus the fertilizing potential of spermatozoa, by increasing NO bioavailability	Otasevic <i>et al.</i> , 2013
Sildenafil (Viagra™)	Improves erectile function through inhibition of cGMP-dependent phosphodiesterase	Schwartz and Kloner, 2010
	Increase velocity, capacitation and amplitude of lethal head displacement in spermatozoa (mechanism unclear)	Lefièvre <i>et al.</i> , 2000
Tadalafil (Cialis™)	Improves erectile function through inhibition of cGMP-dependent phosphodiesterase	Schwartz and Kloner, 2010
Vardenafil (Levitra™)	Improves erectile function through inhibition of cGMP-dependent phosphodiesterase	Schwartz and Kloner, 2010
Ginsenoside R <sub>e</sub>	Improves sperm motility through induction of NOS activity (increase of NO production)	Zhang <i>et al.</i> , 2006

**Figure 3**

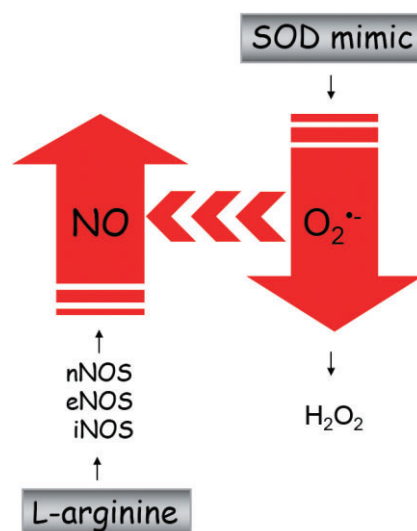
Structure of M40403, a SOD mimic.

encoded subunits of both complexes I and IV of the ETC, while subunits encoded by the mitochondrial genome were restored to the control level. These data undoubtedly showed improvement of functional status of sperm mitochondria, and thus the fertilizing potential of spermatozoa by M40403, while definitive proof of improvements in sperm-fertilizing potential was shown by the finding that the mimic increased sperm motility, a crucial parameter of semen quality.

This is the very first time that M40403 has been shown to improve the molecular basis of sperm mitochondrial function, as well as increase sperm motility, and thus has a positive effect on the functionality of spermatozoa. Although the precise mechanism of this M40403-acting remains to be elucidated, the increase in NO content in spermatozoa after treatment with M40403 implies the involvement of NO in the observed beneficial effects of this drug. This seems quite plausible considering that NO affects mitochondria on several levels and that NO and its precursors increase sperm motility by increasing energy production in the mitochondrial compartment (Hellstrom *et al.*, 1994). In addition, M40403 increased eNOS gene expression, which strongly indicates that an increase in the NO level after M40403 may depend, at least partly, on drug-elicited induction of eNOS and an increase in endogenous NO production. Thus, in future studies designed to define the precise mechanism underlying the M40403-driven increase in the population of sperm with active mitochondria and motile spermatozoa, the involvement of NO warrants further consideration (Figure 4).

Nevertheless, what is certain and clearly shown by this study is that the redox modulator, M40403, is a promising pharmacological tool for the improvement of sperm function during assisted fertilization and for the treatment of infertile states accompanied by mitochondrial impairments and/or a disturbed sperm redox state.

In addition, another potentially important fact that could be significant for determination of NO bioavailability and roles that NO plays in male (in)fertility is NOS uncoupling (Alderton *et al.*, 2001). That is, it is known that in a number of pathological conditions NOS enzymatic activity becomes uncoupled, resulting in the production of  $O_2^{\cdot-}$  instead of NO, and leading to a decrease in NO bioavailability. A number of potential mechanisms are responsible for uncoupling of

**Figure 4**

Strategies to modify NO concentration in spermatozoa. As a substrate for NOSs, L-arginine increases NO production. In contrast, the SOD mimic removes  $O_2^{\cdot-}$ , and by decreasing the bioavailability of  $O_2^{\cdot-}$  for interaction with NO, the amount of NO consequently increases.

NOSs, although the most consistent evidence suggests it is due to tetrahydrobiopterin deficiency (Kietadisorn *et al.*, 2012). NOS uncoupling also occurs when there is lack of other NOS cofactors, substrate – L-arginine, when they are dephosphorylated or redistributed to the cytosolic fraction. Thus, this could be potentially very important from the aspect of male infertility because, theoretically, modulation of NOS uncoupling (by providing their substrate, cofactors, or stabilizing their activity) could be an attractive therapeutic approach to increase NO synthesis and consequently improve sperm functions.

In line with this, it has been shown that when eNOS activity is uncoupled, treatments with redox-active substances, that is, redox changes in endothelium, can substantially ameliorate NO production and thus achieve beneficial effects in cardiovascular system (Visioli and Hagen, 2011). Specifically, it was shown that vitamin C facilitates eNOS-derived NO synthesis by maintaining/increasing intracellular tetrahydrobiopterin in a highly reduced state, necessary for NOS activity, thereby promoting or maintaining eNOS coupling in endothelium (Heller *et al.*, 1999; Carr *et al.*, 2000; Huang *et al.*, 2000). In addition, GSH and stimulators of GSH synthesis, such as (R)- $\alpha$ -lipoic acid, markedly increase eNOS activity by promoting/preserving eNOS phosphorylation, especially at the S1176 residue (Visioli *et al.*, 2002; Suh *et al.*, 2004), whereas polyphenols increase endothelial NO synthesis markedly, but the underlying mechanisms are still elusive. Based on these data obtained in vascular dysfunction, there is compelling rationale to use these substances to increase NO production in spermatozoa, at least in conditions that could lead to uncoupled activity of NOSs. In addition, a recently reported link between vascular dysfunction and various forms of infertility (Visioli and Hagen, 2011) further strengthens the rationale for the use of these molecules to increase bioavailability of NO in spermatozoa.



## Conclusions and perspectives

Although infertility is a common global problem, treatment is sometimes ineffective, and the number of couples seeking infertility services has increased dramatically over the last decade. Such an increase in infertility rates clearly indicates the need for the development of new strategies to treat sterility.

It has become evident that redox regulation in reproductive biology, that is, regulation driven by ROS and RNS, represents an inevitable new tool for studying fundamental fertilization processes. Accordingly, the present article dealt with the problem of sterility from the perspective of redox regulation. We aimed here not only to review the known facts of the multiple roles that NO plays in spermatozoa and integrating different aspects of the physiological act, but also to open new fields for future research of its complex action in male infertility, emphasizing it as a potentially useful strategy for prevention and management of infertility. The ubiquitous involvement of NO in the regulation of crucial sperm-fertilizing events may have huge clinical implications in developing new therapeutic strategies to treat fertilization disorders in men.

In this regard, use of new potent substances that modulate sperm redox state by selective production/removal of certain ROS/RNS could be a new power tool to increase male fertility and promote conception.

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## Conflict of interest

The authors have no conflicts of interest to declare.

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