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Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway.

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Running head: cGMP signaling and sperm motility

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Abstract

Nitric oxide (NO), a modulator of several physiological processes, is involved in different human sperm functions. We have investigated whether NO may stimulate the motility of human spermatozoa via activation of the soluble guanylate cyclase (sGC)/cGMP pathway. Sperm samples obtained by masturbation from seventy normozoospermic patients were processed by the swim-up technique. The kinetic parameters of the motile sperm-rich fractions were assessed by computer-assisted sperm analysis. After a 30-90 min incubation, the NO donor S-nitrosoglutathione (GSNO) exerted a significant enhancing effect on progressive motility (77, 78 and 78% vs 66, 65 and 62% of the control at the corresponding time), straight linear velocity (44, 49 and 48 μm/s vs 34, 35 and 35.5 μm/s), curvilinear velocity (81, 83 and 84 μm/s vs 68 μm/s) and average path velocity (52, 57 and 54 μm/s vs 40, 42 and 42 μm/s) at 5 μM but not at lower concentrations, and in parallel increased the synthesis of cGMP. A similar effect was obtained with the NO donor spermine NONOate after 30 and 60 min. The GSNO-induced effects on sperm motility were abolished by ODQ (a specific sGC inhibitor) and mimicked by 8-Br-cGMP (a cell-permeating cGMP analog): the treatment with Rp-8-Br-cGMPS (an inhibitor of cGMP-dependent protein kinases) prevented both the GSNO- and the 8-Br-cGMP-induced responses. On the opposite, we did not observe any effect of the cGMP/PKG pathway modulators on the onset of hyperactivated sperm motility. Our results suggest that NO stimulates human sperm motility via the activation of sGC, the subsequent synthesis of cGMP and the activation of cGMP-dependent protein kinases.

Introduction

Nitric oxide (NO) is a free radical gas which participates as a mediator in several physiopathological events, such as regulation of vascular tone, neurotransmission, apoptosis, and inflammation (Wink & Mitchell 1998). NO is synthesized by nitric oxide synthases (NOS), a family of enzymes catalyzing the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry (Nathan & Xie 1994). Three NOS isoforms have been described: endothelial (eNOS, NOS III), neuronal (nNOS, NOS I) and inducible (iNOS or NOS II) (Nathan & Xie 1994). NO has been demonstrated to play a role in a variety of functions in the human reproductive tract, including sperm motility (Lewis *et al.* 1996), chemotaxis (Miraglia *et al.* 2007), and sperm-zona pellucida binding ability (Sengoku *et al.* 1998). NOS isoforms have been localized in the acrosome and tail of human, mouse and bovine spermatozoa (Herrero *et al.* 1996; Meiser & Schulz 2003), and low motility spermatozoa have been shown to exhibit aberrant patterns of eNOS immunostaining (O'Bryan *et al.* 1998). It has been reported that low concentrations of NO (25-100 nM sodium nitroprusside) enhance the motility of human spermatozoa (Hellstrom *et al.* 1994; Zhang & Zheng 1996). Accordingly, human sperm motility is inhibited by the NOS inhibitor N^G-nitro-L-arginine methyl ester and by the NO scavenger methylene blue (Lewis *et al.* 1996; Donnelly *et al.* 1997). On the other hand, high NO concentrations (25-125 μM pure nitric oxide, 0.25-2.5 mM sodium nitroprusside, 12-600 μM S-nitroso-N-acetylpenicillamine, 100-125 μM 3-morpholinosydnonimine) seem to exert opposite effects on the motility of human spermatozoa *in vitro* (Rosselli *et al.* 1995; Nobunaga *et al.* 1996; Weinberg *et al.* 1995). Studies on sperm capacitation showed that NO (1-100 μM spermine NONOate or diethylamine-NONOate) increases cAMP levels, thus triggering protein kinase A activation and tyrosine phosphorylation (Herrero *et al.* 2000) and is also involved in activation of protein extracellular signal regulated kinases (ERKs) (Thundathil *et al.* 2003; O'Flaherty *et al.*

2006). On the other hand, like in many other cell types, NO activates the soluble guanylate cyclase (sGC) in human spermatozoa (Revelli *et al.* 2002). The NO donors sodium nitroprusside and spermine-NONOate have been shown to increase the intracellular levels of cGMP in human (Zhang & Zheng 1996; Revelli *et al.* 2001) and murine (Herrero *et al.* 1998) spermatozoa, respectively, and recently the sGC has been identified in human sperm by immunoblotting (Willipinski-Stapelfeldt *et al.* 2004). Although its levels in human sperm are about 100-fold lower than the cAMP content (Willipinski-Stapelfeldt *et al.* 2004), cGMP has been implicated in several sperm signaling pathways functions, such as capacitation, acrosome reaction, chemotaxis and sperm-egg interaction (Revelli *et al.* 2001; Revelli *et al.* 2002, Herrero *et al.* 2003; Miraglia *et al.* 2007). cGMP is thought to modulate also sperm motility. Indeed, the cGMP-dependent phosphodiesterase (PDE) inhibitor sildenafil was reported by some authors (Lefievre *et al.* 2000; Cuadra *et al.* 2000), but not by others (Andrade *et al.* 2000; Aversa *et al.* 2000; Burger *et al.* 2000), to increase the velocity and amplitude of lateral head displacement in human spermatozoa. Lefievre *et al.* observed an inhibition of sperm PDE activity with sildenafil at high concentrations, able to inhibit many PDE and causing also an increase of cAMP (Lefievre *et al.* 2000), whereas Cuadra *et al.* reported that sildenafil stimulates sperm motility at much lower concentrations, quite close to the IC_{50} of sildenafil for the cGMP-dependent PDE (Cuadra *et al.* 2000). A recent review of *ex vivo* studies suggests that sildenafil and tadalafil exert a dose-dependent effect on sperm motility which is enhanced at low doses but may be reduced at high concentrations, but further investigations are required to evaluate the mechanisms by which these phosphodiesterase selective inhibitors modulate sperm motility (Dimitriadis *et al.* 2008).

Until now no clear data show a direct relationship between exposure to NO, increase of sperm cGMP levels and changes of human sperm motility. Therefore, aim of this study has been to investigate whether human sperm motility, which is considered one of the most significant

fertility-related sperm features (Hirano *et al.* 2001), may be affected by NO via activation of the sGC/cGMP signaling pathway.

RESULTS

Since the swim-up procedure was performed in SWM containing bicarbonate and albumin, as previously described (Miraglia *et al*. 2010), for a time sufficient to induce capacitation in most sperm cells, the experiments shown in each point of this paper can be considered as performed on capacitated spermatozoa (see also Materials and Methods section). Preliminary experiments of dose-dependence were performed to establish the concentration of the NO donor GSNO able to affect human sperm motility patterns. A progressively motile sperm swims forward in an essentially straight line: rapid progressive motility (A) indicates sperm swimming with a progression velocity > 25 μm/s, while slow progressive motility (B) indicates sperm swimming with a progression velocity = 5-25 μm/s (Krause & Viethen 1999). At the concentration of 5 μM, GSNO exerted a significant enhancing effect on progressive motility (A + B motility classes) at each time period considered, while at 0.1-1 μM it was not effective (Fig. 1A). When the spermatozoa were incubated with 10 μM GSNO, progressive motility (A + B classes) was comparable to those of untreated sperm (Fig 1A). To check how long the effect of 5 μM GSNO takes to develop, time-dependence experiments were performed. The increase of sperm motility induced by GSNO was not significant at 10 and 20 minutes, but only after an at least 30 minutes incubation (Fig 1B). Analyzing each class of motility we observed that the increase of progressive motility (WHO classes A + B) after treatment with GSNO was mainly due to a significant rise in the percentage of A class spermatozoa, which was counterbalanced by a parallel decrease of both C and D class spermatozoa; the amount of spermatozoa exhibiting a B pattern of motility did not change under all the experimental conditions (data not shown). On the

contrary, the motion parameters linearity (LIN) and straightness (STR) were unaffected, and no induction of HA was observed (data not shown).

In the same way, GSNO strongly increased the individual parameters of sperm movement straight linear velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP) when added at 5 μM but not at 0.1-1 μM (data not shown). After the incubation with 10 μM GSNO, VSL, VCL and VAP were comparable to those of untreated sperm, thus suggesting that GSNO at this concentration was not yet toxic, but it neither could improve the sperm motility (data not shown). Spermine NONOate (SPNO) is a faster NO donor than GSNO: when incubated with several samples (n = 6) of spermatozoa, SPNO 0.5 μM significantly increased the progressive motility (A + B classes) after 30 (71.2 $\pm$ 2 % vs. 51 $\pm$ 1 % in controls) and 60 min (69 $\pm$ 3 % vs. 50 $\pm$ 2 % in controls). In the same experimental conditions 0.5 μM SPNO increased significantly also VSL, VCL and VAP (data not shown).

Oxidized glutathione (GSSG), the product of GSNO decomposition, is a powerful chelator of copper ions (Singh *et al.* 1999). Since copper ions can influence the release of NO from GSNO, we performed further experiments to check whether the increased sperm motility that we observed after incubation with GSNO is due to the chelation of copper by GSSG. We measured sperm motility in the presence of 5 μM reduced glutathione (GSH) or GSSG, to exclude that glutathione per se, in any form, could alter the progressive motility: both GSH and GSSG had no significant effect on sperm motility (n = 4; data not shown). To chelate copper we performed also other experiments with 1 mM EDTA, and even in this case we did not observe any significant modification vs. controls and vs. GSNO alone (n = 4; data not shown). After EDTA treatment the level of calcium was about 1 mM.

Thereafter, the 5 μM concentration of GSNO was chosen to perform the subsequent experiments. The NO donor induced a significant increase of sperm progressive motility

165 measured by CASA after incubation with freshly ejaculated human samples for 30, 60 and 90
166 min (Fig. 2). The sGC inhibitor ODQ did not affect the progressive motility when added alone,
167 but completely blunted its GSNO-elicited increase at each time period (Fig. 2). On the other
168 hand, 8-Br-cGMP, a cell-permeating cGMP analog, exerted a significant enhancing effect on
169 progressive motility *per se* and completely reversed the inhibitory effect of ODQ on the GSNO-
170 stimulated increase (Fig. 2). Finally, the PKG inhibitor Rp-8-Br-cGMPS, which *per se* did not
171 modify the sperm progressive motility, abolished the effects of GSNO and 8-Br-cGMP on this
172 motion parameter (Fig. 2).

173 In order to confirm the role of NO in this process, we measured also the progressive motility
174 in the presence of the NO scavenger PTIO. 100 μM PTIO did not affect sperm motility when
175 used alone, but when co-incubated with GSNO (5 μM) it completely reversed the increase of
176 motility induced by GSNO (Fig. 3). In the presence of 20 μl of packed fresh red blood cells, used
177 as reservoirs of the NO scavenger oxyhemoglobin, the motility results were the same observed
178 with PTIO (n = 3; data not shown). To this purpose, we incubated the spermatozoa at the reported
179 concentrations used in the other experiments and for the indicated times (30, 60, 90 min) in the
180 lower compartment of a transwell system (having a polycarbonate transwell insert membrane
181 with pore sizes of 3 μm, in 24 well plates provided by Corning Incorporated, Apton, MA),
182 containing in the upper compartment 20 μl of packed fresh red blood cells in 0.5 ml of SWM.
183 After each incubation time the upper compartment was taken out and the sperm motility
184 parameters were measured as described in the Materials and Methods section.

185 We also evaluated the effect of NO on sperm kinetic parameters assessed by CASA. In the
186 presence of GSNO, the straight linear velocity (VSL) markedly increased, an effect that was
187 abolished by ODQ (which *per se* did not modify this motion parameter), as shown in Fig. 4A; the
188 cGMP analog 8-Br-cGMP significantly stimulated VSL, and bypassed the inhibition exerted by

ODQ on the GSNO-evoked VSL increase (Fig. 4A). The co-incubation with Rp-8-Br-cGMPS completely blunted the positive action of both GSNO and 8-Br-cGMP on VSL (Fig. 4A).

The same pattern of response was observed when considering the curvilinear velocity (VCL) (Fig. 4B) and the average path velocity (VAP) (Fig. 4C) of human spermatozoa treated under the same experimental conditions.

Finally, under the same experimental conditions GSNO significantly increased the synthesis of cGMP in human spermatozoa at each incubation time considered: the absence of a significant time-dependence suggests that GSNO exerts a maximal effect already after 30 min, and that between 30 and 90 min the synthesis of cGMP is maintained in a steady state condition. The effect of GSNO was completely abolished by ODQ; as expected, after incubation with 8-Br-cGMP, both alone and together with GSNO and ODQ, the cGMP intracellular level was significantly higher than the control level (Fig. 5). Also in this case, no time-dependence was observed, suggesting that in our experimental conditions the entry of 8-Br-cGMP into the cells and its degradation were balanced throughout the time of investigation.

Since the measurement of intracellular cGMP was performed in the presence of the phosphodiesterase inhibitor IBMX to inhibit cGMP hydrolysis, we performed further motility experiments on samples pre-treated for 20 min with 200 μM IBMX and then for 30, 60 and 90 min with 5 μM GSNO: we observed that the pre-treatment with IBMX did not influence the enhancement of sperm motility induced by NO (Fig. 3).

DISCUSSION

The nitric oxide/cGMP signaling pathway modulates several physiopathological events of the mammalian reproductive tract (Rosselli *et al.* 1998). As far as sperm functions are concerned, NO released by sodium nitroprusside has been shown to play an important role in mouse sperm

hyperactivation (Herrero *et al.* 1994) and in the maintenance of post-thaw human sperm motility and viability (Hellstrom *et al.* 1994). Moreover, spermatozoa themselves synthesize NO, and the basal release of this free radical by spermatozoa has been observed to be higher in normozoospermic than in asthenospermic sperm samples; accordingly, normal spermatozoa express more eNOS and generate more nitrite than spermatozoa of asthenospermic samples (Lewis *et al.* 1996). Furthermore, the NO scavenger methylene blue and the NOS inhibitor N^G-nitro-L-arginine methyl ester have been shown to inhibit human sperm motility (Lewis *et al.* 1996; Donnelly *et al.* 1997). On the other hand, when female mice null for one of the three NOS isoforms (eNOS, nNOS and iNOS, respectively) mated with null male mice the rate of in vitro fertilization was not inhibited (Yang *et al.* 2005): this observation does not change the meaning of our results, because in the absence of a NOS isoform NO can be produced by another isoform. Furthermore, these results were obtained in mice. Finally, in spermatozoa NO can be generated as a consequence of a direct hydrogen peroxide attack on arginine (Aitken *et al.* 2004).

In the present work we provide further evidence suggesting a role of the cGMP signaling pathway in human sperm motility. The NO donor GSNO significantly increased the sperm forward progressive motility after 30-90 min of incubation: GSNO significantly augmented the percentage of A class sperm without modifying the overall amount of B class sperm; it also decreased the percentage of *in situ* motile (C class) and immotile (D class) cells. In parallel, the NO donor stimulated the sperm kinetic parameters assessed by CASA, straight linear velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP). This is in accordance with a previous study reporting that sodium nitroprusside increased human sperm motility (Zhang & Zheng 1996): such effect was detectable at 25-100 nM but not at 200-400 nM, whereas we observed a significant motility enhancement using 5 μM GSNO. This difference may be due to the different NO donor employed and the different experimental procedure used to measure

sperm motility: indeed, that study evaluated a trans-membrane migration ratio (the proportion of human spermatozoa moving across a membrane separating two chambers) (Zhang & Zheng 1996), whereas CASA calculates the percentage of cells exhibiting a forward progressive motility and the kinetic parameters of each cell.

Compared to GSNO (having an half-life of hours, ranging from 10 to 38 h) (Nikitovic & Holmgren 1996; Mancuso *et al.* 2003), SPNO is a faster NO donor, with a half-life of 39 minutes at 37°C and pH 7.4 (Keefer *et al.* 1996). In further experiments using SPNO as NO donor, we observed that also the incubation with SPNO 0.5 μM significantly increased the progressive motility (A + B classes), VSL, VCL and VAP after 30 and 60 min.

In a previous work we demonstrated that GSNO and 8-Br-cGMP exerted a significant chemotactic effect on human spermatozoa without affecting their motion parameters (Miraglia *et al.* 2007). In that study both substances were used at different concentrations and time periods compared to those employed in this investigation: GSNO exerted a chemoattractant effect at 100 nM, while in this study it was ineffective on motility even at 1 μM. On the other hand, 8-Br-cGMP was used in the previous study at a 1 mM concentration, two-fold higher than the one used in the present work. Moreover, in our previous work we investigated the sperm motion parameters only after 20 min of incubation with GSNO and 8-Br-cGMP, whereas in the present research we employed longer (30-90 min) time periods of observation (Miraglia *et al.* 2007). Since the intracellular levels of cGMP measured after incubation with either GSNO or 8-Br-cGMP were respectively similar in both experimental works, in spite of the different incubation times and concentrations used, it is likely that these compounds exert a significant effect on sperm motility only when the level of intracellular cGMP is maintained increased for a time longer than the one necessary for cGMP to modulate chemotaxis. This suggestion may make sense, since it is reasonable to suppose that at a first time sperm needs to be simply oriented

versus a source of NO and only subsequently, when the increase of cGMP shows to be persistently high, the motility should increase.

The effect of GSNO on sperm motility is indeed mediated by an increased synthesis of cGMP, as the sGC inhibitor ODQ blunted the GSNO-elicited motility and abolished the increase of intracellular cGMP induced by GSNO. The treatment with the cell-permeating cGMP analog 8-Br-cGMP, which augmented by nearly 4-fold the intracellular content of cyclic nucleotide, strongly increased the forward progressive motility and the kinetic parameters VSL, VCL and VAP. Moreover, 8-Br-cGMP reversed the inhibitory effect of ODQ on the GSNO-evoked increase of progressive motility and velocity, confirming that ODQ inhibited sperm motility by lowering the intracellular level of cGMP.

Taken together, these findings suggest that NO stimulates human sperm motility via the activation of sGC and the subsequent synthesis of cGMP. One of the main targets of cGMP in many tissues is a family of serine/threonine kinases, the PKGs (Hofmann 2005). Rp-8-Br-cGMPS, a PKG inhibitor (Kawada *et al.* 1997), abolished the positive effect exerted by both GSNO and 8-Br-cGMP on sperm motility, suggesting that the effect of endogenous or exogenous cGMP on sperm movement is mediated by PKG activity. Thus, from our data PKG seems to play a role in mediating not only the NO-elicited chemotaxis and the acrosome reaction (Miraglia *et al.* 2007; Revelli *et al.* 2001), but also in modulating several sperm motion patterns. On the other hand, we did not observe any effect of the cGMP/PKG pathway modulators on the onset of hyperactivated sperm motility.

It is widely acknowledged that spermatozoa in the human female reproductive tract have close and prolonged contact with a significant array of NO-producing cells (Rosselli *et al*. 1998; Sun *et al.* 2005; Machado-Oliveira *et al.* 2008): the exact sites of NO production in the female genital tract remain to be investigated, but Machado-Oliveira and colleagues (Machado-Oliveira *et al.*

2008) showed that detectable amounts of NO are produced in human cumulus fragments and oviduct explants. This free radical is relatively unreactive, and is able to diffuse from the cell in which it is generated to the neighbor cells, covering long distances in a very short time (Kröncke *et al.* 1997). Moreover, spermatozoa themselves produce and release NO during their trip along the upper female genital tract. This suggests that a complex interaction between spermatozoa, granulosa cells and other cells of the female reproductive tract may submit human sperm to amounts of NO that are sufficient to elicit *in vivo* the changes of motility we have observed *in vitro*.

It is generally accepted that a good sperm motility is a major component of normal male fertility. Men with poorly motile or immotile sperm are typically infertile or sterile (Turner 2006). A deeper knowledge of the role of the NO/cGMP/PKG signaling pathway in the physiopathology of sperm motility could help to pharmacologically improve the fertilization capacity of human sperm or, alternatively, could lead to the development of an effective and safe male contraceptive based on sperm motility impairment.

Materials and methods

Reagents

Sperm Washing Medium (SWM) was supplied by Celbio (Milan, Italy): it is based on the Modified Human Tubal Fluid (Quinn *et al.* 1985), containing sodium bicarbonate (4 mM), Hepes buffer (21 mM), human serum albumin (5 mg/ml). S-nitrosoglutathione (GSNO), 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), 8-bromo-cGMP (8-Br-cGMP), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO). The inhibitor of cGMP-dependent protein kinases (PKGs) 8-bromoguanosine-3',5'-monophosphorothioate Rp-isomer (Rp-8-Br-cGMPS) was from Biolog Life Science Institute

309 (Bremen, Germany). The [^{3}H]cGMP RIA kit was obtained from Amersham International
310 (Buckinghamshire, UK).

311 ***Collection and preparation of sperm samples***

312 Sperm samples were obtained by masturbation after 3-5 days of sexual abstinence from
313 seventy normozoospermic patients belonging to couples presenting for infertility evaluation.
314 Each donor gave informed consent allowing the use of his semen for our experiments. The
315 Institutional Review Board approval was obtained from the internal ethical committee that
316 authorized the use of semen samples submitted to semen examination for experimental purposes.
317 All samples were allowed to liquefy for at least 30 minutes at 37°C, then they were evaluated for
318 sperm concentration, motility and morphology according to World Health Organization
319 guidelines (World Health Organization 2001). Only specimens with normal parameters
320 (concentration > 20 x 10^6 spermatozoa/ml, progressive motility > 50%) were used in the
321 experiments.

322 Motile spermatozoa were capacitated by the swim-up technique (37°C for 75 min in a 5% CO_2
323 atmosphere) using SWM, as previously described (Miraglia *et al*. 2010). The presence of round
324 cells was initially below $1x10^6$ in all sperm samples, and was minimal if not absent after the
325 swim-up technique in the final suspension. After swim-up, the motile sperm-rich fraction was
326 centrifuged at 600 g for 10 min, the supernatant was discarded and the pellet re-suspended in
327 SWM. The concentration of the spermatozoa suspensions was assessed in a Makler counting
328 chamber (Sefi Medical Instruments, Haifa, Israel) under a phase-contrast microscope
329 (magnification 20 X), and adjusted to approximately 100 x 10^6 cells/ml. The dose-dependent
330 effect of GSNO on sperm motility was investigated in the first 25 samples (20×10^6 cells/200 µl),
331 the effect of the modulation of the cGMP pathway on sperm kinetic parameters was studied in the
332 subsequent 40 samples (20×10^6 cells/200 µl), and finally the ability of the cGMP-modulating

agents to modify the intracellular cGMP content was checked in the last 5 samples (15×10^6 cells/500 µl). GSNO was not toxic at the concentrations used, as checked by the eosin Y exclusion test (Cincik *et al.* 2007).

Analysis of Motility Parameters

Aliquots of sperm suspension (200 µl) in SWM, each containing 20×10^6 cells, were incubated under the experimental conditions indicated in Results. Sperm motility parameters were assessed by computer-assisted sperm analysis (CASA) (CGA-WLJY-9000, CGA Distribution, Florence, Italy) after 30, 60 and 90 min of incubation. The following kinetic parameters were measured: percentage of spermatozoa exhibiting a forward progressive motility (A+B WHO classes), in situ motility (C WHO class), or no motility (D WHO class); straight linear velocity (VSL, which represents the average velocity, expressed in µm/s, measured from the beginning to the end of a linear track); curvilinear velocity (VCL, which is the average velocity measured over the actual point-to-point track followed by the cell, expressed as µm/s); average path velocity (VAP, corresponding to the average velocity of smoothed cell's pathway, expressed in µm/s); linearity [LIN = (VSL/VCL) x 100]; straightness (STR, the percentage of correspondence of the cell's pathway to a straight line, with 100% corresponding to the maximal extent of straightness) (Mortimer 1997). Sperm hyperactivation (HA) was also considered, using the following parameters: VCL $\geq$ 70 µm/s, ALH $\geq$ 7 µm, LIN $\leq$ 30%, VSL $\leq$ 30 µm/s (Green & Fishel 1999).

Measurement of intracellular cGMP

The level of intracellular cGMP was measured as previously described (Miraglia *et al.* 2007) Briefly, aliquots of sperm suspensions (500 µl), each containing 15 x 10^6 cells, were pre-treated for 20 min with the phosphodiesterase inhibitor IBMX (200 µM) to inhibit cGMP hydrolysis, and then were co-incubated for 30, 60 or 90 min with the same substances (GSNO, ODQ, 8-Br-cGMP) used for the assessment of motility parameters, alone or differently combined.

Subsequently, the samples were centrifuged at 13,000 g for 1 min, the supernatants were discarded and 50 µl of absolute ethanol were added to the pellets; ethanol was then evaporated by vacuum centrifugation, and 350 µl of Tris/EDTA buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5) were added. After 10 min, 100 µl of supernatant were tested for the cGMP level with a [^{3}H]cGMP immunoassay system. The cGMP content was expressed as pmol/10^6 cells. Cross-reactivity of the [^{3}H]cGMP immunoassay system with cAMP was less than 0.001%.

Statistical analysis

All data are provided as means ± SEM. The results were analyzed by a One-Way Analysis of Variance (ANOVA) and Tukey's and Bonferroni's test (software: SPSS 11.0 for Windows, SPSS Inc., Chicago, IL), including the different times of incubation in the global significance evaluation. A p value < 0.05 was considered significant.

Declaration of interest.

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

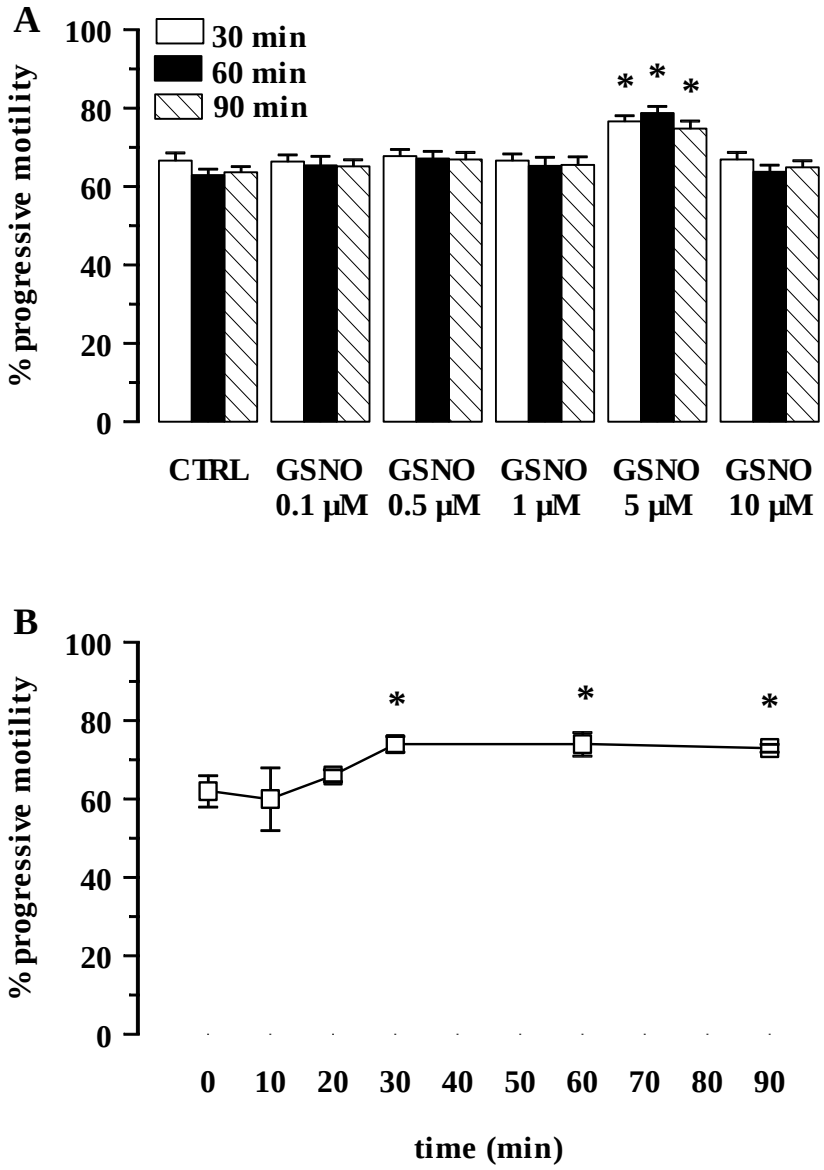

Figure 1. Effect of GSNO on sperm motility patterns.

A. The percentage of spermatozoa exhibiting a forward progressive motility (A + B WHO classes) was recorded by computer-assisted sperm analysis (CASA) after a 30 min (white bars),

60 min (black bars) or 90 min (hatched bars) incubation of 20×10^6 cells /200 µl with 0.1-10 µM S-nitrosoglutathione (GSNO). All data are presented as means ± SEM (n = 25). Significance vs. control at the corresponding incubation time: * $p < 0.05$.

B. The percentage of spermatozoa exhibiting a forward progressive motility (A + B WHO classes) was recorded by CASA after a 10, 20, 30, 60 or 90 min incubation of 20×10^6 spermatozoa/200 µl with 5 µM GSNO. All data are presented as means ± SEM (n = 4). Significance vs. ctrl : * $p < 0.05$.

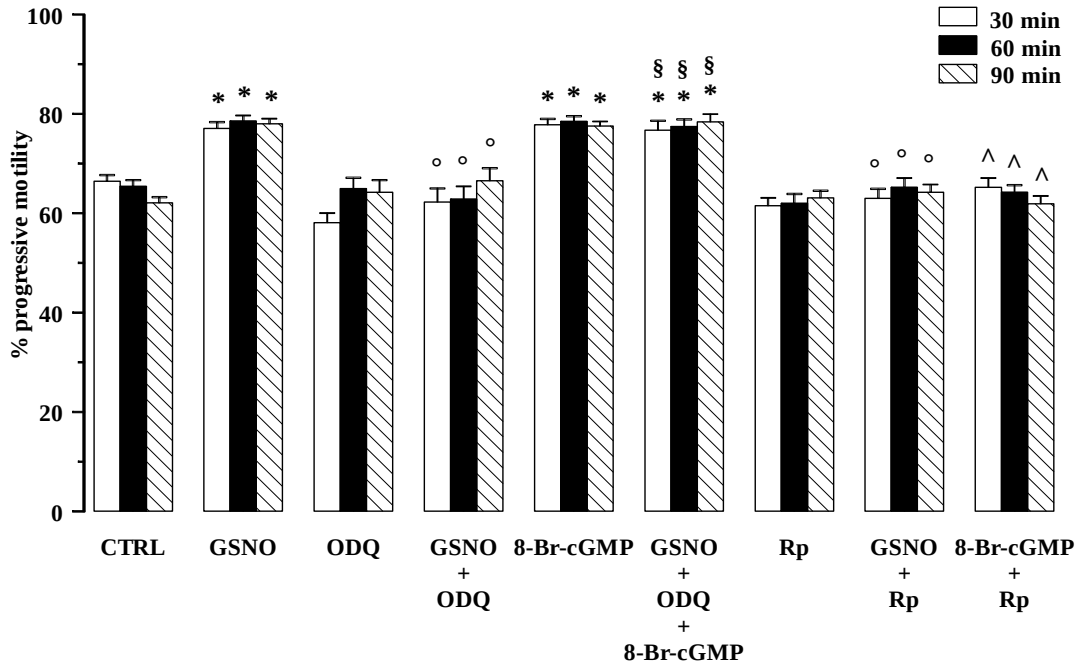

Figure 2. Effect of the modulation of the cGMP pathway on human sperm progressive motility. The forward progressive motility (motility classes A + B) was assessed by CASA in human spermatozoa (20×10^6/200 µl) incubated for 30, 60 or 90 min with the following substances, alone or differently combined: S-nitrosoglutathione (GSNO, 5 µM), 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, 10 µM), 8-bromo-cGMP (8-Br-cGMP, 500 µM), Rp-8-Br-cGMPS (Rp, 10 µM). All data are presented as means ± SEM (n = 40). Significance vs. respective CTRL: * $p < 0.001$; vs. GSNO: ° $p < 0.001$; vs. GSNO+ODQ: § $p < 0.001$; vs. 8-Br-cGMP: ^ $p < 0.001$.

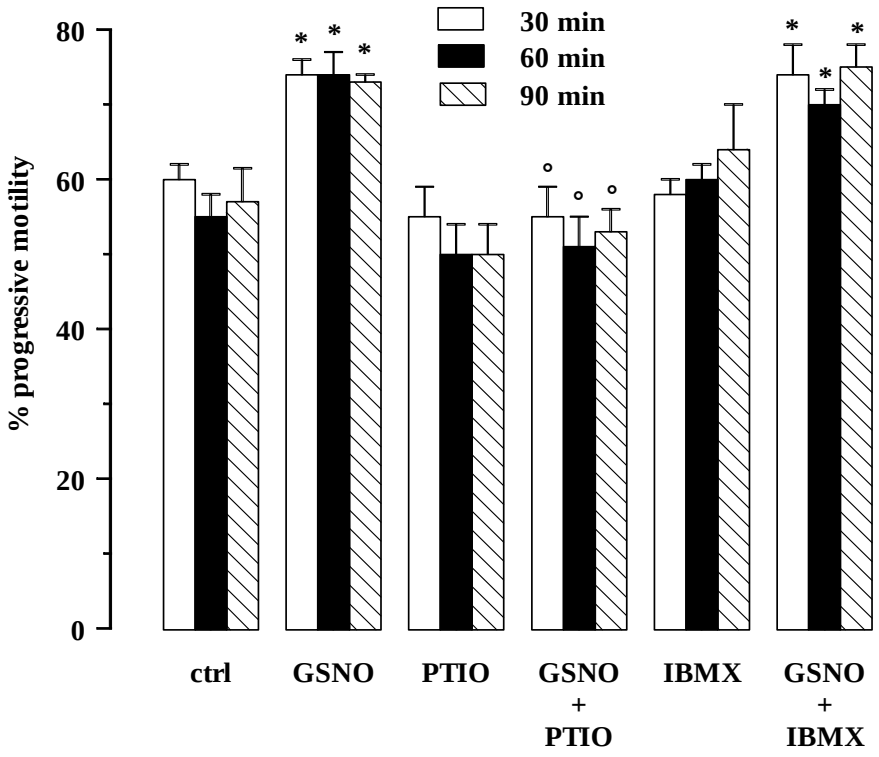

Figure 3. Effect of GSNO, PTIO and IBMX on sperm motility patterns. The forward progressive motility (motility classes A + B) was assessed by CASA in human spermatozoa (20×10^6/200 µl) incubated for 30, 60 or 90 min with the following substances, alone or differently combined: 5 µM GSNO, 100 µM PTIO, 200 µM IBMX. In the case of IBMX, the spermatozoa were pre-treated for 20 min with IBMX before being incubated with 5 µM GSNO

551 for 30, 60 or 90 min. All data are presented as means + SEM (n = 4). Significance vs. respective

552 ctrl: * $p<0.05$; vs. GSNO: ° $p<0.05$.

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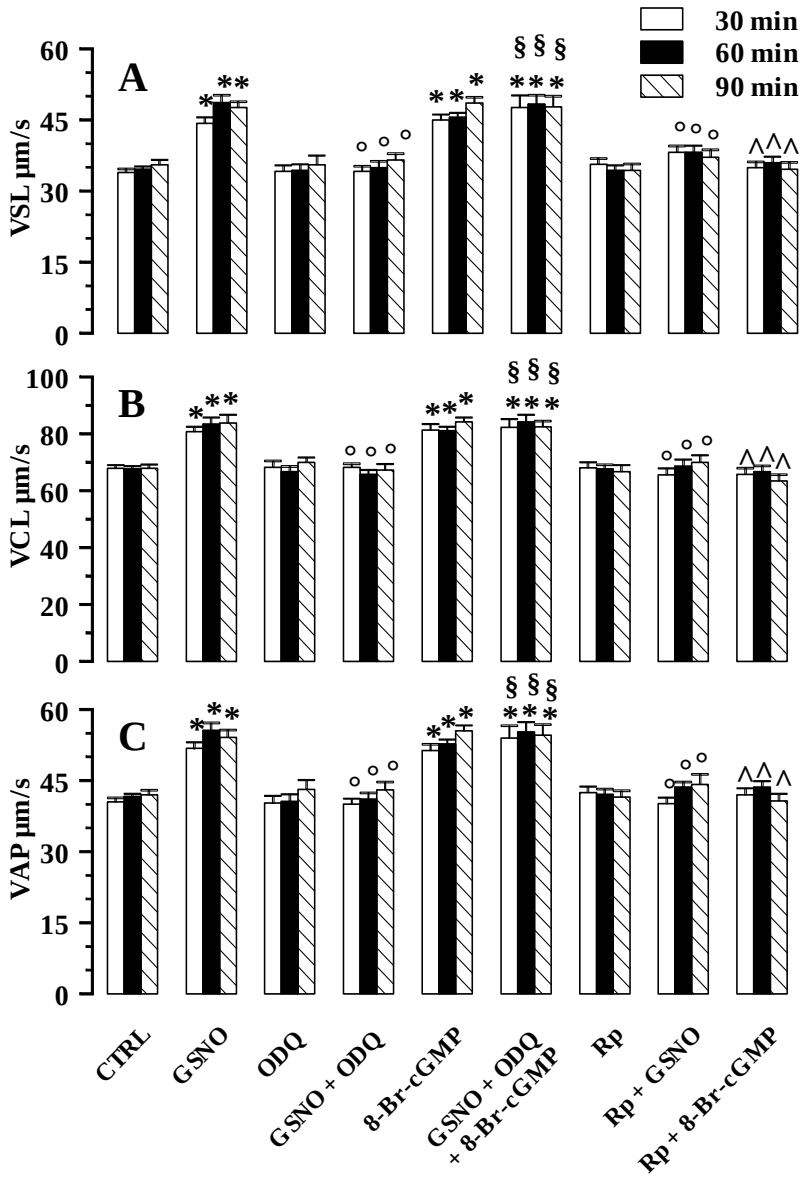

Figure 4. Effects of the modulation of the cGMP pathway on straight linear velocity (VSL, panel A), curvilinear velocity (VCL, panel B) and average path velocity (VAP, panel C) of

559 **human spermatozoa.** VSL, VCL and VAP were measured by CASA on human spermatozoa
560 (20×10^6/200 µl) incubated for 30, 60 or 90 min in the absence (CTRL) or presence of the
561 following agents, alone or differently combined: 5 µM GSNO, 10 µM ODQ, 500 µM 8-Br-
562 cGMP, 10 µM Rp-8-Br-cGMPS (Rp). Results are shown as means ± SEM (n = 40).
563 **A.** Significance vs. CTRL: * $p < 0.001$; vs. GSNO: ° $p < 0.001$; vs. GSNO+ODQ: § $p < 0.001$; vs.
564 8-Br-cGMP: ^ $p < 0.001$.
565 **B.** Significance vs. CTRL: * $p < 0.001$; vs. GSNO: ° $p < 0.005$; vs. GSNO+ODQ: § $p < 0.01$; vs.
566 8-Br-cGMP: ^ $p < 0.005$.
567 **C.** Significance vs. CTRL: * $p < 0.001$; vs. GSNO: ° $p < 0.001$; vs. GSNO+ODQ: § $p < 0.001$; vs.
568 8-Br-cGMP: ^ $p < 0.001$.
569

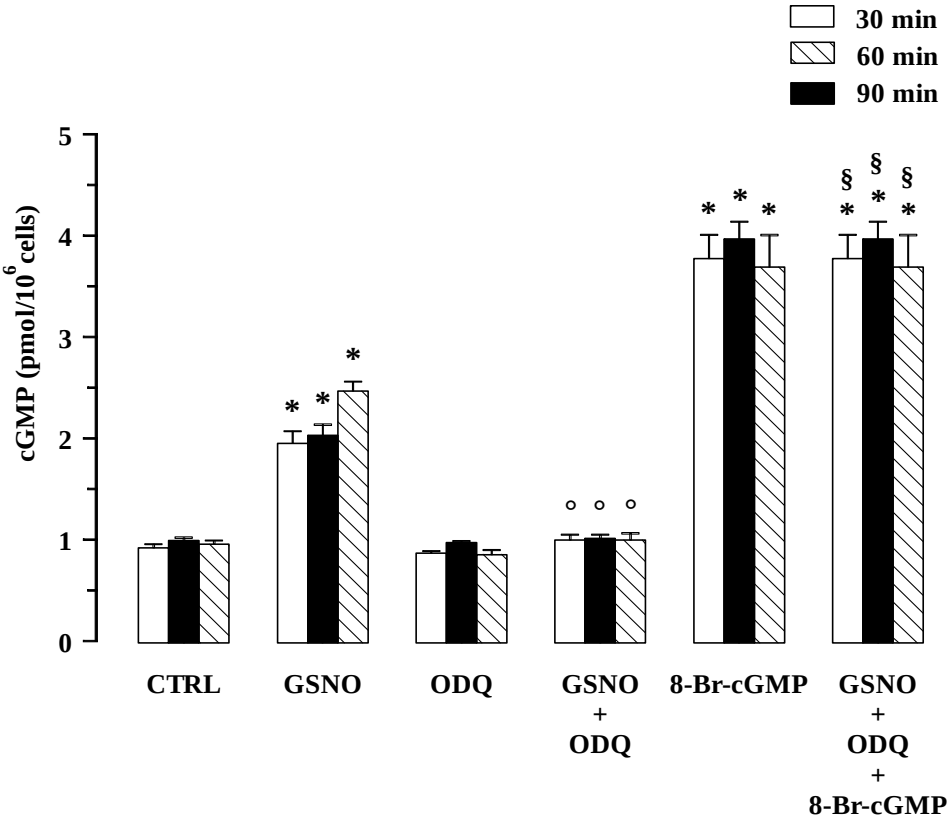

Figure 5. Intracellular cGMP levels in human spermatozoa treated with agents modulating the cGMP pathway. Sperm samples (15 x 10^6 cells/500 µl) were pre-treated with 200 µM IBMX for 20 min, and subsequently they were incubated for 30, 60 or 90 min in the absence (CTRL) or presence of the following substances, alone or in co-incubation: 5 µM GSNO, 10 µM ODQ, 500 µM 8-Br-cGMP. Then, intracellular cGMP concentration was determined as described under the Materials and Methods section. The measurements were performed in triplicate, and data are presented as means ± SEM (n = 5). Significance vs. CTRL: * $p < 0.001$; vs. GSNO: ° $p < 0.005$; vs. GSNO+ODQ: § $p < 0.001$.