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

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29 Erica Miraglia¹, Federico De Angelis², Elena Gazzano¹, Hossain Hassanpour³, Angela Bertagna⁴,
30 Elisabetta Aldieri¹, Alberto Revelli², and Dario Ghigo^{1*}

31
32 ¹ Department of Genetics, Biology and Biochemistry, University of Torino, 10126 Torino, Italy

33 ² Reproductive Medicine and IVF Unit, Department of Obstetrical and Gynecological Sciences,
34 University of Torino, S. Anna Hospital, 10126 Torino, Italy

35 ³ Department of Basic Sciences, College of Veterinary Medicine, University of Shahrekord,
36 Shahrekord, Iran

37 ⁴ Department of Internal Medicine, University of Torino, S. Giovanni Battista Hospital, 10126
38 Torino, Italy

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40 **Running head:** cGMP signaling and sperm motility

41
42 ***Corresponding author:**

43 Dario Ghigo, M.D., Dipartimento di Genetica, Biologia e Biochimica - Sezione di Biochimica,
44 Via Santena, 5/bis - 10126 Torino - Italy (tel. +39-011-6705851; FAX: +39-011-6705845; e-
45 mail: dario.ghigo@unito.it)

46

47 **Abstract**

48 Nitric oxide (NO), a modulator of several physiological processes, is involved in different
49 human sperm functions. We have investigated whether NO may stimulate the motility of human
50 spermatozoa via activation of the soluble guanylate cyclase (sGC)/cGMP pathway. Sperm
51 samples obtained by masturbation from seventy normozoospermic patients were processed by the
52 swim-up technique. The kinetic parameters of the motile sperm-rich fractions were assessed by
53 computer-assisted sperm analysis. After a 30-90 min incubation, the NO donor S-
54 nitrosoglutathione (GSNO) exerted a significant enhancing effect on progressive motility (77, 78
55 and 78% vs 66, 65 and 62% of the control at the corresponding time), straight linear velocity (44,
56 49 and 48 $\mu\text{m/s}$ vs 34, 35 and 35.5 $\mu\text{m/s}$), curvilinear velocity (81, 83 and 84 $\mu\text{m/s}$ vs 68 $\mu\text{m/s}$)
57 and average path velocity (52, 57 and 54 $\mu\text{m/s}$ vs 40, 42 and 42 $\mu\text{m/s}$) at 5 μM but not at lower
58 concentrations, and in parallel increased the synthesis of cGMP. A similar effect was obtained
59 with the NO donor spermine NONOate after 30 and 60 min. The GSNO-induced effects on
60 sperm motility were abolished by ODQ (a specific sGC inhibitor) and mimicked by 8-Br-cGMP
61 (a cell-permeating cGMP analog): the treatment with Rp-8-Br-cGMPS (an inhibitor of cGMP-
62 dependent protein kinases) prevented both the GSNO- and the 8-Br-cGMP-induced responses.
63 On the opposite, we did not observe any effect of the cGMP/PKG pathway modulators on the
64 onset of hyperactivated sperm motility. Our results suggest that NO stimulates human sperm
65 motility via the activation of sGC, the subsequent synthesis of cGMP and the activation of
66 cGMP-dependent protein kinases.

67

68

69 **Introduction**

70 Nitric oxide (NO) is a free radical gas which participates as a mediator in several
71 physiopathological events, such as regulation of vascular tone, neurotransmission, apoptosis, and
72 inflammation (Wink & Mitchell 1998). NO is synthesized by nitric oxide synthases (NOS), a
73 family of enzymes catalyzing the conversion of L-arginine to L-citrulline and NO with a 1:1
74 stoichiometry (Nathan & Xie 1994). Three NOS isoforms have been described: endothelial
75 (eNOS, NOS III), neuronal (nNOS, NOS I) and inducible (iNOS or NOS II) (Nathan & Xie
76 1994). NO has been demonstrated to play a role in a variety of functions in the human
77 reproductive tract, including sperm motility (Lewis *et al.* 1996), chemotaxis (Miraglia *et al.*
78 2007), and sperm-zona pellucida binding ability (Sengoku *et al.* 1998). NOS isoforms have been
79 localized in the acrosome and tail of human, mouse and bovine spermatozoa (Herrero *et al.* 1996;
80 Meiser & Schulz 2003), and low motility spermatozoa have been shown to exhibit aberrant
81 patterns of eNOS immunostaining (O'Bryan *et al.* 1998). It has been reported that low
82 concentrations of NO (25-100 nM sodium nitroprusside) enhance the motility of human
83 spermatozoa (Hellstrom *et al.* 1994; Zhang & Zheng 1996). Accordingly, human sperm motility
84 is inhibited by the NOS inhibitor N^G-nitro-L-arginine methyl ester and by the NO scavenger
85 methylene blue (Lewis *et al.* 1996; Donnelly *et al.* 1997). On the other hand, high NO
86 concentrations (25-125 μ M pure nitric oxide, 0.25-2.5 mM sodium nitroprusside, 12-600 μ M S-
87 nitroso-N-acetylpenicillamine, 100-125 μ M 3-morpholinosydnonimine) seem to exert opposite
88 effects on the motility of human spermatozoa *in vitro* (Rosselli *et al.* 1995; Nobunaga *et al.* 1996;
89 Weinberg *et al.* 1995). Studies on sperm capacitation showed that NO (1-100 μ M spermine
90 NONOate or diethylamine-NONOate) increases cAMP levels, thus triggering protein kinase A
91 activation and tyrosine phosphorylation (Herrero *et al.* 2000) and is also involved in activation of
92 protein extracellular signal regulated kinases (ERKs) (Thundathil *et al.* 2003; O'Flaherty *et al.*

93 2006). On the other hand, like in many other cell types, NO activates the soluble guanylate
94 cyclase (sGC) in human spermatozoa (Revelli *et al.* 2002). The NO donors sodium nitroprusside
95 and spermine-NONOate have been shown to increase the intracellular levels of cGMP in human
96 (Zhang & Zheng 1996; Revelli *et al.* 2001) and murine (Herrero *et al.* 1998) spermatozoa,
97 respectively, and recently the sGC has been identified in human sperm by immunoblotting
98 (Willipinski-Stapelfeldt *et al.* 2004). Although its levels in human sperm are about 100-fold
99 lower than the cAMP content (Willipinski-Stapelfeldt *et al.* 2004), cGMP has been implicated in
100 several sperm signaling pathways functions, such as capacitation, acrosome reaction, chemotaxis
101 and sperm-egg interaction (Revelli *et al.* 2001; Revelli *et al.* 2002, Herrero *et al.* 2003; Miraglia
102 *et al.* 2007). cGMP is thought to modulate also sperm motility. Indeed, the cGMP-dependent
103 phosphodiesterase (PDE) inhibitor sildenafil was reported by some authors (Lefievre *et al.* 2000;
104 Cuadra *et al.* 2000), but not by others (Andrade *et al.* 2000; Aversa *et al.* 2000; Burger *et al.*
105 2000), to increase the velocity and amplitude of lateral head displacement in human spermatozoa.
106 Lefievre *et al.* observed an inhibition of sperm PDE activity with sildenafil at high
107 concentrations, able to inhibit many PDE and causing also an increase of cAMP (Lefievre *et al.*
108 2000), whereas Cuadra *et al.* reported that sildenafil stimulates sperm motility at much lower
109 concentrations, quite close to the IC₅₀ of sildenafil for the cGMP-dependent PDE (Cuadra *et al.*
110 2000). A recent review of *ex vivo* studies suggests that sildenafil and tadalafil exert a dose-
111 dependent effect on sperm motility which is enhanced at low doses but may be reduced at high
112 concentrations, but further investigations are required to evaluate the mechanisms by which these
113 phosphodiesterase selective inhibitors modulate sperm motility (Dimitriadis *et al.* 2008).

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

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

119

120 **RESULTS**

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

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

143 In the same way, GSNO strongly increased the individual parameters of sperm movement
144 straight linear velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP) when
145 added at 5 μM but not at 0.1-1 μM (data not shown). After the incubation with 10 μM GSNO,
146 VSL, VCL and VAP were comparable to those of untreated sperm, thus suggesting that GSNO at
147 this concentration was not yet toxic, but it neither could improve the sperm motility (data not
148 shown). Spermine NONOate (SPNO) is a faster NO donor than GSNO: when incubated with
149 several samples ($n = 6$) of spermatozoa, SPNO 0.5 μM significantly increased the progressive
150 motility (A + B classes) after 30 ($71.2 \pm 2\%$ vs. $51 \pm 1\%$ in controls) and 60 min ($69 \pm 3\%$ vs.
151 $50 \pm 2\%$ in controls). In the same experimental conditions 0.5 μM SPNO increased significantly
152 also VSL, VCL and VAP (data not shown).

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

163 Thereafter, the 5 μM concentration of GSNO was chosen to perform the subsequent
164 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 μ M PTIO did not affect sperm motility when
175 used alone, but when co-incubated with GSNO (5 μ M) it completely reversed the increase of
176 motility induced by GSNO (Fig. 3). In the presence of 20 μ l 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 μ m, in 24 well plates provided by Corning Incorporated, Apton, MA),
182 containing in the upper compartment 20 μ l 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

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

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

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

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

208

209 **DISCUSSION**

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

213 hyperactivation (Herrero *et al.* 1994) and in the maintenance of post-thaw human sperm motility
214 and viability (Hellstrom *et al.* 1994). Moreover, spermatozoa themselves synthesize NO, and the
215 basal release of this free radical by spermatozoa has been observed to be higher in
216 normozoospermic than in asthenospermic sperm samples; accordingly, normal spermatozoa
217 express more eNOS and generate more nitrite than spermatozoa of asthenospermic samples
218 (Lewis *et al.* 1996). Furthermore, the NO scavenger methylene blue and the NOS inhibitor N^G-
219 nitro-L-arginine methyl ester have been shown to inhibit human sperm motility (Lewis *et al.*
220 1996; Donnelly *et al.* 1997). On the other hand, when female mice null for one of the three NOS
221 isoforms (eNOS, nNOS and iNOS, respectively) mated with null male mice the rate of in vitro
222 fertilization was not inhibited (Yang *et al.* 2005): this observation does not change the meaning
223 of our results, because in the absence of a NOS isoform NO can be produced by another isoform.
224 Furthermore, these results were obtained in mice. Finally, in spermatozoa NO can be generated as
225 a consequence of a direct hydrogen peroxide attack on arginine (Aitken *et al.* 2004).

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

237 sperm motility: indeed, that study evaluated a trans-membrane migration ratio (the proportion of
238 human spermatozoa moving across a membrane separating two chambers) (Zhang & Zheng
239 1996), whereas CASA calculates the percentage of cells exhibiting a forward progressive motility
240 and the kinetic parameters of each cell.

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

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

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

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

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

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

285 2008) showed that detectable amounts of NO are produced in human cumulus fragments and
286 oviduct explants. This free radical is relatively unreactive, and is able to diffuse from the cell in
287 which it is generated to the neighbor cells, covering long distances in a very short time (Kröncke
288 *et al.* 1997). Moreover, spermatozoa themselves produce and release NO during their trip along
289 the upper female genital tract. This suggests that a complex interaction between spermatozoa,
290 granulosa cells and other cells of the female reproductive tract may submit human sperm to
291 amounts of NO that are sufficient to elicit *in vivo* the changes of motility we have observed *in*
292 *vitro*.

293 It is generally accepted that a good sperm motility is a major component of normal male
294 fertility. Men with poorly motile or immotile sperm are typically infertile or sterile (Turner
295 2006). A deeper knowledge of the role of the NO/cGMP/PKG signaling pathway in the
296 physiopathology of sperm motility could help to pharmacologically improve the fertilization
297 capacity of human sperm or, alternatively, could lead to the development of an effective and safe
298 male contraceptive based on sperm motility impairment.

299

300 **Materials and methods**

301 ***Reagents***

302 Sperm Washing Medium (SWM) was supplied by Celbio (Milan, Italy): it is based on the
303 Modified Human Tubal Fluid (Quinn *et al.* 1985), containing sodium bicarbonate (4 mM), HEPES
304 buffer (21 mM), human serum albumin (5 mg/ml). S-nitrosoglutathione (GSNO), 1H-
305 [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), 8-bromo-cGMP (8-Br-cGMP), and 3-
306 isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO).
307 The inhibitor of cGMP-dependent protein kinases (PKGs) 8-bromoguanosine-3',5'-
308 monophosphorothioate Rp-isomer (Rp-8-Br-cGMPS) was from Biolog Life Science Institute

309 (Bremen, Germany). The [³H]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 10⁶ 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% CO₂
323 atmosphere) using SWM, as previously described (Miraglia *et al.* 2010). The presence of round
324 cells was initially below 1x10⁶ 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 10⁶ cells/ml. The dose-dependent
330 effect of GSNO on sperm motility was investigated in the first 25 samples (20x10⁶ 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 (20x10⁶ cells/200 µl), and finally the ability of the cGMP-modulating

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

336 ***Analysis of Motility Parameters***

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

351 ***Measurement of intracellular cGMP***

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

357 Subsequently, the samples were centrifuged at 13,000 g for 1 min, the supernatants were
358 discarded and 50 µl of absolute ethanol were added to the pellets; ethanol was then evaporated by
359 vacuum centrifugation, and 350 µl of Tris/EDTA buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5)
360 were added. After 10 min, 100 µl of supernatant were tested for the cGMP level with a
361 [³H]cGMP immunoassay system. The cGMP content was expressed as pmol/10⁶ cells. Cross-
362 reactivity of the [³H]cGMP immunoassay system with cAMP was less than 0.001%.

363 ***Statistical analysis***

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

368

369 **Declaration of interest.**

370 There is no conflict of interest that could be perceived as prejudicing the impartiality of the
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372

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376

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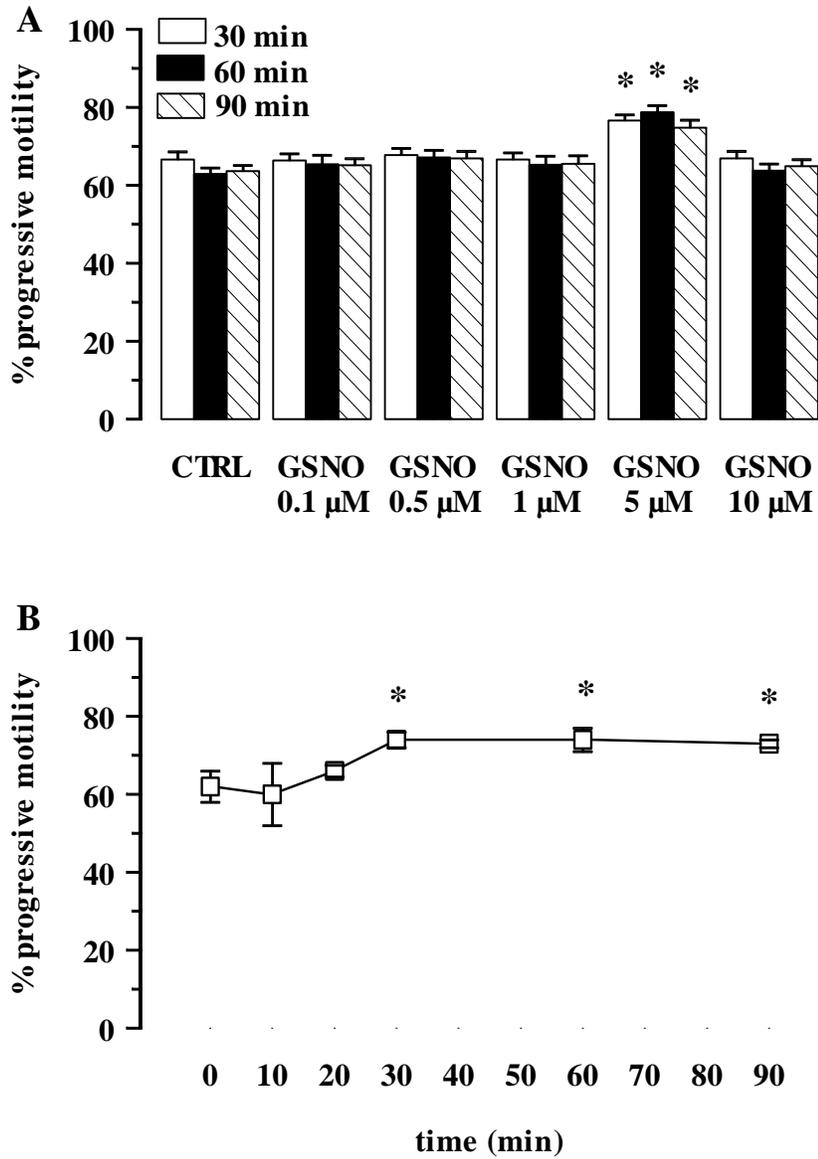
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517 **Figure legends**

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521 **Figure 1. Effect of GSNO on sperm motility patterns.**

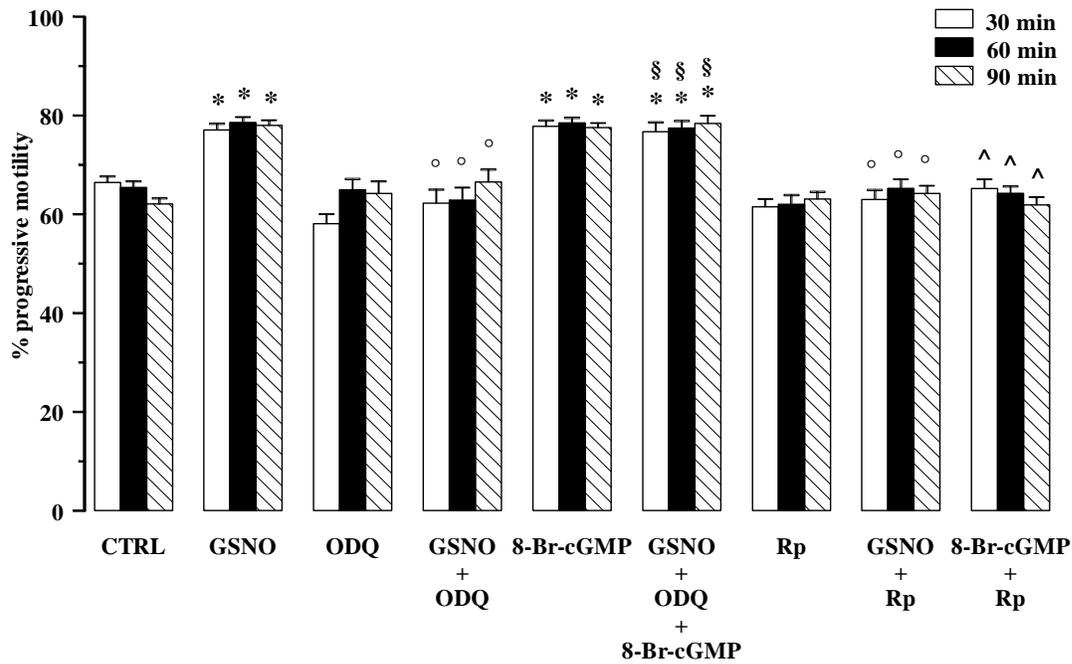
522 A. The percentage of spermatozoa exhibiting a forward progressive motility (A + B WHO

523 classes) was recorded by computer-assisted sperm analysis (CASA) after a 30 min (white bars),

524 60 min (black bars) or 90 min (hatched bars) incubation of 20×10^6 cells /200 μ l with 0.1-10 μ M
525 S-nitrosoglutathione (GSNO). All data are presented as means \pm SEM (n = 25). Significance vs.
526 control at the corresponding incubation time: * $p < 0.05$.

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

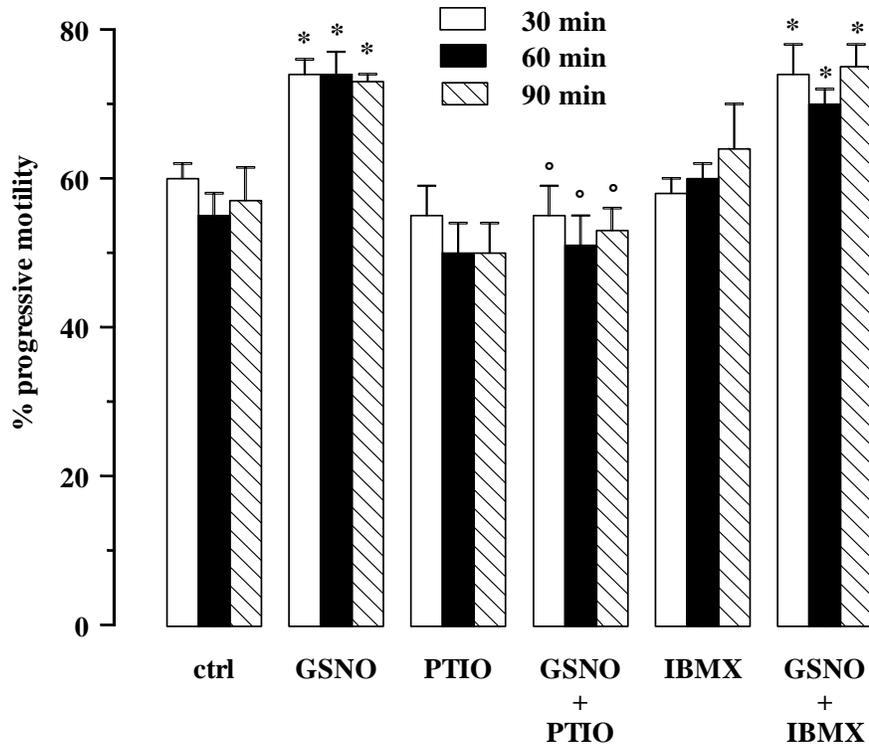
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535 **Figure 2. Effect of the modulation of the cGMP pathway on human sperm progressive**
 536 **motility.** The forward progressive motility (motility classes A + B) was assessed by CASA in
 537 human spermatozoa ($20 \times 10^6/200 \mu\text{l}$) incubated for 30, 60 or 90 min with the following
 538 substances, alone or differently combined: S-nitrosoglutathione (GSNO, 5 μM), 1H-
 539 [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ, 10 μM), 8-bromo-cGMP (8-Br-cGMP, 500
 540 μM), Rp-8-Br-cGMPS (Rp, 10 μM). All data are presented as means \pm SEM (n = 40).
 541 Significance vs. respective CTRL: * $p < 0.001$; vs. GSNO: ° $p < 0.001$; vs. GSNO+ODQ: § $p <$
 542 0.001; vs. 8-Br-cGMP: ^ $p < 0.001$.



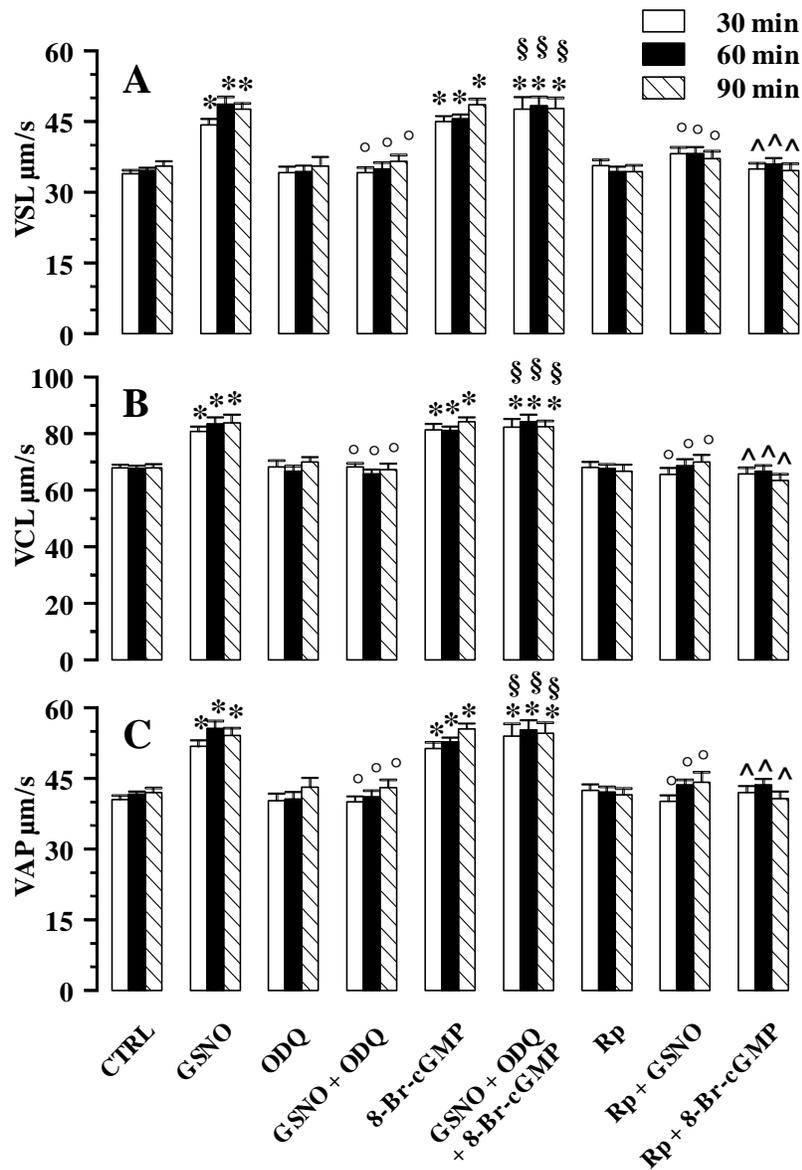
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546 **Figure 3. Effect of GSNO, PTIO and IBMX on sperm motility patterns.** The forward
 547 progressive motility (motility classes A + B) was assessed by CASA in human spermatozoa
 548 ($20 \times 10^6 / 200 \mu\text{l}$) incubated for 30, 60 or 90 min with the following substances, alone or
 549 differently combined: 5 μM GSNO, 100 μM PTIO, 200 μM IBMX. In the case of IBMX, the
 550 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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557 **Figure 4. Effects of the modulation of the cGMP pathway on straight linear velocity (VSL,**

558 **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 ($20 \times 10^6 / 200 \mu\text{l}$) 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 \pm SEM (n = 40).

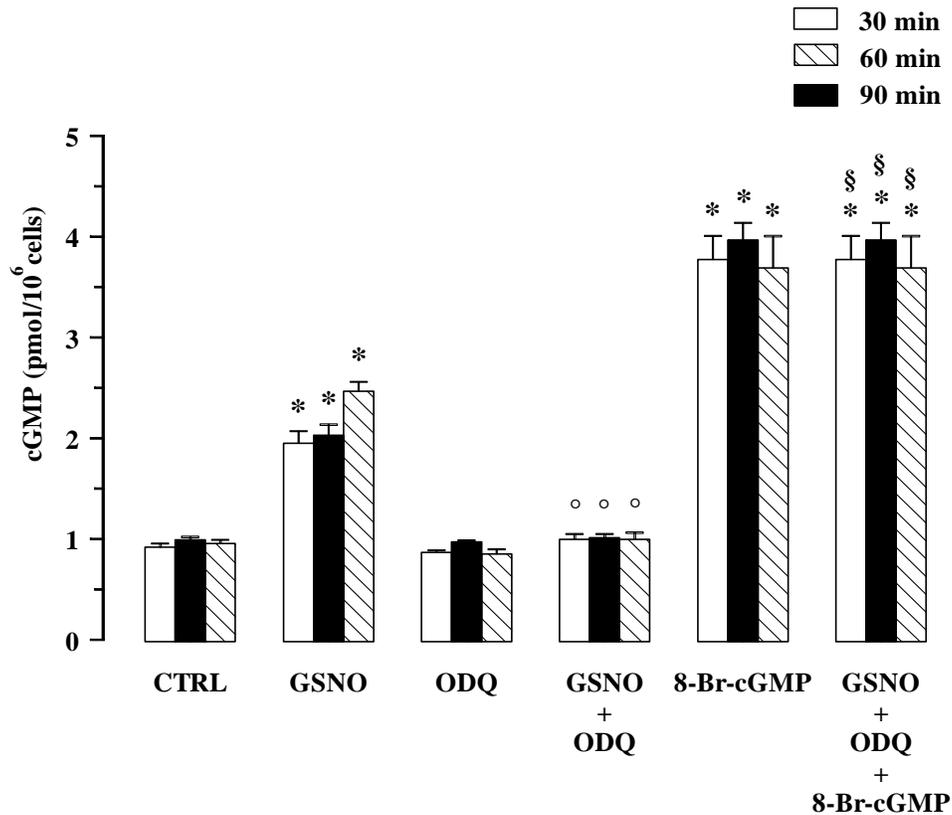
563 **A.** Significance vs. CTRL: * $p < 0.001$; vs. GSNO: $^{\circ} p < 0.001$; vs. GSNO+ODQ: $^{\S} p < 0.001$; vs.
564 8-Br-cGMP: $^{\wedge} p < 0.001$.

565 **B.** Significance vs. CTRL: * $p < 0.001$; vs. GSNO: $^{\circ} p < 0.005$; vs. GSNO+ODQ: $^{\S} p < 0.01$; vs.
566 8-Br-cGMP: $^{\wedge} p < 0.005$.

567 **C.** Significance vs. CTRL: * $p < 0.001$; vs. GSNO: $^{\circ} p < 0.001$; vs. GSNO+ODQ: $^{\S} p < 0.001$; vs.
568 8-Br-cGMP: $^{\wedge} p < 0.001$.

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573 **Figure 5. Intracellular cGMP levels in human spermatozoa treated with agents modulating**

574 **the cGMP pathway.** Sperm samples (15×10^6 cells/500 μ l) were pre-treated with 200 μ M IBMX

575 for 20 min, and subsequently they were incubated for 30, 60 or 90 min in the absence (CTRL) or

576 presence of the following substances, alone or in co-incubation: 5 μ M GSNO, 10 μ M ODQ, 500

577 μ M 8-Br-cGMP. Then, intracellular cGMP concentration was determined as described under the

578 Materials and Methods section. The measurements were performed in triplicate, and data are

579 presented as means \pm SEM (n = 5). Significance vs. CTRL: * $p < 0.001$; vs. GSNO: ° $p < 0.005$;

580 vs. GSNO+ODQ: § $p < 0.001$.

581