

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Effect of relaxin on semen quality variables of cryopreserved stallion semen

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1737766> since 2022-02-17T13:15:51Z

Published version:

DOI:10.1016/j.anireprosci.2020.106351

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **Effect of relaxin on semen quality parameters of cryopreserved stallion semen**

2 Ahmed Reda Elkhawagah¹, Tiziana Nervo², Mariagrazia Poletto², Nicola Antonio
3 Martino^{2*}, Davide Gallo³, Alessia Bertero⁴ Leila Vincenti²

4 ¹*Theriogenology Department, Faculty of Veterinary medicine, Benha University, Egypt.*

5 ²*Department of Veterinary Science, University of Torino, Grugliasco, Italy.*

6 ³*Laboratory of Endocrinology, Diabetology and Metabolism. Division of Endocrinology
7 Diabetology and Metabolism, Department of Medical Sciences, University of Torino, Italy*

8 ⁴*Department of Veterinary Science, University of Milan, Via Celoria 10, 20133 Milano -
9 Italy*

10 *Corresponding author: NA Martino, email address: nicolaantonio.martino@unito.it

11 **Abstract**

12 The aim of the study was to test the effect of different concentrations of relaxin, added in
13 the extender medium during pre-freezing incubation time, on sperm quality parameters of
14 equine frozen-thawed spermatozoa. Semen samples, collected from 3 proven fertility
15 stallions, were filtered, diluted with BotuSemen[®] and centrifuged at 600 g for 10 min. The
16 sperm pellets were resuspended in freezing medium BotuCrio[®] to a final concentration of
17 50x10⁶ sperm/ml. The diluted semen was divided into 5 experimental groups supplemented
18 with 0 (control), 12.5, 25, 50 and 100 ng/ml of relaxin. Semen samples were packed in 0.5
19 ml straws, equilibrated at 5°C for 30 min, exposed to vapor of liquid nitrogen (LN₂) for 15
20 min and plunged into LN₂. After thawing, semen samples were evaluated for motility and
21 velocity parameters, sperm vitality, mitochondrial membrane potential, apoptosis in
22 addition to plasma membrane and DNA integrities. Sperm motility parameters and the
23 percentage of viable spermatozoa were significantly improved in relaxin-treated samples
24 immediately after thawing and after 30, 60, 90 and 120 min. of incubation, with highest
25 values recorded when 12.5 and 25 ng/ml relaxin were used. Moreover, relaxin, at all tested
26 concentrations, significantly improved the sperm mitochondrial membrane potential and
27 decreased the percentage of apoptotic cells compared to the control group. Plasma
28 membrane and DNA integrities were not affected by relaxin addition. In conclusion, the
29 supplementation of relaxin in the extender before semen cryopreservation, especially at
30 12.5 and 25 ng/ml, has a positive stimulatory effect on semen quality parameters of frozen-
31 thawed stallion semen.

32 *Keywords;* Stallion semen; Cryopreservation; Relaxin; Sperm quality

33 **1. Introduction**

34 Artificial insemination, with fresh, cooled, or frozen semen, is one of the assisted
35 reproductive techniques commonly used in global equine industry (Freitas et al., 2016).
36 Therefore, semen quality, intended as the ability to accomplish fertilization, is the most
37 important factor for successful horse breeding programs (Magistrini et al., 1996; Parlevliet
38 and Colenbrander, 1999; Gadella et al., 1999; Stradaioli, 2004). The advances in stallion
39 semen cryopreservation resulted in an improvement of equine breeding industry by
40 allowing the worldwide distribution of superior genetic resources avoiding the risks

41 associated with transportation and natural mating (Neild et al., 2003; Miller, 2008; Arruda
42 de Oliveira et al., 2013). Despite all the precautions, the fertility of frozen thawed stallion
43 semen remains low compared to fresh or cooled semen (Gibb and Aitken, 2016). Many of
44 the deleterious effects induced by cryopreservation may be attributed to the osmotic stress
45 induced by the ice crystals formation (Gibb and Aitken, 2016). Frozen-thawed
46 spermatozoa have demonstrated various degree of damage such as reduced viability and
47 motility as well as perturbations in membrane integrity with consequent loss of sperm
48 fertilizing ability or even sperm death (Arruda de Oliveira et al., 2013; Watson, 2000;
49 Uysal and Bucak, 2007). Furthermore, stallion spermatozoa contain high level of
50 polyunsaturated fatty acids making these cells highly susceptible to reactive oxygen
51 species (ROS) therefore inducing membrane lipid peroxidation (Neild et al., 2003; García
52 et al., 2011; Gibb et al., 2013). In order to improve the quality of frozen-thawed semen,
53 researchers attempts to refine the extender compositions by testing new additives to
54 improve the sperm activity, the plasma membrane integrity and sperm fertility (Arruda de
55 Oliveira et al., 2013; Ghallab et al., 2017).

56 Relaxin, an insulin superfamily regulatory peptide, has been identified in boar
57 testes (Kohsaka et al., 2009) and in human seminal plasma (Ferlin et al., 2012). It has been
58 suggested to have a physiological influence on sperm motility and fertility via specific cell-
59 surface receptors on spermatozoa (Kohsaka et al., 2003). Relaxin has been demonstrated to
60 improve motility of human (Ferlin et al., 2012), bovine (Miah et al., 2007) and porcine
61 (Miah et al., 2008; Feugang et al., 2015) spermatozoa. In addition, relaxin has been found
62 to induce capacitation and acrosome reaction in fresh and frozen-thawed porcine (Miah et
63 al., 2008) and bovine semen (Miah et al., 2011). Furthermore, relaxin improved the
64 fertilizing ability of porcine (Han et al., 2006) and buffalo spermatozoa (Elkhawagah et al.,
65 2013; Elkhawagah et al., 2015). However, to the best of our knowledge, no studies have
66 been published to date on the effects of relaxin on quality parameters of equine
67 spermatozoa. In previous studies, conducted in other species, the effect of relaxin
68 supplementation in the sperm-thawing media has been investigated at the end of the
69 cryopreservation process (Miah et al., 2008; Miah et al., 2011; Elkhawagah et al., 2013;
70 Elkhawagah et al., 2015). In the present study, a different methodological approach was
71 used, indeed different concentrations of relaxin were added before sperm cryopreservation
72 procedures and different fertility parameters were investigated. This methodological
73 approach may be more suitable for in field applications, avoiding any type of treatment of
74 the semen after thawing procedures and/or before the artificial insemination.

75

76 **2. Material and methods**

77 *2.1. Semen collection and dilution*

78 Three commercial proven fertility stallions (10- to 13-year-old ages) used for
79 commercial purpose, were enrolled in this study. They were in good general condition
80 without reporting any current or past health problems. Horses were housed in the same
81 stud (Vigone, Turin, Italy) and were managed similarly for feeding and activity. Physical
82 examination of the genitalia by palpation and ultrasonography identified no abnormalities.
83 The stallions showed good libido. Semen samples were obtained once per week for 6

84 consecutive weeks towards the end of the breeding season when the commercial request
85 was reduced. In detail, a total of 18 ejaculates (6 ejaculates for each stallion) were
86 collected using artificial vagina (Colorado model Equine Artificial Vagina; ARS, Chino-
87 CA, USA) pre-warmed at 45-50 °C. Semen samples were collected in a plastic bottle and
88 filtered immediately after collection to separate gel fraction. Sperm motility, concentration,
89 viability and sperm morphology were evaluated. Samples were diluted using double
90 amount of skimmed milk and kept in water bath at 24 °C for 10 min then centrifuged at
91 600 g for 10 min. After centrifugation, supernatant was removed and the sperm pellets
92 were resuspended using freezing medium (BotuCrio[®], Botupharma, USA) to a final
93 concentration of 50×10^6 sperm/ml. The diluted semen samples from the three stallions
94 were always pooled together. This procedure was aimed to eliminate individual stallion
95 variability (Seifi-Jamadi et al., 2016; Shojaeian et al., 2018; Nouri et al., 2018). Samples
96 were divided into 5 experimental groups and supplemented with relaxin (SRP3147, Sigma-
97 Aldrich, Italy) at different concentrations: 0 (control), 12.5, 25, 50 and 100 ng/ml. Semen
98 of different experimental groups was packed in 0.5 ml polyvinyl straws (IMV, France) and
99 kept in refrigerator at 5 °C for 30 min for equilibration. Then, straws were placed 4 cm
100 over liquid nitrogen (LN₂) vapor for 15 min with an approximate temperature of vapor of
101 130 °C and then directly plunged into LN₂ for storage (Cristanelli et al. 1985). After one
102 week, frozen straws were thawed in water bath at 37 °C for 60 s for subsequent procedures.

103

104 2.2. Assessment of sperm motility and velocity parameters

105 After thawing, semen was incubated at 37 °C and motility and velocity parameters were
106 evaluated at 0, 30 min, 60 min, 90 min and 120 min of incubation using the Computer
107 Assisted Sperm Analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) by using a
108 10x objective at 37 °C and the SETUP specific for the equine species. In detail, the
109 parameters were set as follows: 40 frames acquired at 60 frames/s; minimum contrast 80;
110 minimum cell size 5 pixels; trajectory speed cutoff 20 µm/s; progressive motility cutoff 50
111 µm/s and linear motility 60%; linear motility cutoff 0 µm/s. CASA analyses were
112 conducted by loading 10 µl of semen specimen onto a pre-warmed Makler chamber and
113 submitted to evaluation. The values of total, progressive and rapid motility were recorded
114 and expressed in percentages. Additional velocity parameters including Average Path
115 Velocity (VAP, µm/s), Straight Linear Velocity (VSL, µm/s), Curvilinear Velocity (VCL,
116 µm/s), Amplitude of Lateral Head displacement, (ALH, µm), Beat Cross Frequency (BCF,
117 Hz), Linearity (LIN, $[VSL/VCL] \times 100$) and Straightness (STR, $[VSL/VAP] \times 100$), were
118 determined. In all the trials, 8 randomly-selected microscopic fields were analyzed for each
119 sample.

120 2.3. Assessment of sperm plasma membrane integrity

121 The sperm plasma membrane integrity was determined using the hypo-osmotic
122 swelling assay (HOS) according with the study of Nie and Wenzel, (2001). In detail, the
123 HOS solution was settled to ~ 100 mOsm/kg and contained 1.712 g of sucrose dissolved in
124 50 ml of sterile deionized water. Semen sample - 10 µl - was mixed with 100 µl pre-

125 warmed (37 °C) HOS solution and incubated at 37 °C for 60 min. In all six trials, for the
126 evaluation of each sample, two hundred spermatozoa were counted and the percentage of
127 cells with curled tails (swollen i.e intact plasma membrane) were recorded as HOS
128 positive.

129 *2.4. Assessment of sperm DNA integrity using SCSA*

130 The DNA integrity of spermatozoa was assessed by sperm chromatin structure
131 assay (SCSA), that utilizes the metachromatic properties of acridine orange (AO, Sigma-
132 Aldrich, USA) to distinguish between denatured and native DNA in sperm, according with
133 the procedure reported in the study of Evenson and Jost, (2000). Semen was thawed at 37
134 °C for 60 s and washed once using PBS solution by centrifugation at 500 g for 10 min.
135 Aliquots of the thawed semen were diluted to a final concentration of 2×10^6 sperm/mL
136 with TNE buffer (0.01 M Tris-Cl, 0.15 M NaCl, 1 mM EDTA, disodium pH 7.4). Then,
137 400 µl of acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% (w/v) Triton X-100, pH
138 1.2) was added. After 30 s, 1200 µl of AO staining solution containing 6 µg AO (2% in
139 H₂O,) per ml staining buffer (0.037 M citric acid, 0.126 M Na₂HPO₄, 1.1 mM EDTA
140 disodium, 0.15 M NaCl, pH 6.0), was added. Flow cytometric evaluation was conducted
141 for n=4 trials (3rd, 4th, 5th and 6th).

142

143 *2.5. Assessment of sperm mitochondrial activity*

144 The sperm mitochondrial status was assessed using JC-10 (lipophilic cation).
145 According to the manufacturer (JC-10 Assay for Flow Cytometry, Sigma-Aldrich, USA),
146 JC-10 changes reversibly its fluorescence from green (monomeric status) to orange
147 (multimeric status) when mitochondrial membrane potential is high. Frozen straws were
148 thawed at 37 °C for 60 s. and the sperm suspension was collected into polypropylene tubes
149 at a final concentration of 1×10^6 sperm/ml. One group of semen was induced for
150 apoptosis using carbonyl cyanide m-chlorophenyl hydrazine (CCCP) 1 mM and incubated
151 at 37 °C for 15 min and served as positive control. All groups were washed in 1 ml PBS by
152 centrifugation at 600 g for 10 min, then resuspended in 500 µl of JC-10 (200x JC-10 in
153 DMSO) and incubated 1 h at 37 °C, after that samples were centrifuged and diluted in 1 ml
154 PBS. Flow cytometric evaluation was conducted for n=4 trials (3rd, 4th, 5th and 6th).

155

156 *2.6. Evaluation of sperm for apoptosis (Annexin-V/PI-binding assay)*

157 Translocation of phosphatidylserine (PS) phospholipids and sperm plasma
158 membrane integrity was detected using Alexa Fluor 488 Annexin-V Apoptosis Kit
159 (V13245, Thermo Fisher Scientific, Waltham, MA, USA) and Propidium Iodide (PI)
160 according to Anzar et al., (2002) with some modification. Semen was thawed at 37 °C for
161 60 s, and washed once using PBS by centrifugation at 500 g for 10 min. Aliquots of semen
162 were diluted in Annexin-V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂,
163 pH 7.4) to a final concentration of 1×10^6 spermatozoa/ml. Aliquots of diluted semen (100
164 µl) from each group were transferred to a 5 ml culture tubes and supplemented with 5 µl of

165 Annexin-V and 1 μ l of PI (100 μ g/ml). The tubes were gently mixed and incubated for 15
166 min at room temperature in the dark. Additional 400 μ l of Annexin-V-binding buffer was
167 added to each tube prior to flow cytometric evaluation. Flow cytometric evaluation was
168 conducted immediately after the end of the staining procedure. The analyses were
169 conducted for n=4 trials (3rd, 4th, 5th and 6th).

170

171 2.7. Flow cytometric analysis

172 Samples were analyzed by a FacsStar Plus flow cytometer (Becton Dickinson
173 Immunochemistry, San Jose, CA, USA), equipped with standard optics and an air-cooled
174 argon laser operated at 488 nm excitation and 15 mW.

175 In SCSA assay, after passing a 560 nm short pass dichroic mirror, the green
176 fluorescence (FL1) was evidenced through a 515-545 nm band pass filter. The red
177 fluorescence (FL3) was evidenced after passing a 640 nm long pass filter followed by a
178 650 nm long pass filter. The sheath/sample was set on “low”, adjusted to a flow rate of
179 200 events/s when analyzing a sample with a concentration of 1×10^6 sperm/ml.
180 Immediately after the addition of the AO staining solution, the sample was placed in the
181 flow cytometer. Recording of the red and green fluorescence was started exactly 3 min
182 after the beginning of the staining procedure. In each sample 10×10^3 cells were collected
183 using the Cellquest software (Becton Dickinson Immunochemistry, San Jose, CA, USA).
184 The X-mean (red) and Y-mean (green) values of each sample were recorded.

185 In Annexin-V/PI-binding assay: for each cell, forward light scatter (FSC),
186 orthogonal light scatter (SSC), FITC fluorescence (FL1) and PI fluorescence (FL3) were
187 evaluated using the Cellquest software. Acquisition gate applied in the FSC/SSC two-
188 dimensional histogram was used to restrict the analysis to spermatozoa and to eliminate
189 small debris and other particles for further analysis. For the gated sperm cells, four
190 different kinds of sperm were observed. The percentages of viable spermatozoa (Annexin-
191 V^- , PI^-), necrotic sperm (annexin- V^- , PI^+), apoptotic sperm (annexin- V^+ , PI^+) and early
192 apoptotic (annexin- V^+ , PI^-) were evaluated, based on regions determined from single-
193 stained and unstained control samples.

194 In mitochondrial activity assessment by JC-10: a total of 10.000 gated events/s
195 were analyzed per sample. The sample was adjusted to a flow rate of 200 events/set. A 488
196 nm filter was used for excitation of JC-10. Emission filters of 535 nm and 595 nm were
197 used to quantify the population of spermatozoa with green (JC-10 monomers) and orange
198 (JC-10 aggregates) fluorescence, respectively. Frequency plots were prepared for FL1
199 (green) and FL2 (orange) to determine the percentage of the population stained green and
200 orange. Percentage of orange stained cells was recorded, being considered as a population
201 of cells with High Mitochondrial Membrane Potential (HMMP).

202

203 2.8. Statistical analysis

204 Data were analyzed using the Generalized Linear Model (GLM) procedure for
205 repeated measurements (SPSS, Ver. 16), and presented as mean \pm SEM. Pearson

206 correlations have been used to find the correlations between the different experimental
207 parameters. Values with $P < 0.05$ were considered as statistically significant.

208

209 **3. Results**

210 *3.1. Effect of relaxin on motility parameters of frozen-thawed stallion semen*

211 The mean values of sperm motility parameters evaluated by CASA analysis are
212 presented in Table 1. Relaxin incorporation at different concentrations in extender medium
213 during cryopreservation improved the motility parameters of frozen-thawed stallion sperm.
214 In detail, relaxin significantly improved the total sperm motility after thawing at any
215 examined times of incubation and the highest values were recorded with semen samples
216 treated with 12.5 ng/ml relaxin after 90 min. post thawing, or at 25 ng/ml relaxin
217 concentration after 30 and 60 min. post thawing (up to $P < 0.001$; Table 1). Whereas,
218 samples treated with 50 ng/ml relaxin showed the highest value after 120 min. post
219 thawing ($P < 0.05$; Table 1). Similarly, the progressive motility was significantly improved
220 in relaxin-treated samples immediately after thawing and after 30, 60, 90 and 120 min. of
221 incubation and the highest values were recorded when 12.5 ng/ml relaxin was added
222 ($P < 0.05$; Table 1). Regarding the rapid motility, it was significantly improved by relaxin
223 addition with highest values obtained at the same concentrations and times identified for
224 progressive motility (up to $P < 0.001$; Table 1).

225

226 *3.2. Effect of relaxin on velocity parameters of frozen-thawed stallion semen*

227 The effects of relaxin on sperm velocity parameters are detailed in Table 2. Relaxin
228 incorporation at different concentrations in extender medium during cryopreservation
229 significantly improved several velocity parameters of stallion semen after thawing and
230 incubation for 0, 30, 60 and 120 min. at 37 °C. In detail, VAP, VSL and VCL, velocity
231 parameters also associated with a capacitated state of spermatozoa, were significantly
232 improved by relaxin addition especially at 12.5 and 25 ng/ml from 0 up to 120 min. of
233 incubation (up to $P < 0.001$; Table 2). As well as, STR and LIN, which provide important
234 information about the linearity of the sperm velocity path, were significantly improved
235 after thawing by relaxin addition and highest values were recorded especially at 12.5 ng/ml
236 from 0 up to 120 min. of incubation (up to $P < 0.001$; Table 2). Similarly, ALH and BCF,
237 other velocity parameters correlated with sperm head movement, were also affected by
238 relaxin addition.

239 *3.3. Effect of relaxin on plasma membrane and DNA integrity and mitochondrial* 240 *membrane potential*

241 The effects of relaxin on sperm vitality are detailed in Table 3. On the basis of the
242 results obtained on sperm motility, the timing point of 60 min. was selected as the one in
243 which best results were obtained, thus it was used for further analyze the effect of relaxin
244 on other sperm quality parameters. Interestingly, relaxin at all tested concentrations,

245 significantly improved the sperm mitochondrial membrane potential (HMMP) compared to
246 the control group ($P<0.01$). No statistical differences were found on the effects of relaxin
247 on plasma membrane and DNA integrities at any tested concentrations (Table 3).

248 *3.4. Effect of relaxin on semen apoptosis*

249 The effects of relaxin on sperm apoptosis are presented in Table 4. At all tested
250 concentrations, relaxin significantly ($P<0.02$) decreased the percentage of apoptotic cells.
251 Higher values of normal viable sperm were found in relaxin-treated samples at 12.5, 50
252 and 100 ng/ml, even if there were not significant differences (Table 4). Whereas, no
253 statistical differences were found between the percentage of normal viable and necrotic
254 sperm cells compared with controls.

255 *3.5. Correlations between the different sperm quality parameters*

256 The correlations between the different semen quality parameters, calculated on the
257 basis of the effects of relaxin, are summarized in Table 5. The total sperm motility has a
258 significant positive correlation with progressive motility, rapid motility, intact DNA
259 ($P<0.01$) and HMMP ($P<0.05$), whereas it is negatively correlated with apoptotic sperm,
260 VAP ($P<0.05$), VCL, VSL, ALH, STR and LIN ($P<0.01$). The progressive motility is
261 positively correlated with rapid motility ($P<0.01$) and HMMP ($P<0.05$), whereas it is
262 negatively associated with VAP, ALH, BCF and STR ($P<0.01$).

263

264 **4. Discussion**

265 Semen cryopreservation plays an important role in preserving genetic materials in
266 humans and domestic animals (Axner et al., 2004). However, the cryopreservation process
267 induces detrimental structural effects on spermatozoa during freezing and thawing
268 procedures, as a result of exposure to different stressful factors including thermal,
269 chemical, osmotic, mechanical and oxidative stress (Holt et al., 1992). These changes
270 result in perturbations to the sperm organelles, changes in membrane fluidity and
271 enzymatic viability, loss of plasma membrane and acrosome integrity and finally decreased
272 sperm motility (Alvarez and Storey, 1983; Woelders et al., 1997). All these alterations
273 contribute to decrease the semen fertilizing capability (Tekin et al., 2006) and in particular,
274 the equine sperm appear to be extremely sensitive to alterations generated by the
275 cryopreservation process (Ball, 2008; Gibb and Aitken, 2016). However, differences exist
276 in the ability of sperm to survive cryopreservation, also between individual males within a
277 species. Thus, with the aim to eliminate the potential individual stallion variability, the
278 semen samples from all three stallions were pooled together, as it was also reported in
279 previous studies in the same species (Seifi-Jamadi et al., 2016; Shojaeian et al., 2018;
280 Nouri et al., 2018).

281 In different species, relaxin has been demonstrated to improve sperm motility
282 (Ferlin et al., 2012, Miah et al., 2007, Miah et al., 2008; Feugang et al., 2015), capacitation
283 and acrosome reaction (Miah et al., 2008; Miah et al., 2011), and fertilizing ability of
284 spermatozoa (Han et al., 2006; Elkhawagah et al., 2015). However, in previous studies, the

285 effects of relaxin have been investigated on fresh semen (Han et al., 2006) or on
286 cryopreserved semen with this compound added during thawing procedures (reviewed by
287 Miah et al., 2015). Instead, in the present study, by using a different methodological
288 approach we improved several sperm quality parameters of cryopreserved stallion sperm
289 by the incorporation of relaxin at different concentrations (12.5, 25, 50 and 100 ng/ml) in
290 the extender medium before the cryopreservation process.

291 Sperm motility, evaluated by CASA analysis, is one of the most reliable parameter
292 associated with sperm fertilizing potential (Verstegen et al., 2002). In addition, Voss et al.,
293 (1981) stated that spermatozoa motility is the most reliable method to estimates field
294 fertility. Our results revealed that 12.5 and 25 ng/ml relaxin significantly improved the
295 total and the progressive sperm motility after thawing at different incubation times.
296 Moreover, other sperm velocity parameters were also improved by relaxin addition, and
297 these parameters such as VCL, VSL and ALH positively correlate with sperm capacitation
298 and fertility. However, semen samples did not show excellent post-thawing motility and it
299 might depend from both the use of sperm collected and frozen at the end of the commercial
300 season and the use of nitrogen vapors instead of programmable freezers for
301 cryopreservation procedure. The improvement of the semen quality, even with these
302 conditions, strengthens the consideration on the positive effects of the relaxin addition.

303 Our result are in agreement with other studies performed in different species. In
304 detail, relaxin has been found to improve sperm motility of boar (Feugang et al., 2015;
305 Miah et al., 2006; Kohsaka et al., 2001), bovine (Miah et al., 2007) and buffalo
306 (Elkhawagah et al., 2015) spermatozoa. The influence of relaxin on sperm motility and
307 fertility has been suggested to occur through specific cell-surface receptors on sperm head
308 and tail (Kohsaka et al., 2003; Feugang et al., 2015). Our result in the equine species could
309 be compared with the study reported by Burns and Fleming, (1989), in which a significant
310 improvement in the total sperm motility of frozen-thawed stallion semen treated with 400
311 ng/ml relaxin for 1 hr at room temperature, was identified. However, also in this study,
312 relaxin was supplemented in the sperm-thawing medium.

313 It has been stated that the relationship between motility and fertility of stallion
314 frozen semen is not the only measure of the fertilizing potential (Bataille et al., 1990). In
315 fact, Graham, (1996) and Katila, (2001) recommended that spermatozoa should possess
316 several quality parameters including motility, normal morphology, plasma membrane
317 integrity (Andrabi et al., 2016; Aurich, 2005; Baumber et al., 2005) sufficient metabolism
318 for energy production and membrane integrity to acquire the fertilizing ability. Therefore,
319 HOS test was performed to test the effect of relaxin on sperm quality parameters of stallion
320 spermatozoa. However, HOS test did not reveal any difference within the experimental
321 groups in accordance with that reported by Feugang et al., (2015) who denied the
322 relationship between relaxin treatment and boar sperm plasma membrane integrity.

323 By the evaluation of the effects of relaxin on sperm DNA integrity by using SCSA,
324 our results did not show any differences within experimental groups. It has been stated that
325 the loss of sperm fertilizing ability after freezing/thawing in boar could be attributed to
326 factors other than sperm chromatin structural damage, as it is very resistant (Evenson et al.,
327 1994). However, Neild et al., (2003) and Ortega-Ferrusola et al., (2009) stated that
328 apoptosis-like mechanisms and lipid peroxidation of plasma membrane are associated with
329 cryopreserved equine sperm premature aging and DNA fragmentation.

330 Semen cryopreservation interferes with sperm-membrane functions (Chaveiro et al.,
331 2007) inducing membrane phospholipids asymmetry and progressively damaging the
332 cellular integrity (Martin et al., 1995) with the induction of apoptotic-like changes (Crabo,
333 2001). Our results showed that all concentrations of relaxin significantly decreased the
334 percentage of apoptotic spermatozoa compared to the control group, and the lowest value
335 was recorded with relaxin 100 ng/ml. These results are in agreement with that reported by
336 Ferlin et al., (2012) who found that treatment of human sperm with 100 nM relaxin prevent
337 apoptosis and increased the percentage of viable sperm.

338 Sperm mitochondria are considered to be the site for production of the adenosine-
339 triphosphate (ATP), which is essential for sperm motility (Silva and Gadella, 2006; Amaral
340 et al., 2013). Therefore, the sperm mitochondrial status is an important factor for sperm
341 fertilizing ability. Osmotic shock is a major factor in sperm damage during
342 cryopreservation (Prien and Iacovides, 2016) that leads to a loss in viability by decreasing
343 the mitochondrial membrane potential (Papa et al., 2011). In our study, we improved the
344 mitochondrial membrane potential of cryopreserved stallion semen by the incorporation of
345 relaxin in the freezing medium at different concentrations. This is in agreement with that
346 reported by Ferlin et al., (2012) who found that relaxin at 10 and 100 nM preserved
347 HMMP of human sperm. Moreover, we found a significant positive correlation between
348 motility and quality parameters such as mitochondrial membrane potential and sperm
349 apoptosis.

350 These results reveal that relaxin could be added in the extender medium, before the
351 sperm cryopreservation procedures rather than in the post-thawing media or even in the in
352 vitro fertilization/embryo culture media. Our methodological approach could be more
353 suitable for in field applications, avoiding any type of treatment of the semen after thawing
354 procedures and/or before the artificial insemination and we can recommend the use also in
355 reduced quality sperm ejaculate
356

357 **5. Conclusions**

358 In the present study we improved the post-thawing fertility parameters of
359 cryopreserved stallion semen by incorporation of relaxin at different concentrations (12.5,
360 25, 50 and 100 ng/ml) in extender medium, before cryopreservation procedures. Our
361 results revealed that 12.5 and 25 ng/ml relaxin had a positive stimulatory effect on
362 different quality parameters of frozen-thawed semen including an improvement of sperm
363 motility and velocity parameters in addition to an increase of the percentage of sperm with
364 higher mitochondrial membrane potential and a reduction of sperm apoptosis.

365

366 **Conflict of interest**

367 No conflict of interest was reported by the authors.

368 **Acknowledgements**

369 The authors thank the personnel of the equine stud “Le Fontanette” for their assistance
370 during collection of semen samples. Special thanks to the Laboratory of Endocrinology of
371 the Molinette hospital for their help in flow cytometric analysis.

372

373 **References**

374 Alvarez, J.G., Storey, B.T., 1983. Taurine, hypotaurine, epinephrine and albumin inhibit
375 lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol.*
376 *Reprod.* 29, 548-555.

377 Amaral, A., Lourenço, B., Marques, M., Ramalho-Santos, J., 2013. Mitochondria
378 functionality and sperm quality. *Reproduction* 146, 163–174.

379 Andrabi, S.M.H., Khan, L.A., Shahab, M., 2016. Isolation of bacteria in semen and
380 evaluation of antibiotics in extender for cryopreservation of buffalo (*Bubalus*
381 *bubalis*) bull spermatozoa. *Andrologia* 48, 1166–1174.

382 Anzar, M., He, L., Buhr, M.M., Kroetsch, T.G., Pauls, K.P., 2002. Sperm apoptosis in
383 fresh and cryopreserved bull semen detected by flow cytometry and its relationship
384 with fertility. *Biol. Reprod.* 66, 354–360.

385 Arruda de Oliveira, R., Wolf, C.A., Viu, M.A., Gambarini, M.L., 2013. Addition of
386 glutathione to an extender for frozen equine semen. *J. Equine Vet. Sci.* 33, 1148-
387 1152.

388 Aurich, C., 2005. Factors affecting the plasma membrane function of cooled-stored stallion
389 spermatozoa. *Anim. Reprod. Sci.* 89, 65-75.

390 Axner, E., Hermansson, U., Linde-Forsberg, C., 2004. The effect of Equex STM paste and
391 sperm morphology on post-thaw survival of cat epididymal spermatozoa, *Anim.*
392 *Reprod. Sci.* 84, 179-191.

393 Ball, B., 2008. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function
394 and preservation in the horse. *Anim. Reprod. Sci.* 107, 257–267.

395 Bataille, B., Magistrini, M., Palmer, E., 1990. Analyse objective de la mobilite du sperme
396 congele-decongele d'etalon. Essay de correlation avec la fertilite. (Objective
397 determination of sperm motility in frozen-thawed stallion semen. Correlation with
398 fertility). *Anim. Breed Abstr.* 1990, 96-106.

399 Baumber, J., Ball, B.A., Linfor, J.J., 2005. Assessment of the cryopreservation of equine
400 spermatozoa in the presence of enzyme scavengers and antioxidants. *Am. J. Vet.*
401 *Res.* 66, 772-779.

402 Burns, P.J., Fleming, S.A., 1989. Computerized analysis of sperm motion: effects of
403 relaxin on cryopreserved equine spermatozoa. *J. Androl.* 10, 31.

404 Chaveiro, A., Santos, P., Da Silva, F.M., 2007. Assessment of sperm apoptosis in
405 cryopreserved bull semen after swim-up treatment: a flow cytometric study.
406 *Reprod. Dom. Anim.* 42, 17-21.

407 Crabo, B.G., 2001. Physiological aspects of stallion semen cryopreservation. *Proceedings*
408 *of the Annual Convention – AAEP.* 47, 291-295.

409 Cristanelli, M.J.A., Amann, R.P., Squires, E.L., Pickett, B.W., 1985. Effects of egg yolk
410 and glycerol level in lactose-EDTA-egg yolk extender and of freezing rate on the
411 motility of frozen-thawed stallion spermatozoa. *Theriogenology* 23, 25-38.

412 Elkhawagah, A.R., Longobardi, V., Neglia, G., Salzano, A., Zullo, G., Sosa, G.A.,
413 Campanile, G., Gasparrini, B., 2015. Effect of relaxin on fertility parameters of
414 frozen-thawed buffalo (*Bubalus bubalis*) sperm. *Reprod. Domest. Anim.* 50, 756-
415 762.

- 416 Elkhawagah, A.R., Longobardi, V., Sosa, G.A., Albero, G., Salzano, A., Zullo, G., Sosa,
417 G.A., Campanile, G., Gasparini, B., 2013. Effect of relaxin on fertilizing ability of
418 buffalo sperm. *Reprod. Fertil. Develop.* 26(1), 186-186.
- 419 Evenson, D., Jost, L., 2000. Sperm chromatin structure assay is useful for fertility
420 assessment. *Methods Cell. Sci.* 22, 169-89.
- 421 Evenson, D.P., Thompson, L., Jost, L., 1994. Flow cytometric evaluation of boar semen by
422 the sperm chromatin structure assay as related to cryopreservation and fertility.
423 *Theriogenology* 41, 637-651.
- 424 Ferlin, A., Menegazzo, M., Gianesello, L., Selice, R., Foresta, C., 2012. Effect of Relaxin
425 on human sperm functions. *J. Androl.* 33, 474-482.
- 426 Feugang, J.M., Rodríguez-Muñoz, J.C., Dillard, D.S., Crenshaw, M.A., Scott, T., Willard,
427 S.T., Ryan, P.L., 2015. Beneficial effects of relaxin on motility characteristics of
428 stored boar spermatozoa. *Reprod. Biol. Endocrinol.* 13, 24-33.
- 429 Freitas, M.L., Bouéres, C.S., Pignataro, T.A., Gonçalves de Oliveira, F.J., de Oliveira, Viu,
430 M.A., Arruda de Oliveira R., 2016. Quality of fresh, cooled, and frozen semen from
431 stallions supplemented with antioxidants and fatty acids. *J. Equine Vet. Sci.* 46, 1-6.
- 432 Gadella, B.M., Flesch, F.M., van Golde, L.M., Colenbrander, B., 1999. Dynamics in the
433 membrane organization of the mammalian sperm cell and functionality in
434 fertilization. *Vet. Q.* 21, 142-6.
- 435 García, B.M., Fernández, L.G., Ferrusola, C.O., Rodríguez, A.M., Bolaños, J.M.G.,
436 Martínez, H.R., Tapia, J.A., Morcuende, D., Peña, F.J., 2011. Fatty acids and
437 plasminogen of the phospholipids of the sperm membranes and their relation with
438 the post-thaw quality of stallion spermatozoa. *Theriogenology* 75, 811-818.
- 439 Ghallab, A.M., Shahat, A.M., Fadl, A.M., Ayoub, M.M., Moawad, A.R., 2017. Impact of
440 supplementation of semen extender with antioxidants on the quality of chilled or
441 cryopreserved Arabian stallion spermatozoa. *Cryobiology* 79, 14-20.
- 442 Gibb, Z., Aitken, R.J., 2016. The impact of sperm metabolism during in vitro storage: the
443 stallion as a model. *Biomed. Res. Int.* 9380609.
- 444 Gibb, Z., Butler, T.J., Morris, L.H., Maxwell, W.M., Grupen, C.G., 2013. Quercetin
445 improves the post thaw characteristics of cryopreserved sex-sorted and not sorted
446 stallion sperm. *Theriogenology* 79, 1001-1009.
- 447 Graham, J.K., 1996. Analysis of stallion semen and its relation to fertility. *Vet. Clin. North*
448 *Am. Equine Pract.* 12, 119-129.
- 449 Han, Y.J., Miah, A.G., Yoshida, M., Sasada, H., Hamano, K., Kohsaka, T., Tsujii, H.,
450 2006. Effect of relaxin on in vitro fertilization (IVF) of porcine oocytes. *J. Reprod.*
451 *Dev.* 52, 657-662.
- 452 Holt, W.V., Head, M.F., North, R.D., 1992. Freeze-induced membrane damage in ram
453 spermatozoa is manifested after thawing: observations with experimental
454 cryomicroscopy. *Biol. Reprod.* 46, 1086-1094.
- 455 Katila, T., 2001. In vitro evaluation of frozen-thawed stallion semen: a review. *Acta Vet.*
456 *Scand.* 42, 201-217
- 457 Kohsaka, T., Hamano, K., Sasada, H., Watanabe, S., Ogine, T., Suzuki, E., Nishida, S.,
458 Takahara, H., Sato, E., 2003. Seminal immunoreactive relaxin in domestic animals
459 and its relationship to sperm motility as a possible index for predicting the
460 fertilizing ability of sires. *Int. J. Androl.* 26, 115-120.
- 461 Kohsaka, T., Kato, S., Qin, S., Minagawa, I., Yogo, K., Kawarasaki, T., Sasada, H., 2009.
462 Identification of boar testis as a source and target tissue of relaxin. *Ann. N. Y.*
463 *Acad. Sci.* 1160, 194-196.
- 464 Kohsaka, T., Sasada, H., Takahara, H., Sato, E., Bamab, K., Sherwood, O.D., 2001. The
465 presence of specific binding sites on boar spermatozoa for porcine relaxin and its
466 action on their motility characteristics. *J. Reprod. Dev.* 47, 197-204.

- 467 Magistrini, M., Vidamet, M., Clement, F., Palmer, E., 1996. Fertility prediction in
468 stallions. *Anim. Reprod. Sci.* 42, 181–8.
- 469 Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., La Face,
470 D.M., Green D.R., 1995. Early redistribution of plasma membrane
471 phosphatidylserine is a general feature of apoptosis regardless of the initiating
472 stimulus: inhibition by over expression of Bcl-2 and Abl. *J. Exp. Med.* 182, 1545-
473 1556.
- 474 Miah, A., Hossain, M.S., Tareq, K., Salma, U., Hammano, K., Kohsaka, T., Tsujii H.,
475 2006. Effect of relaxin on motility, acrosome reaction and viability of
476 cryopreserved boar spermatozoa. *Reprod. Med. Biol.* 5, 215-220.
- 477 Miah, A.G., Salma, U., Sinha, P.B., Hölker, M., Tesfaye, D., Cinar, M.U., Tsujii, H.,
478 Schellander, K., 2011. Intracellular signaling cascades induced by relaxin in the
479 stimulation of capacitation and acrosome reaction in fresh and frozen-thawed
480 bovine spermatozoa. *Anim. Reprod. Sci.* 125, 31-40.
- 481 Miah, A.G., Salma, U., Takagi, Y., Hamano, K., Kohsaka, T., Tsujii, H., 2008. Effect of
482 relaxin and IGF-I on capacitation, acrosome reaction, cholesterol efflux and
483 utilization of labeled and unlabeled glucose in porcine spermatozoa. *Reprod. Med.*
484 *Biol.* 7, 29-36.
- 485 Miah, A.G., Salma, U., Tareq, K.M., Kohsaka, T., Tsujii, H., 2007. Effect of relaxin on
486 motility, acrosome reaction, and utilization of glucose in fresh and frozen-thawed
487 bovine spermatozoa. *Anim. Sci. J.* 78, 495–502.
- 488 Miller CD., 2008. Optimizing the use of frozen-thawed equine semen. *Theriogenology* 70,
489 463-8.
- 490 Neild, D.M., Gadella, B.M., Chaves, M.G., Miragaya, M.H., Colenbrander, B., Agüero, A.,
491 2003. Membrane changes during different stages of a freeze-thaw protocol for
492 equine semen cryopreservation. *Theriogenology* 59, 1693-1705.
- 493 Nie, G.J., Wenzel, J.G.W., 2001. Adaptation of the hypo-osmotic swelling test to assess
494 functional integrity of stallion spermatozoal plasma membranes. *Theriogenology*
495 55, 1005-1018.
- 496 Nouri, H., Shojaeian, K., Samadian, F., Lee, S., Kohram, H., Lee, J., 2018. Using
497 resveratrol and epigallocatechin-3-gallate to improve cryopreservation of stallion
498 spermatozoa with low quality. *J. Equine Vet. Sci.* 70, 18-25.
- 499 Ortega Ferrusola, C., Gonzalez Fernandez, L., Macias Garcia, B., Salazar-Sandoval, C.,
500 Rodriguez, A.M., Martinez, H.R., Tapia, J.A., Peña, F.J., 2009. Effect of
501 cryopreservation on nitric oxide production by stallion spermatozoa. *Biol. Reprod.*
502 81, 1106-1111.
- 503 Papa, F.O., Felício, G.B., Melo-Oña, C.M., Alvarenga, M.A., De Vita, B., Trinque, C.,
504 Puoli-Filho, J.N., Dell'Aqua, J.A. Jr., 2011. Replacing egg yolk with soybean
505 lecithin in the cryopreservation of stallion semen. *Anim. Reprod. Sci.* 129, 73-77.
- 506 Parlevliet, J.M., Colenbrander, B., 1999. Prediction of first season stallion fertility of 3-
507 year-old Dutch Warmblood with prebreeding assessment of percentage of
508 morphologically normal live sperm. *Equine Vet. J.* 31, 248–51.
- 509 Prien, S., Iacovides, S., 2016. Cryoprotectants & cryopreservation of equine semen: a
510 review of industry cryoprotectants and the effects of cryopreservation on equine
511 semen membranes. *J. Dairy Vet. Anim. Res.* 3, 00063.
- 512 Seifi-Jamadi, A., Kohram, H., Zareh-Shahne, A., Dehghanizadeh, P., Ahmad, E., 2016.
513 Effect of various concentrations of butylated hydroxyanisole and butylated
514 hydroxytoluene on freezing capacity of Turkman stallion sperm. *Anim. Reprod.*
515 *Sci.* 170, 108e13.

- 516 Shojaeian, K., Nouri, H., Kohram, H., 2018. Does MnTBAP ameliorate DNA
517 fragmentation and in vivo fertility of frozen-thawed Arabian stallion sperm?
518 *Theriogenology* 108, 16-21
- 519 Silva, P.F., Gadella, B.M., 2006. Detection of damage in mammalian sperm cells.
520 *Theriogenology* 65, 958-978.
- 521 Stradaoli, G., Sylla, L., Zelli, R., Chiodi, P., Monaci, M., 2004. Effect of L-carnitine
522 administration on the seminal characteristics of oligoasthenospermic stallions.
523 *Theriogenology* 62, 761-77.
- 524 Tekin, N., Uysal, O., Akcay, E., Yavas, I., 2006. Effects of different taurine doses and
525 freezing rate on freezing of ram semen. *Ank. Univ. Vet. Fak. Derg.* 53, 179-184.
- 526 Uysal, O., Bucak, M.N., 2007. Effects of oxidized glutathione, bovine serum albumin,
527 cysteine and lycopene on the quality of frozen-thawed ram semen. *Acta Vet. Brno.*
528 76, 383-390.
- 529 Verstegen, J., Iguer-Ouada, M., Onclin, K., 2002. Computer assisted semen analyzers in
530 andrology research and veterinary practice. *Theriogenology* 57, 149-179.
- 531 Voss, J.L., Pickett, B.W., Squires, E.L., 1981. Stallion spermatozoal morphology and
532 motility and their relationship to fertility. *J. Am. Vet. Med. Assoc.* 178, 287-289.
- 533 Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. *Anim.*
534 *Reprod. Sci.* 60-61, 481-492.
- 535 Woelders, H., Matthijs, A., Engel, B., 1997. Effects of trehalose and sucrose, osmolality of
536 the freezing medium and cooling rate on viability and intactness of bull sperm after
537 freezing and thawing. *Cryobiology* 35, 93-105.