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

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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; Stradaoli, 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

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

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

2. Material and methods

2.1. Semen collection and dilution

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

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

103

2.2. Assessment of sperm motility and velocity parameters

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

2.3. Assessment of sperm plasma membrane integrity

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

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

2.4. Assessment of sperm DNA integrity using SCSA

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

2.5. Assessment of sperm mitochondrial activity

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

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

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

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372

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