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Effect of relaxin on semen quality variables of cryopreserved stallion semen

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ABSTRACT

The aim of the study was to ascertain effects of different concentrations of relaxin added to extender medium during the pre-freezing incubation periods on quality variables of stallion frozen-thawed spermatozoa. Semen samples collected from three stallions were filtered, diluted with skim milk, and centrifuged at 600*g* for 10 min. Sperm pellets were suspended in BotuCrio freezing medium to a final concentration of 50 × 10^6 sperm/mL. The diluted semen was divided into five experimental groups supplemented with 0 (control), 12.5, 25, 50, or 100 ng/mL of relaxin. The semen samples were transferred into 0.5 mL straws, equilibrated at 5 °C for 30 min, and placed in liquid nitrogen (LN_2) vapour for 15 min before being plunged into LN_2. After thawing, sperm samples were evaluated for motility and velocity variables, mitochondrial membrane potential, apoptosis, and plasma membrane and DNA integrities. For sperm motility variables, there were dose- and time-dependent effects, with the largest values recorded when 12.5 and 25 ng/mL relaxin were used for 0–120 min of incubation. Furthermore, at all of the concentrations at which there were evaluations, relaxin additions to semen diluent led to a marked improvement in sperm mitochondrial membrane potential and a lesser percentage of apoptotic cells compared to the control group. Plasma membranes and DNA integrities were not affected by relaxin supplementations to the diluent. In conclusion, supplementation of relaxin in extender before semen cryopreservation, especially at 12.5 and 25 ng/mL, had a positive effect on the sperm quality variables.

1. Introduction

Artificial insemination (AI) with fresh, cooled, or frozen semen is one of the assisted reproductive techniques commonly used in the equine industry globally (Freitas et al., 2016). Sperm quality, the capacity for fertilization, is the most important factor for successful horse AI breeding programmes (Magistrini et al., 1996; Parlevliet and Colenbrander, 1999). The advances in stallion semen cryopreservation has resulted in an improved genetic foundation in the horse industry as a result of enabling the worldwide distribution of superior genetic resources while avoiding the risks associated with transportation and natural mating (Miller, 2008;

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Arruda de Oliveira et al., 2013). Even though there are precautions, the quality of frozen-thawed stallion semen remains less compared to fresh or cooled semen. Many of the deleterious effects induced by cryopreservation may be attributed to the osmotic stress induced by ice crystal formation (Gibb and Aitken, 2016). Results evaluating frozen-thawed spermatozoa have indicated various amounts of damage such as reduced viability and motility as well as perturbations in membrane integrity with consequent loss of sperm fertilizing capacity or even sperm death (Watson, 2000; Uysal and Bucak, 2007; Arruda de Oliveira et al., 2013). Furthermore, stallion spermatozoa contain large amounts 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). To improve the quality of frozen-thawed sperm, researchers have attempted to refine the extender compositions by testing new additives to improve the sperm activity, 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 human seminal plasma (Ferlin et al., 2012). It has been suggested to have a physiological effect on sperm motility and fertility via specific cell surface receptors on spermatozoa (Kohsaka et al., 2003). Relaxin improves the motility of human (Ferlin et al., 2012), bull (Miah et al., 2007), and boar (Miah et al., 2008; Feugang et al., 2015) spermatozoa. Relaxin also induces sperm capacitation and the acrosome reaction in fresh and frozen-thawed boar (Miah et al., 2008) and bull (Miah et al., 2011) semen. Furthermore, relaxin treatments led to an improvement in the fertilizing capacity of boar (Han et al., 2006) and buffalo bull (Elkhawagah et al., 2013, 2015) spermatozoa. To the best of our knowledge, there have been no studies published focusing on the effects of relaxin on the quality of stallion spermatozoa. In previous studies conducted with animals of other species, the effect of relaxin supplementation to sperm-thawing media was investigated at the end of the cryopreservation process (Miah et al., 2008, 2011; Elkhawagah et al., 2013, 2015). In the present study, varying concentrations of relaxin were added before sperm cryopreservation and different fertility variables were investigated after freezing-thawing. This methodological approach may be more suitable for field applications, avoiding any type of treatments of the semen after thawing and/or before artificial insemination.

2. Materials and methods

2.1. Semen collection and dilution

Three commercial proven fertile stallions (ages 10–13 years) were utilised in this study. The horses were housed in the same stud (Vigone, Turin, Italy) and managed similarly for feeding and physical activity. Physical examination of the genitalia by palpation and using ultrasonography occurred for assessment of any reproductive tract abnormalities and none were detected. Semen samples were obtained once a week for 6 consecutive weeks toward the end of the breeding season when commercial demand for semen was relatively less. A total of 18 ejaculates (six ejaculates for each stallion) were collected using an artificial vagina (Colorado model equine artificial vagina; ARS, Chino, CA, USA) pre-warmed to 45–50 °C. The semen samples were collected in a plastic bottle and filtered immediately after collection to separate the gel fractions. Sperm motility, concentration, viability, and sperm morphology were evaluated. The samples were diluted using twice the amount of skim milk as the semen volume and were maintained in a water bath at 24 °C for 10 min and then centrifuged at 600 *g* for 10 min. After centrifugation, the supernatant was removed and the sperm pellets were re-suspended using freezing medium (BotuCrio, Botupharma, Phoenix, AZ, USA) to a final concentration of 50×10^6 sperm/mL. The diluted semen samples from the three stallions were always pooled, eliminating individual stallion variability (Seifi-Jamadi et al., 2016; Shojaeian et al., 2018; Nouri et al., 2018). The samples were divided into five experimental groups and were not supplemented or were supplemented with relaxin (SRP3147, Sigma-Aldrich, Milan, Italy) at different concentrations: 0 (no supplementation, control), or supplemented with 12.5, 25, 50, or 100 ng/mL. The semen from the different experimental groups was transferred into 0.5 mL polyvinyl straws (IMV, France) and stored in a refrigerator at 5 °C for 30 min for equilibration. The straws were placed 4 cm above the surface of the liquid nitrogen (LN_2) in the vapour for 15 min at approximately 130 °C and then directly plunged into the LN_2 for storage (Cristanelli et al., 1985). After 1 week, the frozen semen in the straws were thawed in a water bath at 37 °C for 60 s for conducting the subsequently described procedures.

2.2. Assessment of sperm motility and velocity variables

After thawing, the sperm were incubated at 37 °C and the motility and velocity variables were evaluated after 0, 30 min, 60 min, 90 min, and 120 min of incubation using a Computer Assisted Sperm Analyser (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) with a 10x objective at 37 °C and a pre-calibrated configuration adjusted for stallions (setup “equine species”) according to the manufacturer’s instructions (CASA; Hamilton Thorne, Inc., Beverly, MA, USA). In detail, the variables were evaluated using the following procedures: 45 frames acquired at 60 frames/s; minimum contrast 80; minimum cell size 5 pixels; progressive cells were identified if VAP > 90 μm/s and STR > 75 %; the VAP cut-off for slow cells was 20 μm/s and the VSL cut-off was 0 μm/s. CASA analyses were conducted by loading 10 μl of semen samples into a pre-warmed Makler chamber and evaluated. The total, progressive, and rapid motility values were recorded and expressed in percentages. Additional velocity variables including the 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] × 100), and straightness (STR, [VSL/VAP] × 100) were determined. For all of the assessments, the sperm in eight randomly selected microscopic fields were analysed for each sample.

2.3. Assessment of sperm plasma membrane integrity

The sperm plasma membrane integrity was determined using a hypo-osmotic swelling assay (HOS) as reported by Nie and Wenzel (2001). The HOS solution concentration was ~100 mOsm/kg and contained 1.712 g of sucrose dissolved in 50 mL of sterile deionised water. A semen sample was mixed a ratio of 1:10 with pre-warmed (37 °C) HOS solution and incubated at 37 °C for 60 min. With all of the assessments, 200 spermatozoa were counted and the percentage of cells with curled tails (with swollen or intact plasma membranes) was recorded as HOS positive.

2.4. Assessment of sperm DNA integrity using SCSA

The DNA integrity of the spermatozoa was assessed using a sperm chromatin structure assay (SCSA) that utilised the metachromatic properties of acridine orange (AO, Sigma-Aldrich, St. Louis, MO, USA) to distinguish between denatured and native sperm DNA as reported by Evenson and Jost (2000). The semen was thawed at 37 °C for 60 s, washed once using phosphate-buffered saline (PBS) solution, and then centrifuged 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, and disodium pH 7.4). Subsequently, there were 400 µL of acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1 % (w/v) Triton X-100, and pH 1.2) added. After 30 s, 1200 µL of AO staining solution containing 6 µg AO (2% in H_2O) per ml staining buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM EDTA disodium, 0.15 M NaCl, and pH 6.0) were added. Flow cytometric evaluations were conducted immediately after the end of staining.

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, St. Louis, MO, USA), there are reversible changes in JC-10 fluorescence from green (monomeric status) to orange (multimeric status) when the mitochondrial membrane potential is relatively greater. The 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 sample of semen was evaluated for apoptosis using carbonyl cyanide m-chlorophenyl hydrazine (CCCP) 1 mM and incubated at 37 °C for 15 min and served as the positive control. The sperm cells of all of the groups were washed in 1 mL PBS using centrifugation at 600*g* for 10 min, and samples were re-suspended in 500 µL of JC-10 (200x JC-10 in dimethyl sulfoxide), and incubated 1 h at 37 °C. The samples were subsequently centrifuged and diluted in 1 mL of PBS. Flow cytometric evaluations were conducted immediately after the end of staining.

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

Translocations of phosphatidylserine (PS) phospholipids and sperm plasma membrane integrity were detected using an 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 modifications. The semen was thawed at 37 °C for 60 s and washed once using PBS and utilising centrifugation at 500*g* for 10 min. Aliquots of the semen were diluted in annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, and pH 7.4) to a final concentration of 1×10^6 spermatozoa/mL. The aliquots of the diluted semen (100 µL) from each group were transferred to 5 mL culture tubes supplemented with 5 µL of annexin V and 1 µL of PI (100 µg/mL). The tubes were gently mixed and incubated for 15 min at room temperature in the dark. An additional 400 µL of annexin V binding buffer was added to each tube prior to flow cytometric evaluation. Flow cytometric evaluations were conducted immediately after the end of staining.

2.7. Flow cytometric analysis

The samples were analysed using a FacsStar Plus flow cytometer (Becton Dickinson Immunochemistry, San Jose, CA, USA) equipped with standard optics and an air-cooled argon laser operated at 488 nm excitation and 15 mW. For the SCSA assay, the fluorescence from the sperm samples stained with AO was recorded with the use of FL1 (530/15 nm filter) and FL3 (650 nm long pass filter). The sheath/sample was set on "low" and adjusted to a flow rate of 200 events/s to analyse the samples with a concentration of 1×10^6 sperm/mL. Immediately after the addition of the AO staining solution, the samples were placed in the flow cytometer. Recording of the sperm cells with the red and green fluorescence started exactly 3 min after the beginning of staining. For each sample, 10×10^3 cells were collected using CellQuest software (Becton Dickinson Immunochemistry, San Jose, CA, USA). The X mean (red) and Y mean (green) values of each sample were recorded.

The annexin V/PI binding assay was used to evaluate each cell with there being assessment of forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1), and PI fluorescence (FL3) being evaluated using CellQuest software. An acquisition gate, applied in a FSC/SSC two-dimensional histogram, was used to restrict the analysis to spermatozoa and eliminate small debris and other particles for further analysis. For the gated sperm cells, four different kinds of sperm were observed. The percentages of viable (annexin V^- and PI^-), necrotic (annexin V^- and PI^+), apoptotic (annexin V^+ and PI^+), and early apoptotic (annexin V^+ and PI^-) spermatozoa were evaluated based on regions determined from single-stained and unstained control samples.

For the mitochondrial activity, there was assessment using JC-10, a total of 10,000 gated events/s were analysed per sample. The sample was adjusted to a flow rate of 200 events/set. A 488 nm laser was used to excite the JC-10. Emission filters of 535 nm and 595 nm were used to quantify the population of spermatozoa with green (JC-10 monomers) and orange (JC-10 aggregates) fluorescence,

Table 1
Effect of relaxin on sperm motility and velocity variables of cryopreserved stallion semen.

Variables	Control	Relaxin 12.5 ng/mL	Relaxin 25 ng/mL	Relaxin 50 ng/mL	Relaxin 100 ng/mL
Total motility (%)	31.8 ± 1.0 [a]	33.3 ± 1.3 [a]	39.9 ± 1.0 [b]	33.0 ± 1.0 [a]	31.6 ± 1.0 [a]
Progressive motility (%)	4.3 ± 0.2 [a]	5.9 ± 0.2 [b, c]	5.4 ± 0.2 [b]	4.4 ± 0.2 [d]	4.4 ± 0.2 [d]
Rapid motility (%)	4.2 ± 0.3 [a]	5.8 ± 0.3 [b, c]	5.4 ± 0.3 [b, c]	4.4 ± 0.3 [d]	4.4 ± 0.3 [d]
VAP (μm/s)	67.0 ± 0.5	69.0 ± 0.7	67.3 ± 0.5	66.9 ± 0.5	67.4 ± 0.5
VSL (μm/s)	56.4 ± 0.6	59.2 ± 0.8 [a]	56.7 ± 0.6	56.1 ± 0.6 [b]	57.0 ± 0.6
VCL (μm/s)	123.2 ± 0.7	122.6 ± 0.9	122.4 ± 0.7	122.2 ± 0.7	122.7 ± 0.7
ALH (μm)	6.0 ± 0.1 [a]	6.8 ± 0.2 [b]	6.5 ± 0.1 [b]	6.8 ± 0.1 [b]	6.7 ± 0.1 [b]
BCF (Hz)	33.4 ± 0.3	34.4 ± 0.4	33.5 ± 0.3	33.7 ± 0.3	34.2 ± 0.3
STR (%)	83.4 ± 0.4	85.3 ± 0.5 [a]	83.6 ± 0.4	83.2 ± 0.4 [b]	83.8 ± 0.4
LIN (%)	46.7 ± 0.5 [a]	49.4 ± 0.6 [b, c]	47.2 ± 0.5 [d]	47.0 ± 0.5 [d]	47.5 ± 0.5

Data are reported as means (+/− Standard errors); Data are grouped regardless the incubation periods; GLM: within each raw, different superscripts indicate differences: a,b; c,d; e,f: $P < 0.05$.

respectively. Frequency plots were prepared for FL1 (green) and FL2 (orange) to determine the percentage of the population stained green and orange. The percentage of orange stained cells was recorded, with the consideration being that this was the population of cells with high mitochondrial membrane potential (HMMP).

2.8. Statistical analysis

The data were analysed using SPSS 25.0. The data are presented as mean ± SE. The effects of varying relaxin treatment concentrations and different incubation duration periods after thawing were analysed using a full factorial generalised linear model (GLM) for repeated measurements. Relaxin doses were considered fixed factors whereas the time was considered a within-subject variable. Bonferroni's *post hoc* test was conducted for multiple comparisons of the observed means. The interactions among factors were also analysed using the GLM. Pearson's correlations were used to evaluate associations between the different experimental variables. The $P < 0.05$ values was considered to indicate there were differences in values.

3. Results

3.1. Motility and velocity variables of the frozen-thawed sperm

The sperm motility and velocity values evaluated using CASA are presented in Table 1. Regardless of the incubation time, treatments with relaxin at different concentrations (12.5, 25, 50, and 100 ng/mL) in the extender medium during cryopreservation led to an improved motility of sperm after thawing of the semen. There was a greater total sperm motility in the samples treated with 25 ng/mL relaxin ($P < 0.05$). Similarly, there were greater progressive and rapid motility values in the semen samples treated with 12.5 and 25 ng/mL relaxin ($P < 0.05$) than the sperm of other treatment groups. Furthermore, ALH, indicative of the lateral sperm-head displacement associated with the capacitated spermatozoa status, was greater as a result of relaxin treatments in the present study ($P < 0.05$). The LIN, which provides important information about the linearity of the sperm velocity path, was greater as a result of relaxin treatments after thawing of sperm and the greatest values were when there was 12.5 ng/mL relaxin added to the semen extender ($P < 0.05$) compared with the values for the control group. There were no differences in the STR, VAP, VSL, and VCL values between samples for which there were relaxin treatments and samples of the control group. Interestingly, the values for sperm motility and some of the velocity variables (VSL, STR, and LIN) were greater in semen treated with 12.5 and 25 ng/mL relaxin than the samples treated with the greater concentrations of relaxin ($P < 0.05$).

3.2. Different incubation durations and associated values for sperm motility and velocity

There were negative effects of duration of incubation on sperm motility and velocity values that were time-dependent manner. All of the data for incubation durations are included in Table 2. The total, progressive, and rapid sperm motility was less after thawing at all of the time points at which evaluations occurred ($P < 0.05$). The VAP, VSL, VCL, BCF and LIN were also negatively affected by different incubation durations ($P < 0.05$). The ALH and STR were the only two variables for which there were slightly greater values as duration of incubation increased. The greater values for ALH were detected after 30 and 60 min of incubation ($P < 0.05$) whereas the greater STR values were detected after 60, 90, and 120 min of incubation ($P < 0.05$).

3.3. Effect of relaxin on the plasma membrane, DNA integrity, and mitochondrial membrane potential

The data regarding effects of relaxin on the sperm quality variables are included in Table 3. Based on the sperm motility results, there was the greatest motility at 60 min, so the data at this time point were used when there were further analyses of the effect of relaxin on the other sperm quality variables. Interestingly, treatment with relaxin at all of the concentrations evaluated led to improvements the sperm mitochondrial membrane potential (HMMP) compared to the values for the control group ($P < 0.01$). There

Table 2
Effect of different incubation periods on motility and velocity variables of cryopreserved stallion semen treated with relaxin.

Variable	Post-thawing	30 min.	60 min.	90 min.	120 min.
Total motility (%)	47.9 ± 1.1^{a,b}	42.1 ± 1.1^{a,b}	36.5 ± 1.1^{a,b}	23.1 ± 1.1^{a,b}	20.0 ± 1.1^{a,b}
Progressive motility (%)	9.8 ± 0.2^{a,b}	7.3 ± 0.2^{a,b}	4.6 ± 0.2^{a,b}	1.8 ± 0.2^{a,b}	0.9 ± 0.2^{a,b}
Rapid motility (%)	9.6 ± 0.3^{a,b}	7.3 ± 0.2^{a,b}	4.6 ± 0.1^{a,b}	1.8 ± 0.1^{a,b}	0.9 ± 0.1^{a,b}
VAP (μm/s)	74.6 ± 0.2^{a,b}	73.3 ± 0.3^{a,b}	69.2 ± 0.3^{a,b}	62.7 ± 0.4^{a,b}	57.8 ± 0.3^{a,b}
VSL (μm/s)	62.9 ± 0.3^{a,b}	60.9 ± 0.4^{a,b}	58.3 ± 0.3^{a,b}	53.8 ± 0.3^{a,b}	49.4 ± 0.4^{a,b}
VCL (μm/s)	130.8 ± 0.4$^{a,\ b}$	130.9 ± 0.5$^{a,\ c}$	126.1 ± 0.4^{a,b,d}	115.9 ± 0.5^{a,b,d}	109.4 ± 0.5^{a,b,d}
ALH (μm)	6.5 ± 0.0^{a,b}	7.1 ± 0.1^{c,b}	7.0 ± 0.1^{b,e}	6.2 ± 0.2^{a,d,f}	5.9 ± 0.2^{b,d,f}
BCF (Hz)	34.8 ± 0.1^{a,b}	33.0 ± 0.1^{a,b,c}	33.4 ± 0.1^{a,b,c}	33.8 ± 0.2^{a,b,c}	34.3 ± 0.2^{b,d}
STR (%)	82.7 ± 0.3^{a}	82.1 ± 0.3^{c}	83.7 ± 0.2^{b,d,e}	85.5 ± 0.2^{b,d,f}	85.3 ± 0.3^{b,d,f}
LIN (%)	48.5 ± 0.2^{a}	47.4 ± 0.2^{b,c}	47.4 ± 0.2^{b,c}	47.9 ± 0.3^{b,e}	46.5 ± 0.4^{b,d,f}

Data are reported as means (+/− Standard errors); Data are grouped regardless the relaxin doses. GLM: within each raw, different superscripts indicate differences: a,b; c,d; e,f:$P < 0.05$.

Table 3
Effect of relaxin on cryopreserved stallion semen plasma membrane and DNA integrities and mitochondrial activity.

Group	Plasma membrane integrity (%)	DNA integrity (%)	HMMP (%)
Control	61.5 ± 1.1	87.9 ± 3.4	44.0 ± 10.7^{a}
Relaxin 12.5 ng/mL	62.5 ± 1.3	87.6 ± 1.2	73.4 ± 3.7^{b}
Relaxin 25 ng/mL	64.3 ± 1.0	88.3 ± 1.6	75.4 ± 6.2^{b}
Relaxin 50 ng/mL	60.7 ± 1.6	89.3 ± 0.4	69.6 ± 5.5^{b}
Relaxin 100 ng/mL	61.1 ± 1.3	87.0 ± 2.0	69.8 ± 5.4^{b}

Values are presented as mean ± SEM; HMMP: High Mitochondrial membrane Potential; Superscripts within the same column indicate differences among experimental groups: a,b: $P < 0.01$.

were no differences of the effects of relaxin on the plasma membrane and DNA integrity at any concentrations evaluated (Table 3).

3.4. Effects of relaxin on sperm apoptosis

The data for effects of relaxin on sperm apoptosis are presented in Table 4. At all of the concentrations evaluated, relaxin treatments resulted in a decrease in the percentage of apoptotic cells ($P < 0.05$). There values for viable sperm with typical sperm morphology were greater in the relaxin-treated samples at 12.5, 50, and 100 ng/mL supplementations to the diluent even though there were none of these values significantly greater than the values for the control group (Table 4). There were no differences between the percentage of viable sperm with typical sperm morphology and necrotic sperm cells compared with the sperm of the control group ($P > 0.05$).

3.5. Correlations between values the different sperm quality variables

The correlation coefficients among different semen quality variables when considering the effects of relaxin are summarised in Table 5. The values for total sperm motility were positively correlated with the values for sperm progressive motility, rapid motility, intact DNA ($P < 0.01$), and HMMP ($P < 0.05$), whereas the values were negatively correlated with the apoptotic sperm values for VAP ($P < 0.05$), VCL, VSL, ALH, STR, and LIN ($P < 0.01$). The values for progressive motility were positively correlated with the values for rapid motility ($P < 0.01$) and HMMP ($P < 0.05$), whereas these values were negatively associated with the VAP, ALH, BCF, and STR values ($P < 0.01$).

Table 4
Effect of relaxin on cryopreserved stallion semen apoptosis.

Group	Normal viable sperm (%)	Apoptic sperm (%)	Total necrotic sperm (%)
Control	23.1 ± 2.8	7.7 ± 3.0^{a}	71.7 ± 3.4
Relaxin 12.5 ng/mL	25.4 ± 5.4	3.6 ± 0.9^{a,b}	71.0 ± 6.4
Relaxin 25 ng/mL	21.4 ± 2.9	3.1 ± 0.2^{b}	75.5 ± 2.7
Relaxin 50 ng/mL	27.1 ± 6.0	2.3 ± 0.3^{b}	70.5 ± 6.3
Relaxin 100 ng/mL	31.3 ± 2.4	2.5 ± 0.4^{b}	66.1 ± 2.8

Values are presented as mean ± SEM; Superscripts within the same column indicate differences among experimental groups: a,b: $P < 0.05$.

Table 5
Correlation coefficients between the values for different variables of cryopreserved stallion semen supplement with relaxin.

	Group	VAP	VSL	VCL	ALH	BCF	STR	LIN	T. Motility	P. Motility	R. Motility	Intact P.M.	Intact DNA	Apoptic sperm	Necrotic sperm	HMMP
VAP	.119															
VSL	.076	.824**														
VCL	.186*	.695**	.621**													
ALH	.339**	−.193*	−.107-	.151*												
BCF	.048	.577**	.573**	.059	−.308**											
STR	−.034-	.301**	.777**	.303**	−.005-	.316**										
LIN	−.045-	.568**	.861**	.180*	−.186*	.689**	.830**									
T. Motility	−.052-	−.185*	−.608**	−.503**	−.213**	−.049-	−.803**	−.496**								
P. Motility	−.014-	.341**	−.092-	−.120-	−.289**	.381**	−.516**	−.098-	.764**							
R. Motility	−.034-	.292**	−.186*	−.126-	−.294**	.270**	−.614**	−.215**	.820**	.979**						
Intact P.M.	−.048-	.008	.017	−.081-	−.171-	.224	.030	.133	.130	.099	.093					
Intact DNA	.412*	−.561**	−.792**	−.304-	.486*	−.512*	−.654**	−.872**	.551**	−.024-	.114	−.106-				
Apoptotic sperm	−.491-	.511	.872**	.388	−.516*	.219	.723**	.879**	−.574*	−.051-	−.178-	.307	−.830**			
Necrotic sperm	.286	−.423-	−.747**	−.184-	.424	−.372-	−.604*	−.866**	.458	−.039-	.090	−.299-	.963**	−.856**		
HMMP	.457*	.189	−.288-	−.272-	−.020-	.282	−.457*	−.200-	.528*	.487*	.473*	.069	−.043-	−.250-	−.001-	

VAP: Average path velocity; VSL: Straight linear velocity; VCL: Curvilinear velocity; ALH: Amplitude of lateral head displacement; BCF: Beat cross frequency; STR: straightness; LIN: Linearity; T. Motility: Total motility; P. Motility: Progressive motility; R. Motility, Rapid motility; Intact P.M.: Intact Plasma membrane; HMMP: High Mitochondrial Membrane Potential; *Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level.

4. Discussion

In different species, relaxin supplementations to semen diluents led to an improved sperm motility (Burns and Fleming, 1989; Miah et al., 2007; Ferlin et al., 2012; Feugang et al., 2015), capacitation and acrosome reaction (Miah et al., 2008, 2011), and fertilizing capacity (Han et al., 2006; Elkhawagah et al., 2015). In previous studies, the effects of relaxin were investigated in fresh semen (Han et al., 2006) or cryopreserved semen with relaxin being added during the thawing process (reviewed by Miah et al., 2015). In the present study, using a different methodological approach, there were improvements in several sperm quality variables of cryopreserved stallion sperm after thawing as a result of relaxin supplementation at different concentrations (12.5, 25, 50, and 100 ng/mL) in the extender medium before cryopreservation.

Sperm motility, evaluated using CASA analysis, is one of the most reliable variables associated with sperm fertilizing capacity (Verstegen et al., 2002). Voss et al. (1981) reported that spermatozoa motility is the most reliable method of estimating field fertility. Results from the present study indicate that supplementation of the semen diluent with 12.5 and 25 ng/mL relaxin improved the total, progressive, and rapid sperm motility after thawing with prolonged effects over time from 30 to 120 min of incubation as compared with the sperm of the control group. Furthermore, values for other sperm velocity variables, such as the VCL, VSL, ALH, STR, and LIN, were greater as a result of relaxin supplementations with values being consistent with those reported from another study where there were assessments of thawed stallion semen (Seifi-Jamadi et al., 2016). The values for these motility characteristics were positively correlated with sperm capacitation and fertility (Liu et al., 1991). In the present study, sperm motility characteristics, together with the sperm total, progressive, and rapid motility were the primary effects of relaxin on cryopreserved stallion semen. The sperm in the semen samples evaluated in the present study did not have excellent post-thawing progressive motility, which might have been a consequence of the semen processing procedures used with LN_2 vapour being utilised instead of a programmable freezer for cryopreservation of the sperm collected for conducting the present study. Furthermore, there was likely a relatively lesser quality of the semen utilised for the present study as a consequence of the semen being collected and frozen at the end of the commercial season for stallion semen collection as compared with what might have been the case if the semen had been collected earlier in the breeding season. The greater sperm quality in relaxin-treated samples, even considering these short comings in semen processing and timing of semen collection during the breeding season, is encouraging because this further indicates the potential positive effects of the supplementation of semen extenders with relaxin when cryopreserving stallion semen.

The relationship between sperm motility and fertility is not the only variable associated with fertilizing capacity (Bataille et al., 1990). Graham (1996) and Katila (2001) recommended that stallion spermatozoa processed for cryopreservation should have relatively greater values from several variables including motility, normal morphology, membrane integrity (Aurich, 2005; Baumber et al., 2005), and sufficient metabolism for energy production to help ensure the samples have an acceptable fertilising capacity post-thawing. The HOS test, therefore, was used to verify the functionality of the sperm plasma membrane after relaxin supplementations of semen samples. Results of the present study, however, indicated there were no differences for the experimental groups when the data using the HOS test were analysed and these findings are consistent with the results from a previous study of Feugang et al. (2015). In this previous study, there were no effects of relaxin treatment and boar sperm plasma membrane integrity. Evaluation of the effects of relaxin on sperm DNA integrity using SCSA assessments indicated there were no differences among values from the experimental groups of the current study. It has been stated that the loss of sperm fertilizing capacity after freezing/thawing of boar semen could be attributed to factors other than sperm chromatin structural damage, because the sperm chromatin integrity is very resistant to cryopreservation/thawing and other semen processing procedures (Evenson et al., 1994).

Semen cryopreservation disrupts sperm membrane functions (Chaveiro et al., 2007), inducing membrane phospholipid asymmetry and progressively damaging the cellular integrity (Martin et al., 1995) with the induction of apoptotic-like changes (Crabo, 2001). Results from the present study indicate that treatment with all of the concentrations of relaxin markedly decreased the percentage of apoptotic spermatozoa compared to the control group, and the least value was when there was supplementation of extender with 100 ng/mL of relaxin. These results are consistent with those reported by Ferlin et al. (2012), from which it was reported that treatment of human sperm with 100 nM of relaxin prevented apoptosis and increased the percentage of viable sperm.

Sperm mitochondria are considered the site for the production of adenosine-triphosphate (ATP), which is essential for sperm motility (Silva and Gadella, 2006; Amaral et al., 2013) and sperm fertilising capacity. Osmotic shock is a major factor in sperm damage during cryopreservation (Prien and Iacovides, 2016) that leads to a loss of sperm viability by leading to a decreased mitochondrial membrane potential (Papa et al., 2011). In the present study, there was a greater mitochondrial membrane potential of cryopreserved stallion semen when there was supplementation with relaxin in the freezing medium at different concentrations. Results from the present study are consistent with those reported by Ferlin et al. (2012), with relaxin supplementations at 10 and 100 nM preserving the HMMP of human sperm. Interestingly, in the present study there was a positive correlation between motility and other semen quality variables such as the HMMP and sperm apoptosis.

The results of the present study indicate relaxin can be added to the extender medium before sperm cryopreservation and have positive effects on sperm viability rather than the additions of relaxin to post-thawing media or *in vitro* fertilization/embryo culture media as has occurred in previous studies with stallion semen. This methodological approach may be more suitable for field applications, avoiding any type of semen treatment after thawing and/or before artificial insemination.

5. Conclusions

The results of the present study indicate there were improvements in the post-thawing quality of cryopreserved stallion semen as a result of adding relaxin at different concentrations (12.5, 25, 50, and 100 ng/mL) to the extender medium before cryopreservation.

These results indicate that supplementations of semen diluents with 12.5 and 25 ng/mL of relaxin had a positive stimulatory effect on different quality variables of frozen-thawed semen including improvement of the values for sperm motility and velocity variables. In addition, with these relaxin supplementations, there was a larger percentage of sperm with a greater mitochondrial membrane potential and a reduced percentage of sperm undergoing apoptosis.

CRediT authorship contribution statement

Ahmed R. Elkhawagah: Conceptualization, Methodology, Writing - original draft. **Tiziana Nervo:** Data curation, Investigation, Visualization. **Mariagrazia Poletto:** Investigation. **Nicola A. Martino:** Writing - review & editing, Supervision. **Davide Gallo:** Data curation, Investigation. **Alessia Bertero:** Data curation, Investigation. **Leila Vincenti:** Conceptualization, Writing - original draft, Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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