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Effect of relaxin on cryopreserved beef bull semen characteristics

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ABSTRACT

This study aimed to improve the quality of cryopreserved beef bull (Piedmontese) semen by incorporation of relaxin in diluted semen before cryopreservation procedures. Semen samples were collected from 4 proven fertile bulls, using artificial vagina, once per week for 8 consecutive weeks and pooled together then diluted with Bullxcell® extender, and supplemented with different concentrations of relaxin (0 (control), 25, 50 and 100 ng/ml) before cooling, equilibration and freezing procedures. Frozen semen was thawed and assessed for motility by Computer-Assisted Sperm Analysis and vitality parameters such as acrosome, plasma membrane and DNA integrities, apoptosis, mitochondrial membrane potential, mucus penetration and SOD activity. The developmental potential of bovine embryos produced in vitro by using relaxin-treated was also investigated. In the present study, 50 and 100 ng/ml relaxin incorporation in extended bull semen before cryopreservation induced a reduction of sperm motility immediately after thawing (0h), whereas, during long incubation periods (1–2 h), relaxin showed a significant positive effect on sperm quality by improving the sperm motility and velocity parameters. Interestingly, sperm vitality was improved by 25 and 100 ng/ml relaxin and the blastocyst developmental rate was significantly increased in the 25 ng/ml relaxin group compared with controls (52/118, 44.0% vs. 32/116, 27.6%, respectively). These findings suggest a potential use of relaxin at the doses tested in the present study as an additive in the cryopreservation media of bull semen to improve sperm quality.

1. Introduction

Success of Artificial Insemination (AI) depends on the quality of collected semen and its capacity to maintain fertilizing ability after dilution and storage [9]. The final goal of semen cryopreservation is to prolong the life span of spermatozoa and maintaining their capacity to fertilize by preserving metabolic efficiency in order to produce energy for progressive movement, plasma proteins to survive in the female reproductive tract and acrosomal enzymes to penetrate the cumulus cells and the egg membranes [31]. However, cryopreservation is known to induce significant damage in a number of sperm cells including disruption of plasma and acrosomal membranes, considerable production of reactive oxygen species and a reduction of the percentage of motile sperm [38]. In addition, within the motile population, sub lethal damage impairs the function of frozen-thawed spermatozoa [35]. Owing to this damaging effect of cryopreservation, in order to achieve similar fertilization rates in bovine [35] about 2–10 times more cryopreserved spermatozoa compared to fresh ones must be used to inseminate. These considerations encourage researchers to modify the extenders in use and to implement additives for maintaining the sperm activity and plasma membrane integrity by improving the fertilization ability [2].

Relaxin, a regulatory peptide of the insulin superfamily, is an important component of seminal plasma in several species. It is reported that bovine seminal plasma contains the highest concentration (41.9 ± 25.2 ng/ml) of relaxin followed by humans (17.4 ± 2.9 ng/ml), rams (13.1 ± 1.7 ng/ml), boars (2.4 ± 0.5 ng/ml) and bucks (1.0 ± 0.3 ng/ml). It has been demonstrated that relaxin has a physiological influence on sperm motility and fertility through its link with specific sperm-

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surface receptors [22]. In fact, relaxin improved the motility of bovine [27], porcine [29] and human spermatozoa [13]. In addition, relaxin has been found to induce capacitation and acrosome reaction in fresh and frozen–thawed porcine [29] and bovine [28] spermatozoa. Furthermore, relaxin improves the in vitro fertilization rate of porcine [16] and buffalo spermatozoa [11]. However, to the best of our knowledge, there are no previous studies reporting the effects of relaxin, added during the cryopreservation procedures, on semen viability, motility and ability to produce viable embryos up to the blastocyst stage. The present study aimed to improve the quality of cryopreserved beef bull (Piedmontese) semen by incorporation of different concentrations (0 (control), 25, 50, and 100 ng/ml of relaxin in diluted semen before cryopreservation procedures. Moreover, to fully demonstrate its beneficial effect the developmental potential of in vitro produced bovine embryos obtained by using relaxin-treated sperm samples has also been investigated.

2. Material and methods

2.1. Semen collection and dilution

The present study was carried out at the Department of Veterinary Science, Torino University, Italy. Four proven fertile Piedmontese beef bulls, aged between 1.5 and 2 years, located in ANABORAPI Bull Center (Carrù, Piemonte, Italy) were enrolled in the experiment. Semen samples were collected once per week from each bull with the bovine artificial vagina for 8 consecutive weeks. All samples that had a concentration of $\geq$800 $\times$ 10^6/ml, and motility $\geq$70% were used as semen source. Semen samples were pooled and diluted with Bullxcell® extender (IMV, France), according to instructions, and supplemented with relaxin (SRP3147, Sigma-Aldrich, Italy) at different concentrations: 0 (control), 25, 50 and 100 ng/ml. Diluted semen with a final concentration of 30 $\times$ 10^6 spermatozoa/ml was slowly cooled to 5 °C, packed into 0.5 ml polyvinyl straws (Minitub, Germany), equilibrated for 4 h and frozen using a controlled-rate freezer (SY LAB Gerate GmbH, Neupurkersdorf, Austria) following the standard procedure. In detail, temperature was decreased from +4 °C to −9 °C at a rate of 4 °C/min, - from −9 °C to −25 °C at a rate of 50 °C/min, from −25 °C to −100 °C at 35 °C/min, from −100 °C to −144 °C at a rate of 20 °C/min and from −144 °C to −150 °C at a rate of 4 °C/min. Afterwards, the straws were plugged into liquid nitrogen. After 24 h of storage in liquid nitrogen, frozen straws were thawed (N = 4/trial/treatment) in water bath at 37 °C for 40 s for subsequent procedures.

2.2. Semen evaluation

2.2.1. Assessment of sperm motility and velocity parameters

Preliminary semen quality evaluations were performed to define the best incubation times in the presence of relaxin. Post thawing, semen was incubated at 37 °C and the different parameters of motility and velocity were evaluated at 0, 1, 2, 3 and 4 h of incubation using the computer assisted sperm analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) with a 10× objective at 37 °C and the SETUP specific for the bull semen, as presented in Table 1. An aliquot of 10 μl from diluted semen specimen was put onto a pre-warmed Makler chamber and evaluated. The values of motility parameters (total, progressive and rapid motility) were recorded in percentages. 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] × 100) and straightness (STR, [VSL/VAP] × 100) were determined. The sperm motilities were calculated with speed standards set as: fast >80 μm/s, medium >60 μm/s, slow >20 μm/s and static. For each evaluation, randomly selected 8 microscopic fields were analyzed.

Table 1
Computer Assisted Sperm Analyzer (CASA) setup used to evaluate bull spermatozoa.

Variables	Settings
Frame rate (Hz)	60
Frames acquired	30
Minimum contrast	40
Minimum cell size (pixels)	8
Threshold straightness (%)	64
Medium VAP cut-off (m/s)	80
Low VAP cut-off (m/s)	15
Low VSL cut-off (m/s)	4.4
Non-motile head intensity	80
Static size limit – minimum	0.38
Static size limit – maximum	1.49
Static intensity limit – minimum	0.42
Static intensity limit – maximum	1.35
Static elongation limit – minimum	12
Static elongation limit – maximum	81

2.2.2. Assessment of sperm viability and acrosomal status

Sperm viability and acrosomal status were evaluated by using the dual staining technique (Trypan Blue/Giemsa) according to Boccia et al. [5]. In detail, semen samples were diluted 1:1 with Trypan blue (0.27%), smeared on glass slides and allowed to dry. Then, the slides were fixed in 37% formaldehyde with neutral red for 4 min and stained with Giemsa (7.5%) overnight. Slides were examined by phase contrast microscopy (Advanced Automated Research Microscope System, Nikon Eclipse E200). Based on staining characteristics the sperm cells were differentiated in four categories: acrosome-intact live (AIL), acrosome-intact dead (AID), acrosome lost live (ALL) and acrosome-lost dead (ALD). Spermatozoa displaying both head and tail viable were recorded as alive whereas those with either the head or the tail unviable were classified as dead. At least 200 sperm cells were counted for each group sample and the percentage of live spermatozoa was calculated.

2.2.3. Assessment of sperm plasma membrane integrity

The integrity of sperm plasma membrane was determined by hypo-osmotic swelling test (HOS) according to the study of Akhter et al. [1]. The HOS solution (osmotic pressure ~ 190 mOsm/kg) contained sodium citrate 0.735 g and fructose 1.351 g, dissolved in 100 ml of distilled water was used. Ten μl of semen sample were mixed with 100 μl pre-warmed (37 °C) HOS solution and incubated at 37 °C for 40 min. After incubation, spermatozoa were stained with Eosin-Nigrosin stain and examined by using phase contrast microscopy. Spermatozoa were counted and the percentage of cells with curled tails (swollen/intact plasma membrane) were recorded as HOS positive.

2.2.4. Assessment of sperm DNA integrity using SCSA

Sperm DNA integrity was assessed by cytometric sperm chromatin structure assay (SCSA), that utilizes the metachromatic properties of acridine orange (AO) to distinguish between denatured and native DNA in sperm, according to Evenson and Jost [12], with some modifications. Semen samples were thawed at 37 °C for 40 s, (two straws/-group/replicate for 4 replicates) and washed once using phosphate-buffered saline (PBS) by centrifugation at 500×*g* for 10 min. Aliquots of the thawed semen were diluted to a final concentration of 2 $\times$ 10^6 sperm/ml with TNE buffer (0.01 M Tris–Cl, 0.15 M NaCl, 1 mM EDTA, pH 7.4). Then, 400 μl of acid detergent solution (0.08 NHCl, 0.15 M NaCl, 0.1% (w/v) Triton X-100, pH 1.2), was added. After exactly 30 s, 1.2 ml of AO staining solution containing 2% AO dissolved in staining buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM EDTA, 0.15 M NaCl, pH 6.0) was added. Flow cytometric evaluation was conducted immediately after preparation of the sample.

2.2.5. Assessment of sperm mitochondrial activity

The mitochondrial status of spermatozoa was assessed using the

lipophilic cation JC-1. According to the manufacturer (JC-1 Assay for Flow Cytometry, Sigma-Aldrich, USA), JC-1 changes reversibly its fluorescence from green (monomeric status) to orange (multimeric status) when mitochondrial membrane potential is high. Semen straws were thawed at 37 °C in water bath for 40s (two straws/group/replicate for 4 replicates) and distributed to polypropylene tubes at a concentration of 1×10^6 sperm cells. 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 as positive control. All semen samples were washed in 1 ml PBS at 500×*g* for 10 min then spermatozoa were resuspended in 500 μl of JC-1 staining solution (200x JC-1 in DMSO) and incubated at 37 °C for 1h. Finally, samples were centrifuged and resuspended in PBS and assessed by Flow Cytometry.

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

Alexa Fluor 488 Annexin-V Apoptosis Kit (INVITROGEN - V13245) and Propidium Iodide were used to detect the translocation of phosphatidylserine (PS) phospholipids and sperm plasma membrane integrity according to Anzar et al. [4] with some modifications. Semen samples were thawed at 37 °C for 40 s (two straws/group/replicate for 4 replicates), 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 $CaCl_2$, pH 7.4) to a concentration of 2×10^6 spermatozoa/ml. Aliquots of extended semen (100 μl) of each experimental group were transferred into 5 ml culture tubes and 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. Additional 400 μl of Annexin-V-binding buffer was added (final spermatozoa concentration of 1×10^6 cells/ml) to each tube prior to flow cytometric evaluation. Flow cytometric evaluation was conducted immediately after the end of the staining procedure.

2.2.7. Flow cytometric analysis

Samples were analyzed by 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. In Annexin-V/PI-binding assay; for each cell, forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1) and PI fluorescence (FL3) were evaluated using the Cellquest software (Becton Dickinson, San Jose, CA, USA). Acquisition gate applied in the FSC/SSC two-dimensional histogram was used to restrict the analysis to spermatozoa and to 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 spermatozoa (Annexin-V^-, PI^-), apoptotic sperm (annexin-V^+, PI^+), necrotic sperm (annexin-V^-, PI^+), and early necrotic (annexin-V^+, PI^-) were evaluated, based on regions determined from single-stained and unstained control samples.

In SCSA after passing a 560 nm short pass dichroic mirror, the green fluorescence (FL1) was collected through a 515–545 nm band pass filter. The red fluorescence (FL3) was collected after passing a 640 nm long pass filter followed by a 650 nm long pass filter. The sheath/sample was set on ''low'', adjusted to a flow rate of 200 events/sec when analyzing a sample with a concentration of 2×10^6 sperm/ml. Immediately after the addition of the AO staining solution, the sample was placed in the flow cytometer. Recording of the red and green fluorescence was started exactly 3 min after initiation of the staining process. In each sample 10×10^3 cells were collected in list mode using the Cellquest software. The X-mean (red) and Y-mean (green) values of each sample were recorded.

In Mitochondrial activity assessment by JC-1; 10.000 gated events were analyzed per sample. The sample was adjusted to a flow rate of 200 events/set. A 488 nm filter was used for excitation of JC-1. Emission filters of 535 nm and 595 nm were used to quantify the population of spermatozoa with green (JC-1 monomers) and orange (JC-1 aggregates) fluorescence, respectively. Frequency plots were prepared for FL1 (green) and FL2 (orange) to determine the percentage of the population stained green and orange. Percentage of orange stained cells was recorded, being considered as a population of cells with high mitochondrial membrane potential (HMMP).

2.2.8. Assessment of sperm Mucus penetration test

The sperm mucus penetration test was done in a Petri dish according to the technique reported by Hamano et al. [15], with some modification as follow: cervical mucus was collected in conical tubes (50 ml) from Piedmontese cows at estrus and tested with mucus score and fern test. The approved clear mucus was stored at −80 °C until used. The media used for sperm washing and migration analysis was Sperm Tyrode'-s-albumin-lactate-pyruvate (TALP) medium. At the day of experiment, frozen thawed cervical mucus was mixed with an equal volume of TALP supplemented with 2 mg/ml Bovine Serum Albumin (BSA). In a 60 mm Petri dish, a horizontal column of cervical mucus with terminal reservoir was constructed. The column consisted of 7 sequentially connected segments, each segment was 3 cm length and 1–2 mm width. The mucus column was overlaid with mineral oil and kept in incubator at 37 °C until used. Frozen-thawed semen (two straws/group/replicate for 4 replicates) was washed with the FERT-TALP medium by centrifugation at 300×*g* for 5 min. After washing the semen concentration was adjusted to 40×10^6/ml using the FERT-TALP supplemented with BSA and sperm motility was assessed before adding to the column. Twenty-five μl of washed sperm were added to the starting point of the column and incubated for 2 h at 37 °C in 5% CO_2 incubator. After incubation, the Petri dish was scanned under the inverted microscope (OLYMPUS CK40) to measure the sperm across the column. The evaluation of sperm migration was made by measuring the distance progressed by spermatozoa in the mucus column segments.

2.2.9. Assessment of antioxidant activity

The super oxide dismutase (SOD) activity was performed in sperm samples immediately after thawing (two straws/group/replicate for 4 replicates). Samples were prepared according to Sariozkan et al. [33] centrifuged at 800 g for 10 min, and the sperm pellet was separated and washed by resuspending in PBS and re-centrifuging (three times). After the last centrifugation, sperm cells were resuspended in 1 ml deionized water and assessed. The activity of SOD was assessed using SOD Assay Kit-WST (19160 SOD determination kit, Sigma-Aldrich, USA) which depends on utilizing WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt that produces a water-soluble formazan dye upon reduction with a superoxide anion. According to the manufacturer instructions, 20 μl of semen sample were added to sample and blank 2 wells, and 20 μl of double distilled water (ddH2O) were added to blank 1 and blank 3 wells. Then 200 μl of WST Working Solution were added to each well, and mixed. After that, 20 μl of dilution buffer were added to blank 2 and blank 3 wells and 20 μl of Enzyme Working Solution were added to sample and blank 1 wells, then mix thoroughly. The plate was incubated at 37 °C for 20 min and the absorbance was read at 450 nm using a microplate reader. The SOD activity (inhibition rate %) was calculated using the following equation: SOD activity (inhibition rate %) = {[(Ablank 1 - Ablank 3) – (Asample - Ablank 2)]/(Ablank 1 - Ablank 3)} x 100.

2.3. In vitro bovine embryo production

2.3.1. In vitro maturation of bovine oocytes

Ovaries were collected from the slaughterhouse (Manzo Carni, Cuneo, Italy) from Piemontese breed. They were washed with warm saline solution (SS) and transported in a thermos filled up with a warm SS to maintain constant the temperature. Ovaries were individually rinsed with warm SS (38 °C) and dried with gauze. Follicles were selected by size to be between 2 and 8 mm in diameter. Follicular fluid aspiration was performed using a 10 ml syringe with an 18-gauge needle. The follicular fluid was collected in a 50 ml tube and the oocytes were recovered from the bottom of the tube at the end of the procedure. The sediment of the 50 ml tube was aspirated and transferred into a 100

Table 2
Effect of Relaxin on motility parameters of cryopreserved Piedmontese bull semen after different incubation periods.

Parameter	Group	Post-thawing	1hr	2hr	3hr	4hr
Total motility (%)	control	91.30 ± 0.37[a]	77.92 ± 1.09[a]	61.02 ± 1.68[a]	47.12 ± 1.49[a]	41.66 ± 1.37
	Relaxin 25 ng/mL	90.72 ± 0.44	82.73 ± 1.29[b]	64.45 ± 1.05	50.20 ± 1.53	42.59 ± 1.31
	Relaxin 50 ng/mL	88.30 ± 0.79[b]	81.14 ± 0.67	61.66 ± 1.55	52.69 ± 1.63[b]	44.36 ± 1.60
	Relaxin 100 ng/mL	83.48 ± 1.01[b]	86.69 ± 0.92[b]	66.02 ± 1.02[b]	47.91 ± 0.96	44.44 ± 0.94
	Sig.	0.001	0.001	0.05	0.05	NS
Progressive motility (%)	control	54.45 ± 0.56[a]	45.05 ± 0.94[a]	22.45 ± 1.48[a]	6.51 ± 1.21	3.17 ± 0.93
	Relaxin 25 ng/mL	54.50 ± 0.60	45.86 ± 0.93	27.94 ± 1.23[b]	10.23 ± 1.45	3.66 ± 1.10
	Relaxin 50 ng/mL	52.47 ± 0.69	41.92 ± 0.45[b]	27.59 ± 1.34[b]	9.47 ± 1.41	4.39 ± 1.22
	Relaxin 100 ng/mL	49.88 ± 0.65[b]	45.95 ± 0.83	29.05 ± 1.02[b]	6.88 ± 1.01	3.77 ± 1.04
	Sig.	0.001	0.001	0.001	NS	NS
Rapid motility (%)	control	72.00 ± 0.54[a]	59.63 ± 1.09[a]	37.18 ± 1.84[a]	18.41 ± 1.86	9.22 ± 1.89
	Relaxin 25 ng/mL	72.19 ± 0.82	64.30 ± 1.17[b]	41.56 ± 1.17	22.91 ± 2.10	10.77 ± 1.91
	Relaxin 50 ng/mL	69.13 ± 0.86[b]	59.05 ± 0.64	42.20 ± 1.58[b]	23.97 ± 2.14	12.45 ± 2.32
	Relaxin 100 ng/mL	64.47 ± 0.80[b]	66.11 ± 1.08[b]	44.94 ± 1.24[b]	19.97 ± 1.42	11.44 ± 1.70
	Sig.	0.001	0.001	0.01	NS	NS

Values are presented as mean ± SEM; Sig: Significance; NS: Not Significant. The superscripts within the same column indicate significant differences among experimental groups.

mm dish for the research of the cumulus-oocyte complexes (COCs). Only COCs with an intact compact cumulus were selected and moved into a petri dish containing the washing medium constituted by TCM-199 HEPES supplemented with 10% Fetal Calf Serum. Then, COCs were washed 3 times into TCM-199 HEPES medium and 3 times into the in vitro maturation medium, as follow. In vitro maturation (IVM) was performed as reported by Perrini et al. [32] for 24 h in TCM199 Earle's Salt medium supplemented with 10% FCS, 5 μg/ml luteinizing hormone (LH) (Lutropin, Bioniche), 5 μg/ml follicle-stimulating hormone (FSH) (Folltropin, Bioniche), 0.2 mM sodium pyruvate, 10 μg/ml gentamycin and 1 mg/ml estradiol 17β. The oocytes were cultured in 70 μl droplets of IVM medium covered by paraffin oil, at 38.5 °C in 5% CO_2. A maximum of 20 oocytes per droplet were used.

2.3.2. In vitro fertilization of bovine oocytes

In vitro fertilization (IVF) was performed in TALP medium [32] supplemented with 2 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine and 20 μg/ml heparin, 10 μg/ml gentamicin, 0.2 mM pyruvate and 6 mg/ml BSA. Frozen–thawed semen was prepared by Percoll gradient (Amersham Pharmacia Biotec) centrifugation in a 15 ml tube by adding 1 ml Percoll 90% under 1 ml Percoll 45%. Semen was thawed at 37 °C for 40 s, placed on the top of the Percoll gradient and centrifuged for 30 min at 300×*g*. The semen (with or without relaxin treatment) was added to the IVF droplets containing the oocytes at the final concentration of 10×10^6 spermatozoa/ml. The IVF dish with the oocytes and the semen was incubated for 20h. At the end of gametes co-culture, the cumulus cells were completely removed, and cumulus-free presumptive zygotes were washed three times and cultured in synthetic oviductal fluid (SOF; [17]) at 38.5 °C with 5% CO_2 and 5% of O_2 for 7 days.

2.3.3. Embryo fixing and quality assessment after Hoechst staining

Embryos were fixed with 2% paraformaldehyde, mounted on a microscope slide, stained with Hoechst 33258 and covered with a coverslip. Nuclear chromatin was observed under a Nikon Eclipse TE 2000-S fluorescent microscope equipped with a B2A (346 nm excitation/460 nm emission) filter. Embryos were classified according to the chromatin status: regular-shaped nuclei inside each blastomere were considered normal, micronuclei and lobulated nuclei were considered as signs of chromatin damage, if they contained more than 32 cells without an organized outer ring of cells they were classified as morulae, if they contained more than 50 cells and showing an outer ring of cells around a blastocoel cavity, they were classified as blastocysts.

2.4. Statistical analysis

Semen data were analyzed and presented as mean ± SEM using SPSS (Ver. 16). Multiple comparisons of the means were done using General Linear Model (GLM) multivariate followed by Dunnett's post hoc test. Pearson correlations have been used to find the correlations between the different experimental parameters. Proportions of matured oocytes, cleaved embryos, morulae and blastocysts were compared among treatments by the Chi-Square test. Differences were considered to be significant when $P < 0.05$.

4. Results

4.1. Effect of relaxin on motility parameters

The mean values of sperm motility parameters evaluated by CASA analysis are shown in Table 2. Relaxin incorporation at different concentrations in extender medium during cryopreservation had a variable effect on the sperm motility parameters after thawing and incubation for 4h. Semen samples treated with relaxin at 50 and 100 ng/ml decreased the sperm total and rapid motility immediately after thawing compared to the control group ($P < 0.001$), whereas, progressive motility was reduced only at 100 ng/ml ($P < 0.001$). Interestingly, after 1h of incubation, the total and the rapid motility was increased in samples treated with 25 and 100 ng/ml relaxin ($P < 0.001$) compared with controls. On the contrary, the progressive motility was decreased at 50 ng/ml of relaxin ($P < 0.001$). After 2h of incubation, the progressive motility was increased in all relaxin-treated samples compared with controls ($P < 0.001$), whereas, the rapid motility was increased at 50 and 100 ng/ml relaxin ($P < 0.01$) and the total motility was only increased at 100 ng/ml relaxin ($P < 0.05$). Even after 3h of incubation, 50 ng/ml relaxin increased the percentage of total motility ($P < 0.05$). After 4h of incubation, no statistical differences were identified for motility parameters between relaxin-treated samples and controls.

4.2. Effect of relaxin on velocity parameters

The effect of different concentrations of relaxin on sperm velocity parameters is presented in Table 3. Relaxin incorporation in extender medium during cryopreservation modified several velocity parameters after thawing and incubation up to 4h at 37 °C. Relaxin significantly increased the sperm VAP and VSL values immediately after thawing in samples treated with 100 ng/ml relaxin, compared with controls. After 1 of incubation, VAP, VSL and VCL, were significantly improved by 25 and 100 ng/ml relaxin addition. Similarly, after 2h of incubation, VSL values were increased in all relaxin-treated samples ($P < 0.01$), whereas, VAP

Table 3
Effect of Relaxin on velocity parameters of cryopreserved Piedmontese bull semen after different incubation periods.

Parameter	Group	Post-thawing	1hr	2hr	3hr	4hr
VAP (μm/s)	control	87.11 ± 0.33[a]	78.98 ± 0.50[a]	60.77 ± 0.91[a]	53.18 ± 1.27	48.23 ± 1.27
	Relaxin 25 ng/mL	86.62 ± 0.27	81.34 ± 0.59[b]	63.19 ± 1.02	53.62 ± 1.36	48.38 ± 1.26
	Relaxin 50 ng/mL	86.93 ± 0.25	80.48 ± 0.67	63.72 ± 0.94	54.14 ± 1.37	49.89 ± 1.30
	Relaxin 100 ng/mL	88.15 ± 0.32[b]	81.63 ± 0.62[b]	64.77 ± 1.09[b]	53.86 ± 1.28	50.48 ± 1.16
	Sig.	0.01	0.01	0.05	NS	NS
VSL (μm/s)	control	73.00 ± 0.25[a]	65.38 ± 0.30[a]	48.92 ± 0.81[a]	37.54 ± 1.27	31.18 ± 1.26
	Relaxin 25 ng/mL	72.76 ± 0.25	67.00 ± 0.45[b]	51.91 ± 0.91[b]	39.74 ± 1.40	31.82 ± 1.26
	Relaxin 50 ng/mL	73.13 ± 0.28	66.11 ± 0.49	51.88 ± 0.78[b]	39.00 ± 1.35	32.68 ± 1.22
	Relaxin 100 ng/mL	74.68 ± 0.22[b]	66.92 ± 0.45[b]	52.51 ± 0.86[b]	38.53 ± 1.26	32.80 ± 1.24
	Sig.	0.001	0.05	0.01	NS	NS
VCL (μm/s)	control	142.64 ± 0.94	135.15 ± 0.92[a]	104.28 ± 1.49[a]	94.14 ± 2.05	80.59 ± 2.35
	Relaxin 25 ng/mL	142.02 ± 0.56	140.99 ± 1.34[b]	107.85 ± 1.73	94.19 ± 2.16	81.98 ± 2.27
	Relaxin 50 ng/mL	140.86 ± 0.71	139.05 ± 1.42	109.58 ± 1.59	95.25 ± 2.20	83.25 ± 2.45
	Relaxin 100 ng/mL	144.05 ± 1.02	140.31 ± 1.35[b]	111.71 ± 1.88[b]	95.49 ± 2.12	85.26 ± 2.07
	Sig.	NS	0.01	0.01	NS	NS
ALH (μm)	control	5.94 ± 0.04	6.03 ± 0.40[a]	5.73 ± 0.06	5.61 ± 0.26[a]	3.34 ± 0.33
	Relaxin 25 ng/mL	5.96 ± 0.02	6.20 ± 0.05[b]	5.55 ± 0.03	5.42 ± 0.25	3.88 ± 0.55
	Relaxin 50 ng/mL	5.85 ± 0.04	6.14 ± 0.06	5.63 ± 0.06	5.03 ± 0.22	4.22 ± 0.36
	Relaxin 100 ng/mL	5.93 ± 0.06	6.10 ± 0.04	5.70 ± 0.07	6.88 ± 0.13[b]	3.93 ± 0.33
	Sig.	NS	0.05	NS	0.001	NS
BCF (Hz)	control	27.72 ± 0.20	22.33 ± 0.12	18.43 ± 0.24[a]	14.19 ± 0.38[a]	14.49 ± 0.38
	Relaxin 25 ng/mL	27.58 ± 0.17	22.36 ± 0.14	19.30 ± 0.23[b]	16.23 ± 0.33[b]	13.81 ± 0.41
	Relaxin 50 ng/mL	28.09 ± 0.17	22.62 ± 0.14	19.42 ± 0.25[b]	15.51 ± 0.38[b]	13.40 ± 0.35
	Relaxin 100 ng/mL	28.29 ± 0.19	22.72 ± 0.15	19.12 ± 0.29	15.08 ± 0.40	13.68 ± 0.36
	Sig.	NS	NS	0.05	0.01	NS
STR (%)	control	84.19 ± 0.21	83.25 ± 0.30[a]	81.39 ± 0.39[a]	70.78 ± 0.78[a]	64.52 ± 0.71
	Relaxin 25 ng/mL	84.20 ± 0.17	82.72 ± 0.21	82.67 ± 0.23[b]	74.06 ± 0.87[b]	66.05 ± 0.73
	Relaxin 50 ng/mL	84.34 ± 0.25	82.58 ± 0.16	82.17 ± 0.24	72.31 ± 0.87	65.33 ± 0.64
	Relaxin 100 ng/mL	84.98 ± 0.29	82.39 ± 0.21[b]	81.86 ± 0.24	71.91 ± 0.76	64.61 ± 0.76
	Sig.	NS	0.05	0.01	0.05	NS
LIN (%)	control	53.13 ± 0.29	49.80 ± 0.23[a]	48.39 ± 0.38	40.41 ± 0.53[a]	39.06 ± 0.44
	Relaxin 25 ng/mL	53.06 ± 0.21	48.95 ± 0.21[b]	49.30 ± 0.25	42.52 ± 0.61[b]	39.23 ± 0.47
	Relaxin 50 ng/mL	53.77 ± 0.28	48.98 ± 0.18[b]	48.78 ± 0.27	41.50 ± 0.60	39.77 ± 0.47
	Relaxin 100 ng/mL	53.91 ± 0.38	49.09 ± 0.20[b]	48.48 ± 0.22	40.95 ± 0.53	38.48 ± 0.47
	Sig.	NS	0.01	NS	0.05	NS

Values are presented as mean ± SEM. Sig: Significance NS: Not Significant. VAP: Average path velocity (μm/s). VSL: Straight linear velocity (μm/s). VCL: Curvilinear velocity (μm/s). ALH: Amplitude of lateral head displacement(μm). BCF: Beat cross frequency (Hz). STR: straightness ([VSL/VAP] × 100). LIN: Linearity (VSL/VCL] × 100). The superscripts within the same column indicate statistically significant differences versus controls.

and VCL values were statistically increased only after 100 ng/ml relaxin addition compared with controls. ALH values were increased in samples treated with 25 ng/ml relaxin addition after 1h of incubation ($P < 0.05$) and with 100 ng/ml relaxin addition after 3h of incubation ($P < 0.01$). BCF values were increased in samples treated with 25 and 50 ng/ml relaxin compared with controls after 2 ($P < 0.01$) and 3h ($P < 0.05$) of incubation. Although, STR values were reduced in samples treated with 100 ng/ml relaxin after 1h of incubation ($P < 0.05$), they were increased if treated with 25 ng/ml relaxin after 2h ($P < 0.01$) and 3h ($P < 0.05$) of incubation. Similarly, LIN values were reduced in all relaxin-treated samples after 1h of incubation, whereas, they were increased after 3h of incubation in samples treated with 25 ng/ml relaxin ($P < 0.05$).

4.3. Effect of relaxin on sperm vitality, acrosome, plasma membrane and DNA integrities

Sperm vitality, as assessed by eosin-nigrosin staining procedure, was improved by 25 and 100 ng/ml relaxin treatment compared to controls ($P < 0.05$; Table 4). However, no statistical differences were identified regarding sperm acrosome, plasma membrane and DNA integrity between relaxin-treated samples and controls (Table 4). As well as, no statistical difference was found between the experimental groups regarding the percentage of necrotic and apoptotic sperm cells (Table 5).

Table 4
Effect of Relaxin on cryopreserved Piedmontese bull semen vitality, acrosome, plasma membrane and DNA integrities.

Group	Sperm vitality (%)	Acrosome Integrity (%)	Plasma membrane Integrity (%)	DNA Integrity (%)
Control	68.44 ± 1.26[a]	95.00 ± 1.33	60.50 ± 1.76	91.46 ± 1.11
Relaxin 25 ng/mL	73.31 ± 1.34[b]	94.88 ± 1.17	62.75 ± 1.92	92.05 ± 1.39
Relaxin 50 ng/mL	69.94 ± 0.88	95.50 ± 1.02	62.75 ± 1.68	91.62 ± 1.94
Relaxin 100 ng/mL	70.63 ± 1.11[b]	93.63 ± 2.27	58.94 ± 2.11	92.00 ± 1.37

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

Table 5
Effect of Relaxin on cryopreserved Piedmontese bull semen apoptosis.

Group	Normal viable sperm (%)	Necrotic sperm (%) (Early necrotic + Necrotic %)	Apoptotic sperm (%)
Control	45.49 ± 4.74	51.27 ± 4.46	3.25 ± 0.65
Relaxin 25 ng/mL	54.13 ± 3.79	43.37 ± 3.81	2.50 ± 0.95
Relaxin 50 ng/mL	52.53 ± 1.48	44.11 ± 1.14	3.37 ± 1.52
Relaxin 100 ng/mL	54.45 ± 2.17	43.90 ± 2.45	1.65 ± 0.63

Values are presented as mean ± SEM.

Table 6
Effect of Relaxin on cryopreserved Piedmontese bull sperm mitochondrial activity, mucus penetration ability and SOD activity.

Group	HMMP (%)	Mucus penetration distance (cm)	SOD activity (u/mL)
Control	20.59 ± 7.25	8.70 ± 0.41	1.68 ± 0.02
Relaxin 25 ng/mL	26.41 ± 8.92	9.83 ± 0.98	1.68 ± 0.01
Relaxin 50 ng/mL	32.75 ± 9.96	9.08 ± 1.56	1.66 ± 0.01
Relaxin 100 ng/mL	41.87 ± 5.53	6.68 ± 1.55	1.69 ± 0.02

Values are presented as mean ± SEM. HMMP: High Mitochondrial membrane; SOD: Superoxide dismutase.

4.4. Effect of relaxin on sperm mitochondrial membrane potential, mucus penetration distance and superoxide dismutase activity (SOD)

As presented in Table 6, even if a dose-dependent increase of sperm with HMMP was found in all relaxin-treated samples, no significant difference has been identified compared to the control group. Similarly, no significant differences have been found concerning the mucus penetration distance and the SOD activity (Table 6).

4.5. Effect of relaxin-treated semen on developmental potential of bovine embryos

On the basis of the results obtained on semen quality assessment, the 25 ng/ml relaxin concentration was selected to test the effect of relaxin-treated semen on developmental potential of bovine in vitro produced embryos. Seven experiments were conducted and a total of 708 cumulus-oocytes complex (COCs) were recovered and selected as suitable for in vitro culture. Of these, 389 were fertilized with relaxin-added semen whereas, 319 formed the control group. As shown in Table 7, the cleavage rate was not improved by using relaxin-treated semen compared with controls. Interestingly, the blastocyst developmental rate resulted in a significant increase in the relaxin group compared with controls ($P < 0.01$; Table 7)

4.6. Correlations between the different experimental parameters

The correlation coefficients among different semen quality parameters when considering the effects of relaxin are presented in Table 8. The total sperm motility was positively correlated with intact acrosome ($P < 0.05$), progressive motility, rapid motility and intact plasma membrane ($P < 0.01$), whereas it was negatively correlated with ALH ($P < 0.05$), VSL, STR and LIN ($P < 0.01$). The progressive motility correlated positively with intact plasma membrane ($P < 0.05$), rapid motility, BCF, STR and LIN ($P < 0.01$) and negatively with VAP, VCL and ALH ($P < 0.01$). The rapid motility correlated positively ($P < 0.01$) with intact plasma membrane and negatively with VSL and STR ($P < 0.01$). A negative correlation was found between the live sperm %, VAP and VCL ($P < 0.01$). The intact acrosome correlated positively with the intact plasma membrane ($P < 0.01$) and negatively with VSL and ALH ($P < 0.05$). The intact plasma membrane correlated positively with LIN ($P < 0.05$) and negatively with VCL ($P < 0.05$) and ALH ($P < 0.01$). A negative correlation was found between viable sperm and necrotic sperm % ($P < 0.01$). The HMMP correlated positively with viable sperm and BCF ($P < 0.05$) and negatively with apoptotic sperm ($P < 0.05$).

5. Discussion

The use of cryopreserved semen in animal breeding programs could results in reduced fertility when compared to natural mating or raw semen owing to decreased sperm viability and damage in the functional

Table 7
Effect of relaxin-treated semen at 25 ng/ml before cryopreservation on developmental potential of in vitro-produced bovine embryos.

Group	Evaluated COCs	Cleavage (%)	Morula (%, cleaved)	Blastocyst (%, cleaved)
Control	319	116 (36.4)	11 (9.5)	32 (27.6)[a]
Relaxin (25 ng/ml)	389	118 (30.3)	12 (10.2)	52 (44.0)[b]

Chi square test: a, b: $P < 0.01$; COC: Cumulus-oocyte complex.

Table 8
Correlation coefficients between the different parameters of cryopreserved Piedmontese bull semen under the effect of Relaxin.

	Group	VAP	VSL	VCL	ALH	BCF	STR	LIN	T. Motility	P. Motility	R. Motility	Live sperm	Intact acrosome	Intact P.M.	Intact DNA	Viable sperm	Necrotic sperm	Apoptic sperm	HMMP	Mucus penetration
VAP	.159[b]																			
VSL	.282[b]	.681[b]																		
VCL	.051	.839[b]	.311[b]																	
ALH	-.042-	.599[b]	.069	.895[b]																
BCF	.167[b]	-.299[b]	.159[a]	-.664[b]	-.806[b]															
STR	.150[a]	-.327[b]	.450[b]	-.584[b]	-.625[b]	.545[b]														
LIN	.144[a]	-.423[b]	.296[b]	-.795[b]	-.858[b]	.764[b]	.872[b]													
T. Motility	-.454[b]	-.112-	-.418[b]	-.099-	-.129[a]	.076	-.409[b]	-.162[b]												
P.Motility	-.330[b]	-.188[b]	.076	-.433[b]	-.502[b]	.389[b]	.316[b]	.459[b]	.559[b]											
R. Motility	-.420[b]	-.006-	-.242[b]	-.065-	-.118-	.018	-.305[b]	-.102-	.830[b]	.767[b]										
Live sperm	.077	-.333[b]	-.205-	-.337[b]	-.213-	.147	.126	.144	-.083-	.169	.077									
Intact acrosome	-.066-	-.170-	-.284[a]	-.169-	-.288[a]	.099	-.103-	.099	.273[a]	.045	.142	.198								
Intact P.M.	-.070-	-.196-	-.199-	-.283[a]	-.394[b]	.133	-.010-	.269[a]	.489[b]	.316[a]	.371[b]	-.024-	.409[b]							
Intact DNA	.051	-.144-	-.174-	-.126-	.262	-.009-	-.029-	-.028-	-.129-	-.037-	-.037-	.102	.047	.151						
Viable sperm	.417	-.422-	-.096-	-.430-	.165	.372	.458	.264	-.164-	.072	-.268-	.459	-.257-	-.290-	.083					
Necrotic sperm	-.369-	.471	.166	.459	-.142-	-.328-	-.446-	-.238-	.107	-.049-	.269	-.434-	.145	.322	-.156-	-.961[b]				
Apoptic sperm	-.234-	-.101-	-.228-	-.029-	-.108-	-.213-	-.120-	-.134-	.227	-.093-	.040	-.165-	.430	-.062-	.237	-.301-	.027			
HMMP	.488	.004	.213	-.081-	-.032-	.546[a]	.348	.241	-.396-	-.105-	-.376-	.250	-.030-	-.398-	-.465-	.499[a]	-.371-	-.525[a]		
Mucus penetration	-.316-	-.302-	-.371-	-.248-	.072	-.413-	-.139-	-.103-	.386	.104	.137	-.233-	.126	.273	.156	.158	-.199-	.117	-.105-	
SOD activity	.115	.107	.224	-.013-	-.236-	.028	.219	.203	-.106-	.273	.077	-.050-	.066	-.174-	-.261-	-.049-	.107	-.192-	.253	.236

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, SOD: Superoxide dismutase.

[a] Correlation is significant at the 0.05 level.

[b] . Correlation is significant at the 0.01 level.

capability of live spermatozoa [6,38]. In the present study, the effect of different concentrations of relaxin, added into the extender before the cryopreservation process, were tested on Piedmontese bull spermatozoa up to 4h of incubation. We found that relaxin incorporation in extended bull semen before cryopreservation induced a reduction of sperm motility immediately after thawing (0h), whereas, during long incubation periods (1–2 h), relaxin showed a significant improvement of the sperm motility and velocity parameters. Moreover, sperm vitality and blastocyst developmental rate were significantly improved by relaxin addition.

Sperm motility assessed by CASA is one of the most significant features linked with the fertilizing potential [37]. Progressive motility and velocity parameters (VAP and VSL) have been positively correlated with in vitro fertility potential of bull spermatozoa [20]. Although, in a previous study [24] the addition of relaxin (16.7 nM) to human semen before freezing did not improve the sperm motility, others studies suggested a potential role of relaxin in enhancing sperm quality in different species [13,27,29]. In the present study, 50 and 100 ng/ml relaxin induced a reduction of sperm motility immediately after thawing. Unfortunately, these data cannot be compared with previous studies, as no study verified the effects of relaxin immediately after thawing. Generally, the effects of relaxin have been assessed during long incubation periods (1–4 h) as relaxin attenuated the natural decline in sperm motility identified in the control samples, whereas it remained higher in samples incubated with relaxin [13,27,29]. Similarly, in our study, relaxin increased the total, the rapid motility and the velocity variables (VAP, VSL and VCL) of bovine spermatozoa after 1 and 2 h of incubation. Our results are in line with previous studies. In fact, it was found that the supplementation of 20 ng/ml relaxin in the thawing solution improved the boar sperm motility after 1–3h of incubation [26]. In a study on fresh porcine semen, 20 ng/ml of relaxin significantly increased sperm progressive motility [29]. In another study, sperm motility of buffalo semen was improved in samples treated with 50 and 100 ng/ml relaxin [10]. Similarly, Feugang et al. [14] investigated the beneficial effects of relaxin on motility characteristics of stored boar spermatozoa and demonstrated that relaxin at 100 ng/ml increased motile, progressive and rapid spermatozoa up to day 2 of incubation whereas 500 ng/ml had beneficial effects up to day 4. In human medicine, it was stated that relaxin attenuated the natural decline in sperm motility during 4 h of incubation mainly with concentration of 100 nM [13].

Regarding the sperm vitality, our results revealed that different doses of relaxin (25 and 100 ng/ml) significantly improved the percentage of viable sperm. These parameters are useful to predict the sperm fertilizing ability [3] in bovine [18] and buffaloes [23]. The addition of relaxin before the sperm cryopreservation process might had a positive effect on the stabilization of the phospholipid bilayer of the sperm membranes inducing a protective role during the freezing process that have been associated with excessive production of reactive oxygen species (ROS) associated with a decrease in the quality of frozen-thawed spermatozoa [36]. Semen processing is stressful for spermatozoa because it affects sperm membranes function [7], which begins by asymmetry of the membrane phospholipids before the integrity is progressively damaged [25], leading to the onset of oxidative stress, apoptosis and eventually cellular necrosis. However, in the present study, DNA integrities, SOD activity and apoptosis did not reveal any difference between relaxin-treated samples and controls. Conversely, in the study of Ferlin et al. [13] 100 nM relaxin prevent apoptosis and increased the percentage of viable human sperm.

Sperm mitochondrial transmembrane potential is considered as one of the most sensitive tests for predicting sperm fertilizing capacity in buffalo [34] and human [19]. In the present study we did not find any changes in sperm HMMP in relaxin-treated compared with controls, however, we recorded a significant ($P < 0.05$) positive correlation between viable sperm and HMMP and both have negative correlation with necrotic and apoptotic sperm. On the contrary, it was found that relaxin at 10 and 100 nM improved human sperm HMMP up to 4h of incubation [13].

The passage of the sperm through the female reproductive tract is affected by several factors and sperm motility is one of them [27]. Sperm mucus penetration test might reveal another factor affecting bull fertility rather than sperm motility alone as the extent of mucus penetration by spermatozoa was better associated with the conception rate than subjectively estimated sperm motility [30]. It has been employed in the diagnosis of human male infertility [8] and assessment of bull fertility [15,30]. In the present study, we did not identify any significant differences in sperm mucus penetration distance after relaxin treatments.

In order to determine the real benefit of increased motility we investigated the developmental potential of in vitro produced bovine embryos obtained by using relaxin-treated sperm samples. Interestingly, in our study, we demonstrated that the blastocyst formation rate was significantly increased by supplementation of relaxin to semen before the cryopreservation procedure. To the best of our knowledge, few studies have investigated the relaxin effects on in vitro embryo production. In a previous study [16], relaxin was added after the swim-up procedure of porcine fresh semen at the concentrations of 20 and 50 ng/ml and during the in vitro fertilization medium at the concentration of 5, 10, 15, 20 ng/ml. In that study, relaxin improved sperm motility and acrosome reaction rate, and the percentage of normospermic penetration. In another study, Elkhawagah et al. [10] supplemented with 50 and 100 ng/ml relaxin the buffalo semen after thawing and showed that relaxin was beneficial to the semen in vitro fertilizing ability. In fact, relaxin increased the cleavage rate and normospermic penetration compared to the control group. Similarly, Kim et al. [21] investigated relaxin effects on in vitro fertilization of porcine oocyte by adding relaxin (1, 10, and 100 ng/ml) during IVF. Their results showed that relaxin the blastocyst formation rate, and the GSH content (an indicator of cytoplasmic maturity) were increased with 1 and 10 ng/ml of relaxin. Our data are in line with this study, however we believe that the advantage of our study is that relaxin has been added during the semen cryopreservation procedures and not after thawing procedure or during the in vitro fertilization. Our experimental procedure is more practical in order to be applied on field conditions, avoiding any type of post-thawing treatments. Further studies are needed to determine the effect of relaxin in bulls with reduced fertility.

Declaration of competing Interest

No conflict of interest was reported by the authors.

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Appendix A. Supplementary data

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