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Original Research Article

Influence of hydroxytyrosol in semen extenders on fertilizing capacity and embryo development using cooled and cryopreserved bull sperm

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ABSTRACT

This study investigated the effects of hydroxytyrosol (HT), a potent antioxidant polyphenol, on sperm functionality, fertilizing ability, and embryo development when added to semen extenders using cooled and cryopreserved bull semen. In a preliminary trial, 1 and 25 μM HT were identified as the most effective concentrations and subsequently used in the main experiments. Semen from 12 Angus bulls was divided into aliquots and stored either refrigerated at 4 °C for 24 h or cryopreserved. Sperm parameters (motility, viability, membrane and acrosomal integrity), malondialdehyde (MDA) levels, total antioxidant capacity (TAC), zona pellucida (ZP) binding, and *in vitro* fertilization outcomes were assessed. In cooled semen, HT enhanced functional membrane integrity and acrosomal status, and increased sperm–ZP binding. Supplementation with 1 μM HT improved embryo quality, increasing hatching rate and blastocyst cell number. In cryopreserved semen, 25 μM HT improved ZP-binding capacity, blastocyst rates, and blastocyst cell numbers. These findings suggest that HT supplementation improves sperm functionality and embryo development under both cooled and cryopreserved storage conditions, highlighting its potential to enhance assisted reproductive technologies in cattle.

1. Introduction

Reproductive efficiency is a key determinant of the economic sustainability of cow-calf systems [1]. Assisted reproductive technologies (ARTs), such as fixed-time artificial insemination (FTAI) and *in vitro* embryo production (IVP), significantly enhance livestock productivity by accelerating genetic gain and enabling more efficient herd management [2–4]. Among the various factors that influence the success of these technologies, semen quality is particularly critical, especially when preserved spermatozoa are used [5–7].

Two main strategies are employed for semen preservation at low temperatures: refrigeration at 4–5 °C and cryopreservation at −196 °C in liquid nitrogen. Cooled semen avoids freezing-related injuries and typically maintains higher sperm viability than frozen-thawed semen [8,9]. Consequently, this improvement can optimize the use of genetically superior sires in AI programs by reducing the required insemination dose [10]. However, its short shelf life limits its application to local and short-term use [11]. In contrast, cryopreservation enables long-term storage and worldwide distribution, but the freezing and thawing processes induce substantial structural and functional damage to spermatozoa, leading to reduced motility, membrane integrity, and fertilizing potential [12,13].

Oxidative stress plays a central role in the deterioration of sperm quality during preservation [14]. The imbalance between oxidants and antioxidants can impair sperm viability and functionality in cooled and cryopreserved samples [14,15]. In cooled semen, reactive oxygen species (ROS) progressively accumulate as a result of continued metabolic activity [15], whereas in cryopreserved semen, ROS are predominantly generated during the freeze–thaw process [14]. Excessive ROS production causes lipid peroxidation, DNA fragmentation, and damage to sperm proteins, ultimately compromising fertilizing ability [16]. Given the limited endogenous antioxidant capacity of mammalian spermatozoa [17], the inclusion of effective antioxidants in semen extenders could play a crucial role in mitigating oxidative damage and preserving

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sperm function during storage.

Hydroxytyrosol (HT), a potent polyphenol derived from olive oil, has demonstrated strong antioxidant properties, including ROS scavenging, upregulation of antioxidant enzymes, and metal-chelating activity [18,19]. Several studies have reported beneficial effects of HT supplementation in semen extenders for cryopreservation across various species, including humans [19], stallions [20], rams [21], and bulls [22]. These studies have demonstrated that HT supplementation improves sperm viability and motility post-thaw [19–22]. However, enhancements in conventional sperm parameters do not necessarily translate into improved fertilizing ability or embryonic development [23]. To date, no study has investigated the effect of HT supplementation in semen extenders on the fertilizing capacity and embryonic developmental potential of bovine sperm, whether cooled or cryopreserved.

This study aimed to evaluate the effect of HT supplementation in semen extenders on sperm binding to the zona pellucida (ZP), fertilization outcomes, and embryo development to the blastocyst stage using cooled and frozen-thawed bull semen. Additionally, sperm viability, motility, acrosomal integrity, and antioxidant status were evaluated after both storage conditions.

2. Materials and methods

2.1. Reagents and media

The semen extender BIOXcell was obtained from IMV (L'Aigle, France), and the hydroxytyrosol was obtained from Nova Mentis Ltd. (1-HT® Hydroxytyrosol, Nova Mentis Ltd., Dublin, Ireland). All *in vitro* embryo production (IVP) media preparation reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The maturation medium was TCM-199 supplemented with 10 % (v/v) FCS (Internegocios S.A., Buenos Aires, Argentina) and 1 μg/mL FSH. The fertilization medium consisted of Tyrode's albumin lactate pyruvate (TALP) supplemented with 6 mg/mL fatty acid-free bovine serum albumin (FAF-BSA), 20 μM penicillamine, 10 μM hypotaurine, and 100 μg/mL heparin sulfate. The medium for *in vitro* culture (IVC) was synthetic oviduct fluid (SOF) supplemented with 2 % (v/v) minimum essential medium (MEM)-essential amino acids, 1 % (v/v) MEM-nonessential amino acids, and 8 mg/mL FAF-BSA.

2.2. Animals and semen collection

The experimental procedures were approved by the School of Veterinary Sciences Institutional Animal Care and Use Committee (National University of La Plata, Buenos Aires, Argentina; Protocol No. 241122-1). For this study, Angus bulls aged between 15 and 40 months were selected from three commercial farms in Buenos Aires, Argentina. Semen collection was performed using the electro-ejaculation method. Fresh semen samples were assessed for concentration using a hemocytometer chamber, for motility employing the iSperm® mCASA mobile computer-assisted sperm analyzer (Aidmics Biotechnology, Taipei, Taiwan), and for morphology using a phase-contrast microscope. Samples meeting the criteria of $\geq$65 % motility and $\leq$15 % abnormal morphology were chosen for the experiments. Throughout the process of fresh semen evaluation and dilution, the semen was maintained at 37 °C in a water bath.

2.3. Extender preparation and cryopreservation (cooling and freezing)

The semen was diluted with BIOXcell at an ambient temperature (~25 °C) to a final concentration of 25 × 10^6 spermatozoa per 0.25 mL straw and maintained at 4 °C for 4 h. The cooling rate was approximately 0.55 °C/min. Subsequently, the semen was either stored at 4 °C for 20 h (cooled semen) or frozen for later thawing (frozen-thawed semen). For the latter, after the 4-h equilibration period, the semen was loaded into 0.25 mL French straws and frozen using a Digital Frozen Semen Machine (INTA-22, Sistel, Argentina) with a programmed freezing curve of −15 °C/min, starting at 4 °C and continuing until −80 °C. Once this temperature was reached, the freezing rate was set to −20 °C/min until −140 °C, at which point the straws were immersed in liquid nitrogen.

2.4. Analysis of sperm kinetic parameters

Motility and kinetic parameters were evaluated using iSperm® mCASA (Aidmics Biotechnology), following the manufacturer's instructions for software use. The following motion parameters were determined: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), straightness (STR, %), linearity (LIN, %), beat-cross frequency (BCF, Hz), the amplitude of lateral head displacement (ALH, μm/s), and wobble (WOB, %).

2.5. Analysis of viability, functional plasma membrane integrity, and acrosomal status

Viability was assessed using eosin-nigrosin staining. Functional sperm membrane integrity was evaluated using the hypo-osmotic swelling test (HOST). For this, semen samples were diluted in 1:10 (v/v) HOST solution (100 mOsm/L, 57.6 mM fructose, and 19.2 mM sodium citrate) and incubated at room temperature (RT). A wet mount was prepared by placing 10 μL of the mixed sample onto a microscope slide and covering it with a coverslip. For each semen sample, a total of 200 sperm were counted in at least five different microscopic fields at 400 × magnification, recording the percentage of sperm with swollen and curved tails. Acrosomal status (AS) was assessed using fluorescein isothiocyanate-labeled Pisum sativum agglutinin (FITC-PSA) staining. Sperm smears were allowed to air-dry for 10 min, fixed in methanol for 30 s, and then labeled using 50 mg/mL FITC-PSA in phosphate-buffered saline (PBS) for 30 min in the dark at RT. The slides were then rinsed with distilled water and mounted. A total of 200 sperm per sample were counted with an Olympus BX40 epifluorescent microscope (Olympus, Tokyo, Japan) using excitation wavelengths of 450–490 nm and a 1000x magnification. Acrosome-intact sperm exhibited FITC-PSA-positive staining in the acrosomal region, appearing green, whereas acrosome-damaged sperm displayed an equatorial fluorescent band with minimal or no staining in the anterior head region.

2.6. Measurement of lipid peroxidation

Lipid peroxidation levels were determined using the TBARS assay and expressed as malondialdehyde (MDA) levels. The TBARS concentration was reported as μmol MDA/mg protein, using tetramethoxypropane (TMP) as the standard. A semen aliquot underwent three freeze-thaw cycles before being centrifuged at 4220 g for 5 min. Subsequently, 100 μL of the resulting supernatant was combined with 100 μL of an 8.1 % SDS solution and 750 μL of a 20 % acetic acid solution. After adding 750 μL of a 0.8 % TBA solution and 2 mL of distilled water, the mixture was heated at 95 °C for 1 h. It was then cooled to RT and centrifuged at 4220 g for 15 min. Finally, the absorbance of the supernatant was recorded at 532 nm using a spectrophotometer.

2.7. Total antioxidant status

Total antioxidant capacity was assessed using the Randox Total Antioxidant Status kit (cat. no. NX2332, Randox Laboratories, Ltd, Crumlin, UK) with minor modifications. A semen aliquot underwent three freeze-thaw cycles and was subsequently centrifuged at 4220 g for 5 min. After centrifugation, 20 μL of the resulting supernatant was mixed with 1 mL of the chromogen ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]). In total, 20 μL Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) at a concentration of 2.27 mmol/L was used

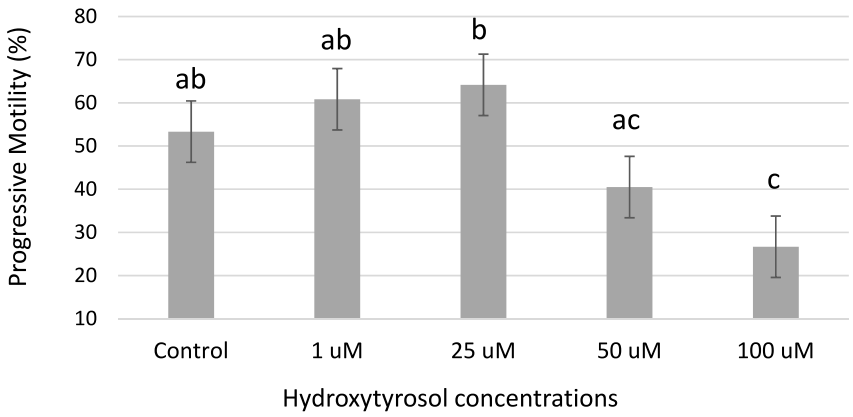

Fig. 1. Effect of different hydroxytyrosol concentrations added to the semen extender on progressive motility after 3 h of incubation. Results are expressed as LSM ± SE. [a,b,c]Bars with different letters differ statistically ($P < 0.05$).

as standard, whereas 20 μL deionized water was used as the blank. Chromogen (1 mL) was added to standard and blank samples. The absorbance was measured 3 min after substrate addition at 600 nm with a spectrophotometer. Results were expressed as μM/mg protein. Measurements in duplicate were used to calculate intra-assay variability.

2.8. Spermatozoa-ZP binding test

The sperm-zona pellucida (ZP) binding was assessed following the protocol described by Anchordoquy et al. [24]. Prior to each experiment, immature cumulus-oocyte complexes (COC) were denuded by vortexing in 0.1 % (w/v) hyaluronidase prepared in HEPES-TALP, washed twice, and incubated in 90 μL droplets of IVF medium for 2 h at 38.5 °C under 5 % CO_2 in air. Subsequently, oocytes were co-incubated with 2×10^6 spermatozoa/mL at 38.5 °C under the same atmospheric conditions for 2 h. After incubation, the oocytes were washed five times in phosphate-buffered saline (PBS) to remove loosely bound spermatozoa and then fixed and stained with Hoechst 33342. The number of spermatozoa bound to each oocyte was determined by observation at × 400 magnification under an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a 365 nm excitation filter, a 400 nm barrier filter, and a 400 nm emission filter.

2.9. In vitro maturation, fertilization, and culture

In vitro maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture of embryos (IVC) were performed according to Anchordoquy et al. [25] with some modifications. Bovine ovaries were obtained from an abattoir and transported to the laboratory within 3 h after slaughter. COCs were aspirated from 3 to 8 mm follicles, using an 18-G needle attached to a sterile syringe. Groups of 10 COC were transferred into 50 mL of IVM medium under mineral oil (Squibb, Princeton, NJ, USA), which had been pre-equilibrated in a CO_2 incubator. The incubations were performed at 38.5 °C in an atmosphere of 5 % CO_2 in air with saturated humidity for 24 h. For IVF, oocytes were washed twice in HEPES-TALP and transferred into 50 μL drops of IVF medium under mineral oil. For cooled semen, a 250 μL aliquot containing 40×10^6 spermatozoa was used. In the case of frozen semen, a straw with the same sperm concentration was thawed in a 37 °C water bath before use. Spermatozoa were washed in a discontinuous Percoll gradient prepared by depositing 400 μL of 90 % Percoll under 400 μL of 45 % Percoll in a 1.5 mL microcentrifuge tube. Semen samples were deposited on the top of the Percoll gradient and centrifuged for 15 min at 300 g. The pellet was removed and resuspended in 800 μL of IVF medium and centrifuged at 40 g for 4 min. After removal of the supernatant, spermatozoa were resuspended in IVF medium, counted in a hemocytometer chamber, and further diluted. The final sperm concentration in IVF was 2×10^6 sperm/mL. Incubations were conducted at 38.5 °C in 5 % CO_2 in air with saturated humidity for 24 h. Presumptive zygotes were denuded of cumulus cells by pipetting, washed twice in HEPES-TALP, and subsequently cultured in SOFm. Embryo culture was conducted in 40 μL drops of medium under mineral oil, with 10 presumptive zygotes per drop, at 38.5 °C in an atmosphere of 5 % O_2, 5 % CO_2, and 90 % N_2 with saturated humidity. The medium was changed every 48 h, and embryos were incubated for 8 days (day 0 = day of fertilization). Cleavage rates were recorded 48 h after insemination. Cleavage rate was determined as the percentage of cleaved embryos relative to the number of presumptive zygotes, whereas blastocyst rate was assessed as the proportion of blastocysts to the total number of presumptive zygotes. At the end of the incubation period, embryos were assessed for total blastocyst cell number using Hoechst 33342 staining and for the hatching rate.

2.10. Experimental design

A preliminary experiment was conducted with fresh semen to define the concentrations of HT to be used throughout the study. For this purpose, ejaculates from 8 bulls (n = 8) were used, with each ejaculate divided into 5 aliquots. For each aliquot, the semen extender was supplemented with 0, 1, 25, 50, and 100 μM of HT; the concentrations used are according to Sharafi et al. [22]. Progressive motility of the fresh semen was evaluated using the iSperm® mCASA (Aidmics Biotechnology) after 3 h of incubation.

To evaluate the beneficial effect of HT on cooled and frozen-thawed semen, the BIOXcell extender was supplemented with three concentrations of HT: 0 μM HT (no supplementation; Control) and the two most effective concentrations identified in the preliminary experiment. Ejaculates from 12 bulls (n = 12) from three commercial farms were used. Each semen sample was split into three aliquots, which were then diluted with extenders supplemented with different concentrations of HT. For cooled semen, samples were stored at 4 °C for 24 h. After this period, sperm kinetic parameters, viability, HOST, AS, MDA, TAC, sperm-ZP binding, and *in vitro* fertilization capacity were evaluated, as previously described. For frozen-thawed semen, samples were cryopreserved. After at least one week of storage, frozen straws were thawed in a 37 °C water bath for 40 s before analysis. The same variables assessed for cooled semen were evaluated.

Table 1
Effect of different hydroxytyrosol concentrations added to semen extender on viability, sperm membrane integrity (HOST), and acrosomal status of cooled and frozen-thawed bull sperm.

Hydroxytyrosol concentrations	Viability (%)	HOST positive (%)	Acrosome integrity (%)
Cooled semen			
0 μM	81.66 ± 0.96[a]	77.54 ± 0.90[a]	88.46 ± 0.70[a]
1 μM	82.11 ± 0.86[a]	82.72 ± 0.82[b]	91.21 ± 0.63[b]
25 μM	83.19 ± 0.83[a]	84.42 ± 0.78[b]	91.11 ± 0.63[b]
Frozen semen			
0 μM	39.02 ± 7.80[a]	54.25 ± 5.70[a]	87.91 ± 3.23[a]
1 μM	43.88 ± 8.04[a]	50.86 ± 5.74[a]	90.18 ± 2.71[a]
25 μM	39.34 ± 7.80[a]	51.52 ± 5.74[a]	88.51 ± 3.10[a]

For each test, at least 7200 spermatozoa were evaluated (at least 200 spermatozoa per treatment and bull; bull n = 12); [a-b] Within a column means without a common superscript differed ($P < 0.05$). Results are expressed as least-squares means ± SEM.

2.11. Statistical analysis

A completely randomized design with a split-ejaculate approach was used. The statistical model included bull (n = 12) as a random effect and HT treatment (0, 1, and 25 μM) as a fixed effect. The effect of farm (considered as replicate; n = 3) was initially included as a fixed factor in the statistical model but was removed when non-significant. Kinetic parameters, TAC, and MDA were evaluated with Proc Glimmix of SAS (SAS Inst. Inc., US) with normal distribution and identity link function. Viability, HOST, AS and cleavage, blastocyst, and hatching rates were evaluated with Proc Glimmix of SAS (SAS Inst.) with binomial distribution and logit link function. The analysis of sperm-zona binding and blastocyst cell number was run using the Glimmix procedure (SAS Inst.) using a Poisson distribution and a Log link function. Data were expressed as least squared means ± SEM. Statistical significance was set at $P < 0.05$, while a trend for statistical significance was set between $P > 0.05$ and ≤0.10.

3. Results

In the preliminary experiment, HT concentrations of 1 and 25 μM improved PM (numerically and significantly, respectively; $P < 0.05$; Fig. 1), and were consequently selected for subsequent experiments.

3.1. Effects of hydroxytyrosol on cooled semen

The addition of HT to the semen extender increased the percentage of HOST-positive sperm and improved acrosome integrity ($P < 0.05$; Table 1). However, it did not have a significant effect on sperm viability ($P > 0.05$; Table 1) or sperm kinetic parameters ($P > 0.05$; Table 2). Sperm lipid peroxidation, measured by MDA levels, was not affected by adding HT to the semen extender ($P > 0.05$; Fig. 2A). Similarly, no difference was observed in TAC between control and HT treatments ($P > 0.05$; Fig. 3A).

The effect of HT on sperm-zona binding is shown in Fig. 4A. Significantly more sperm bound to the ZP when HT was added at 25 μM ($P < 0.05$; Fig. 4A). Moreover, supplementation with 1 μM HT tended to increase the number of bound sperm compared to the control ($P = 0.06$; Fig. 4A).

The effect of HT on subsequent embryo development is shown in Table 3. No differences were detected in cleavage and blastocyst rates when HT was added to the semen extender ($P > 0.05$). However, supplementation with 1 μM HT improved the hatching rate compared to the control group ($P < 0.05$; Table 3). In addition, the number of blastocyst cells was increased by adding HT ($P < 0.05$; Table 4).

Table 2
Effect of different hydroxytyrosol concentrations added to semen extender on kinetic parameters of cooled and frozen-thawed bull sperm.

	HT Concentrations	TM (%)	PM (%)	VCL μm/s	VAP μm/s	VSL μm/s	STR (%)	LIN (%)	BCF Hz	ALH μm/s	WOB (%)
Cooled semen	0 μM	69.25 ± 2.3[a]	47.5 ± 1.9[a]	166.0 ± 3.0[a]	87.0 ± 1.6[a]	76.6 ± 1.5[a]	84.5 ± 0.6[a]	44.9 ± 0.7[a]	27.9 ± 0.4[a]	8.4 ± 0.2[a]	51.6 ± 0.7[a]
	1 μM	71.0 ± 2.3[a]	47.3 ± 1.9[a]	164.6 ± 3.0[a]	85.5 ± 1.6[a]	74.8 ± 1.5[a]	83.6 ± 0.6[a]	44.0 ± 0.7[a]	27.5 ± 0.4[a]	8.7 ± 0.2[a]	51.2 ± 0.7[a]
	25 μM	69.3 ± 2.3[a]	48.0 ± 1.9[a]	170.0 ± 3.0[a]	85.5 ± 1.6[a]	76.0 ± 1.5[a]	83.5 ± 0.6[a]	43.7 ± 0.7[a]	27.5 ± 0.4[a]	9.0 ± 0.2[a]	49.6 ± 0.7[a]
Frozen semen	0 μM	34.69 ± 6.4[a]	22.53 ± 4.7[a,*]	145.6 ± 3.8[a]	76.7 ± 2.9[a]	67.7 ± 3.1[a]	82.7 ± 1.1[a]	44.6 ± 1.2[a]	31.7 ± 0.6[a]	7.7 ± 0.1[a]	51.6 ± 0.9[a]
	1 μM	42.53 ± 6.4[b]	27.76 ± 4.7[a,*,†]	146.9 ± 3.8[a]	78.6 ± 2.9[a]	70.0 ± 3.1[a]	83.4 ± 1.1[a]	44.9 ± 1.2[a]	32.2 ± 0.6[a]	7.7 ± 0.1[a]	51.7 ± 0.9[a]
	25 μM	34.23 ± 6.4[a]	22.61 ± 4.7[a,†]	144.2 ± 3.8[a]	73.7 ± 2.9[a]	65.4 ± 3.1[a]	82.9 ± 1.1[a]	43.1 ± 1.2[a]	31.3 ± 0.6[a]	8.0 ± 0.1[a]	49.8 ± 0.9[a]

Kinetic parameters were evaluated using iSperm® mCASA. TM, total motility; PM, progressive motility; VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity; STR, straightness; LIN, linearity; BCF, beat-cross frequency; ALH, amplitude of lateral head displacement; WOB, wobble. [a-b] Within a column, means without a common superscript differed ($P < 0.05$). Results are expressed as least-squared means ± SEM.

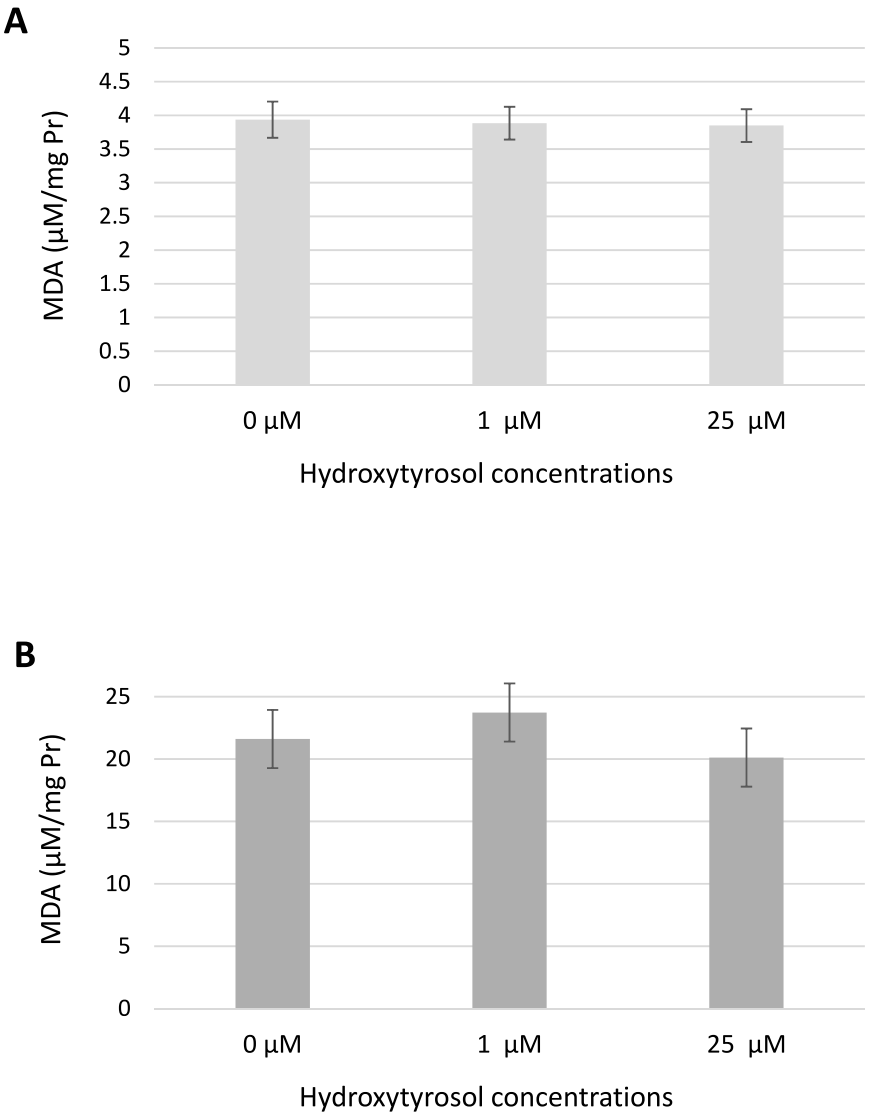

Fig. 2. Effect of different hydroxytyrosol (HT) concentrations added to the semen extender on malondialdehyde (MDA) levels of cooled (A) and frozen-thawed (B) bull sperm. Results are expressed as LSM ± SE. There were no differences in MDA levels at any HT concentrations evaluated ($P > 0.05$).

3.2. Effects of hydroxytyrosol on frozen-thawed semen

The addition of 1 µM HT increased TM compared to the control ($P < 0.05$; Table 2) and showed a tendency to increase MP ($P = 0.09$; Table 2). However, HT did not affect sperm viability, the percentage of HOST-positive sperm, or acrosome integrity ($P > 0.05$; Table 1). In addition, MDA concentration was not affected by adding HT to the semen extender ($P > 0.05$; Fig. 2B). Similarly, no difference was observed in TAC between control and HT treatments ($P > 0.05$; Fig. 3B).

The effect of HT on sperm-zona binding is shown in Fig. 4B. Considerably more sperm bound to ZP were observed when HT was added to the extender at all concentrations studied ($P < 0.01$), but the difference was highest when using 25 µM HT ($P < 0.01$; Fig. 4B).

The effect of HT on subsequent embryo development is shown in Table 3. No differences were observed in cleavage rates with HT supplementation ($P > 0.05$). However, HT at any concentration significantly increased the blastocyst yield on Day 8 of culture ($P < 0.05$; Table 3). In addition, the proportion of blastocysts on Days 6 and 7 was higher in the 25 µM HT group compared with the control ($P < 0.05$; Table 3). In contrast, no differences in hatching rates were observed among treatments ($P > 0.05$; Table 3). Furthermore, the number of blastocyst cells was increased by the addition of 25 µM HT to the semen extender ($P < 0.01$; Table 4).

4. Discussion

This study provides novel insights into the effects of HT supplementation on the fertilizing ability and embryonic development potential of bovine semen under cooled and frozen-thawed storage conditions. Additionally, to the best of our knowledge, this is the first study to investigate the effect of HT on cooled bovine semen.

The addition of HT to semen extenders improved several functional parameters of sperm. In cooled semen, HT enhanced the functional integrity of the plasma membrane and preserved acrosomal integrity, without affecting TM or PM. These findings are in agreement with previous reports in other species using non-cryopreserved semen, such as pig [26] and ram [27]. The HT supplementation helped maintain membrane integrity [26] but did not affect sperm motility [27]. In contrast, these beneficial effects were not observed in frozen-thawed semen. This discrepancy may be attributed, at least in part, to the

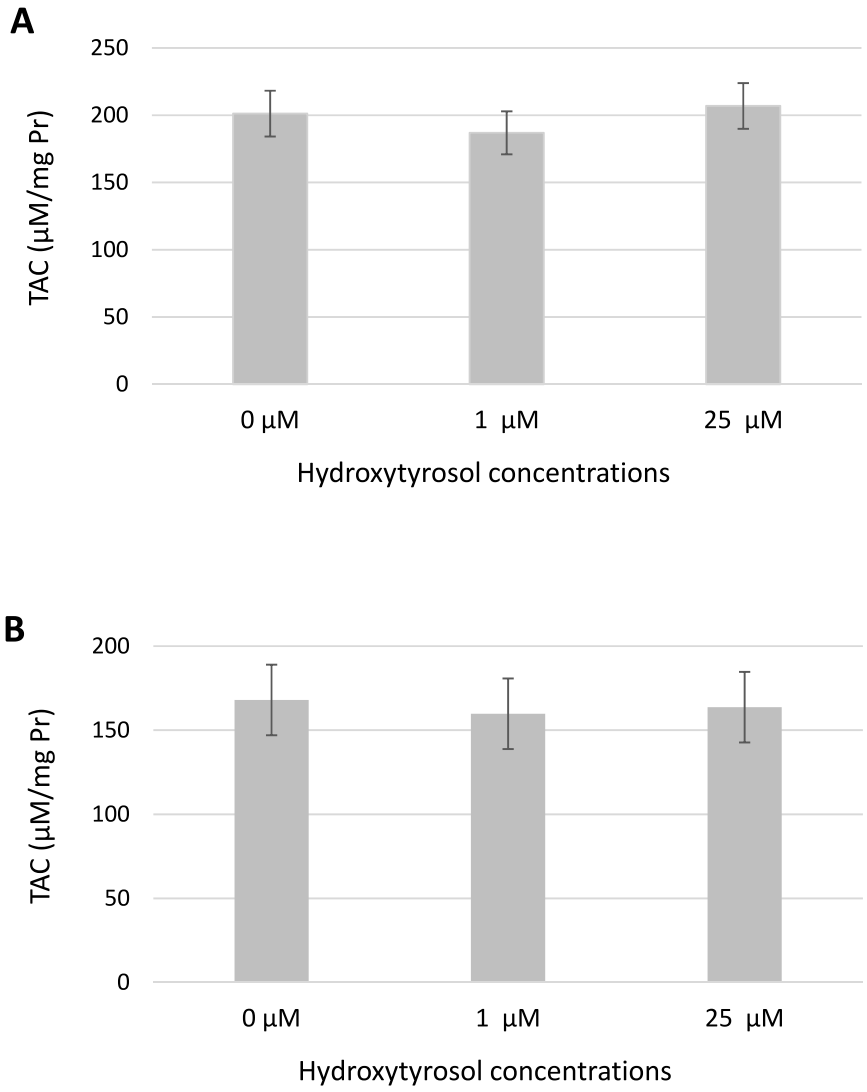

Fig. 3. Effect of different hydroxytyrosol (HT) concentrations added to the semen extender on total antioxidant capacity (TAC) of cooled (A) and frozen-thawed (B) bull sperm. Results are expressed as LSM ± SE. There were no differences in TAC of sperm at any HT concentrations evaluated ($P > 0.05$).

considerable variability in post-thaw semen quality due to inter-bull differences in cryotolerance, which affect sperm susceptibility to cryo-injury and oxidative stress [28]. Recently, Sharafi et al. [22] reported that HT supplementation reduced sperm membrane damage and enhanced sperm motility in frozen-thawed semen from low-cryotolerance bulls, but not in those with high cryotolerance. Unlike that study, bull cryotolerance classification (i.e., 'good' or 'bad' freezers) was not considered in the experimental design of the present study. Similarly, in frozen-thawed semen, HT supplementation did not improve viability or acrosomal integrity in rams [21], nor sperm motility or kinetic parameters in rams [21] or humans [19]. However, in the present study, HT supplementation improved sperm motility; specifically, the addition of 1 µM HT increased TM, with a similar trend observed for PM. Sperm motility has been positively correlated with mitochondrial membrane potential and negatively correlated with lipid peroxidation and DNA damage, all of which are considered key indicators of sperm functionality [29–31]. In the present study, HT treatment did not significantly reduce MDA levels, suggesting that the observed improvement in sperm motility is more likely attributable to decreased DNA damage or enhanced mitochondrial activity, as previously reported by Sharafi et al. [22].

The binding of sperm surface proteins to ZP glycoproteins is a key event in oocyte recognition and the initiation of fertilization [32]. In the present study, HT supplementation increased the number of sperm binding to the ZP, especially at 25 µM, under both storage conditions. Several human studies have reported that sperm capable of binding to the ZP tend to exhibit superior quality [33–35], highlighting the relevance of this interaction as a functional marker of fertilizing competence. The enhanced ZP-binding capacity may result from multiple sperm-related factors, including sperm morphology and hyperactivated motility [34,36], DNA integrity [37,38], acrosomal integrity [39], and the level of protamination and global DNA methylation [36]. The effect of HT on the number of sperm bound could be partially explained by some of these factors, such as improved acrosomal and membrane integrity and enhanced sperm motility. Although DNA integrity and methylation were not evaluated in the present study, recent evidence has shown that HT supplementation can modulate both parameters [19,40].

Embryonic development and embryo quality were improved when HT was added to the semen extender. In cooled semen, 1 µM HT was the most effective concentration, doubling the hatching rate compared to the control and resulting in the highest number of cells per blastocyst. In

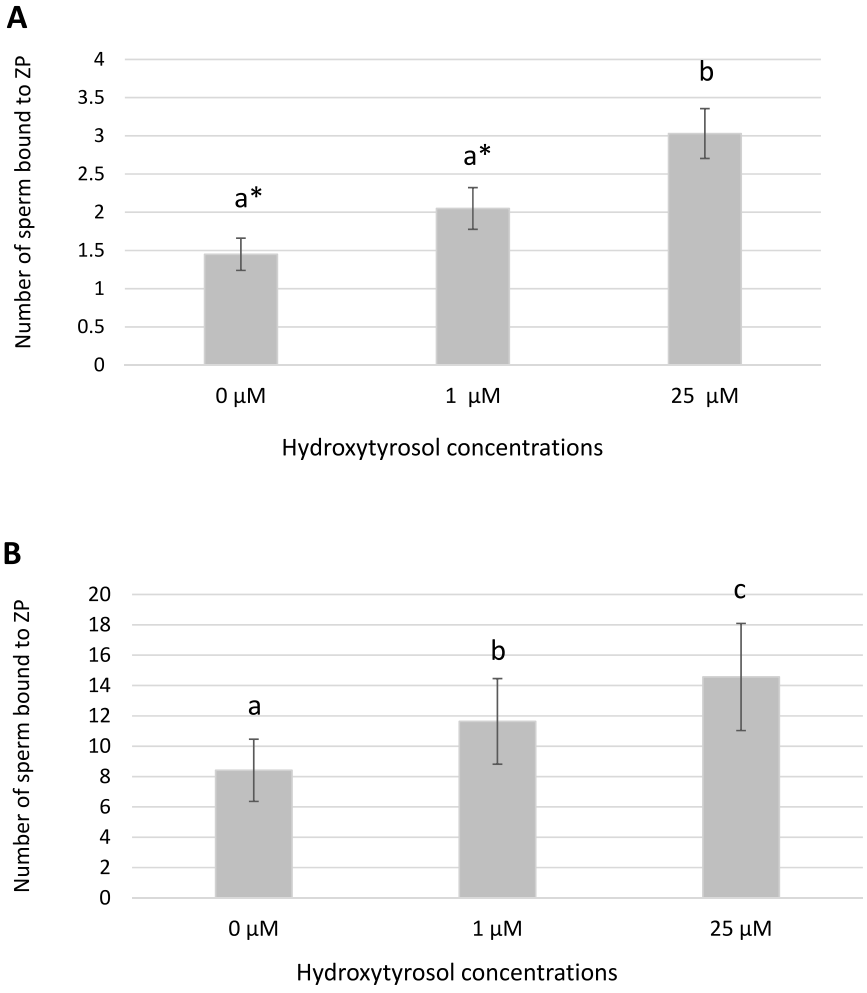

Fig. 4. Effect of different hydroxytyrosol concentrations added to the semen extender on sperm–zona pellucida (ZP) binding in cooled (A) and frozen-thawed (B) bull sperm. Results are expressed as LSM ± SE. [a,b,c]Bars with different letters differ statistically ($P < 0.05$). Bars with (*) tended to be different ($P = 0.06$).

Table 3
Effect of hydroxytyrosol supplementation in semen extender on *in vitro* embryo development with cooled and frozen-thawed bull sperm.

Hydroxytyrosol concentrations	Oocytes (n)	Cleaved/presumptive zygotes (%)	Cumulative blastocysts/presumptive zygotes (%)			Hatched (%)
			Day 6	Day 7	Day 8	
Cooled semen						
0 µM	155	65.75 ± 3.89[a]	10.91 ± 2.52[a]	27.01 ± 4.12[a]	32.16 ± 3.82[a]	34.76 ± 7.51[a]
1 µM	155	71.29 ± 3.69[a]	14.26 ± 2.84[a]	28.19 ± 4.19[a]	34.72 ± 3.90[a]	67.46 ± 6.89[b]
25 µM	180	59.78 ± 3.70[a]	11.00 ± 2.33[a]	21.25 ± 3.50[a]	26.86 ± 3.35[a]	38.62 ± 7.47[a]
Frozen semen						
0 µM	220	64.29 ± 8.32[a]	8.14 ± 3.23[a]	15.19 ± 4.16[a]*	15.64 ± 4.10[a]	21.58 ± 11.3[a]
1 µM	194	65.20 ± 8.28[a]	9.75 ± 3.71[a]	22.97 ± 5.41[a]	27.94 ± 5.79[b]	32.61 ± 12.7[a]
25 µM	212	58.10 ± 8.78[a]	21.17 ± 6.57[b]	26.69 ± 6.18[a]*	28.50 ± 6.17[b]	42.22 ± 15.1[a]

Cleavage rates were recorded 48 h after insemination. Data reported for development to the blastocyst stage included those embryos that progressed to the expanded or hatched blastocyst stages after 8 d in culture. All values for cleavage and development rates are expressed as least-squares means ± SEM. [a-c] Within a row, means without a common superscript differed ($P < 0.05$).

contrast, in cryopreserved semen, 25 µM HT yielded the best outcomes, increasing the blastocyst rate from Day 6 onwards and enhancing the total number of blastocyst cells. This difference in optimal concentration may reflect the greater challenge imposed by cryopreservation on spermatozoa [41], with 1 µM HT being insufficient to counteract the alterations induced by cryopreservation, thereby requiring a higher concentration of HT to exert its protective effects. Interestingly, the increase in blastocyst yield was not due to an enhanced fertilization capacity, as indicated by the cleavage rate. This suggests that HT improved some aspects of sperm function that extended beyond the moment of fertilization. It has been demonstrated that during storage, especially under cryopreservation, epigenetic factors involved in gene expression, such as non-coding RNAs (ncRNAs), DNA methylation, chromatin remodeling, and post-translational histone modifications, can be altered in sperm [42–45]. These factors are known to be essential for pre-implantation embryo development [41–44]. Another factor that can

Table 4
Effect of hydroxytyrosol in semen extender on Day-8 bovine blastocyst cell numbers using cooled and frozen-thawed bull sperm.

	Hydroxytyrosol concentrations		
	0 μM	1 μM	25 μM
Cooled semen			
No. blastocysts	23	22	23
Cell number/ blastocyst	94.05 ± 2.03[a]	138.52 ± 2.53[b]	100.14 ± 2.13[c]
Frozen semen			
No. blastocysts	16	15	18
Cell number/ blastocyst	82.92 ± 11.58[a]	84.32 ± 11.56[a]	134.99 ± 18.62[b]

Values are expressed as least-squares means ± SEM. [a-c] Within a row, means without a common superscript differed ($P < 0.05$).

impair early embryonic development is sperm DNA damage, which has been reported to negatively affect the blastocyst rate without altering fertilization or cleavage rates [45]. HT supplementation may potentially influence sperm epigenetic status or mitigate DNA damage, as suggested by previous studies [19,40]; however, additional research is necessary to confirm this hypothesis.

In this study, the supplementation of the semen extender with HT significantly increased blastocyst formation rates and improved embryo quality, suggesting that this compound may play a key role in preserving sperm functionality, possibly by mitigating molecular or structural alterations induced by the evaluated storage conditions. These findings not only reinforce the biotechnological potential of HT but also support its incorporation as a functional additive in semen extenders used in assisted reproductive technologies. Although further studies are needed to elucidate the underlying mechanisms and validate its effectiveness under field conditions, the results obtained position HT as a promising tool to enhance reproductive efficiency in embryo production programs. Finally, in cooled semen, 1 μM HT was clearly the most effective concentration, whereas performance decreased at 25 μM. In frozen–thawed semen, the opposite pattern was observed, with 25 μM proving to be the most effective. Therefore, the potential effects of concentrations higher than 25 μM in frozen–thawed samples remain uncertain and should be evaluated.

5. Conclusions

In conclusion, HT supplementation to the semen extender improved sperm quality after refrigeration at 4 °C for 24 h and after cryopreservation in liquid nitrogen. In cooled semen, although both HT concentrations enhanced membrane and acrosomal integrity as well as sperm–ZP binding, 1 μM HT improved embryo quality. In frozen–thawed semen, 25 μM HT yielded the best results, increasing the number of sperm bound to the ZP, the blastocyst rate, and embryo quality. Therefore, it can be inferred that the inclusion of HT in the formulation of semen extenders may contribute to enhancing the efficiency of *in vitro* embryo production systems.

CRediT authorship contribution statement

Marianela Balbi: Methodology, Investigation, Formal analysis. **Ana Laura Flaherti:** Investigation, Formal analysis. **Trinidad Otero Fernández:** Investigation. **Nicolás Agustín Farnetano:** Investigation. **Juan Mateo Anchordoquy:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Juan Patricio Anchordoquy:** Writing – original draft, Validation, Methodology, Investigation, Funding acquisition.

Availability of data and materials

The datasets in this study are available from the corresponding author on reasonable request.

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Conflict of 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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