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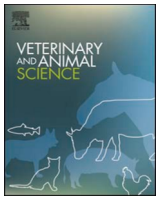

Impact of low-dose ozone supplementation on motility parameters and bacterial growth in horse cryopreserved semen

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ABSTRACT

Two studies were conducted to evaluate the use of medical ozone (O_3) in commercial extenders for equine semen cryopreservation. In the first study (Study 1), 0, 5, and 15 μg/mL of O_3 were added to diluents of refrigerated or frozen semen. Samples were evaluated for sperm kinematics at different time points for the chilled samples and after a thermoresistence test for the frozen/thawed samples. In the second study (Study 2), 0, 5, and 10 μg/mL of O_3 were added to an antibiotic-free diluent for refrigerated semen for comparison with the control group in which semen was diluted in the same diluent enriched with antibiotics. Semen sample kinematics were analyzed and an aliquot was collected after ozonification for bacteriological analyses. For Study 1 no difference was found comparing all the kinematic parameters analyzed over time, in the various treatments ($P > 0.05$). In Study 2 the absence of antibiotics did not affect the kinematic parameters compared to the control ($P > 0.05$). However when antibiotics were added, a smaller number of bacterial colony-forming units were detected compared to samples without antibiotics and without or with different O_3 supplementations. In conclusion, O_3 treatment at low dosages did not affect the semen kinematics, although it was ineffective in preventing bacterial overgrowth. Higher O_3 concentrations should be evaluated to explore the possibility of reducing the use of antibiotics in equine sperm conservation.

1. Introduction

Semen preservation is a biotechnology used not only for the preservation of gametes, but also for the diffusion of genetic material in all animal species, including horses (Amann & Pickett, 1987). Semen storage at low temperatures can cause several changes, including structural reorganization in the sperm cells (Sieme et al., 2015) and oxidative stress, which can lead to damage and a reduction in fertility (Sieme et al., 2015). Interestingly, when oxidative stress is induced under controlled conditions, it has been reported to activate the appropriate antioxidant system of the sperm cells, thereby reacting against oxidative stress and improving semen quality characteristics (Hezavehei et al., 2019; Horváth, Szenci, Nagy, Végh & Pribenszky, 2016).

Ozone (O_3) is an energy-rich, unstable gas derived from three oxygen atoms that bind in a cyclic structure (Bocci, 2006). Ozone generators produce different concentrations from 1 to 100 mcg/ml, however typically for medical purposes from 10 to 40 microgram/ml concentrations are used (Bocci, Borrelli, Travagli & Zanardi, 2009). From a practical point of view, ozone therapy has been proposed as a primary or adjunct therapy for various diseases in both humans (Bocci, 2002; Elvis & Ekta, 2011; Saini, 2011) and veterinary medicine (Đuričić, Valpotić & Samardžija, 2015; Hayashi, 2018; Jyotsana, Bhat & Kuldeep, 2016; Kozat & Okman, 2019; Penido, Lima & Ferreira, 2010; Rocha, de Aguiar Lima & Ferreira, 2010). This is because it seems to increase the antioxidant response of the treated cells (Bocci, 2002).

Regarding male reproduction, the scientific literature on the use of O_3 supplementation in semen is scarce, and results are still very contrasting (Gradil, Eaglesome, Stewart, Garcia & Quimby, 1995; Macêdo et al., 2021; Naserzadeh, Shahi, & Izadi, 2021; Pereira et al., 2022; Reis

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et al., 2023). In the two studies conducted on horses, one author concluded that low doses of O_3 supplementation can ameliorate some sperm parameters in both chilled and frozen semen (Pereira et al., 2022). On the other hand, the other study (Macêdo et al., 2021) reported that O_3 addition decreased the quality of frozen-thawed semen. Besides exerting pro-oxidative activity to stimulate cellular defenses, O_3 can strongly oxidize cell walls and bacterial cytoplasmic membranes, thus representing a valid alternative to the use of other bactericidal agents (Bocci, 2006).

In order to preserve the semen over time and prevent possible contamination in the female genital tract following artificial insemination (AI), semen extenders must not have any negative effect on semen quality.

One of the benefits of AI is the prevention of contact between animals for breeding and therefore reducing the spread of diseases. However if some pathogens are present in the semen, paradoxically AI is one of the ways pathogens may be diffused to susceptible mares. Pathogens can also derive from contamination during laboratory procedures, which can be reduced by good laboratory practices during semen preparation and evaluation (Goularte et al., 2018; Hueston et al., 1988; Schulze, Nitsche-Melkus, Jakop, Jung & Waberski, 2019). To reduce bacterial overgrowth, many commercially available semen extenders include a mixture of antibiotic agents in their formulas that help reduce bacterial activity without interfering with sperm parameters, such as viability and motility (Aurich & Spergser, 2007). Although the addition of antibiotics in commercial extenders may reduce the risk of disease transmission, some bacteria seem to develop resistance (Santos & Silva, 2020; Wentink, Frankena, Bosch, Vandehoek & Van den Berg, 2000), thus representing a global threat that has gained recent interest in all human and veterinary fields.

The aims of the present study were firstly to evaluate the effects of two low-concentration supplementations of medical O_3 on motility parameters after cryopreservation both in chilled semen (with or without seminal plasma) and frozen semen (Study 1); and secondly, to determine whether supplementation with a low concentration of medical O_3 can reduce bacterial overgrowth in chilled semen without the use of antibiotics (Study 2).

2. Materials and methods

2.1. Animals

Five Stallions of different breeds aged 6 to 17 were included in Study 1, while six Standardbred Stallions aged 9 to 17 were included in Study 2. All animals were healthy and with a body condition score of 3 (Carroll & Huntington, 1988). Stallions were kept in boxes and fed with mixed grass and alfalfa hay twice a day and water ad libitum, with a daily supplement of oats (Study 1) or with commercial horse feed (Study 2).

The study was approved by the Ethics Committee of Pisa University (number 43/2022).

2.2. Medium preparation

For Study 1, INRA96 and INRA Freeze Kit equine semen extenders (IMV, IMV Technologies, France) were used. INRA96 was warmed at 37 °C before semen dilution and evaluation, while the INRA Freeze Kit was kept at room temperature. For Study 2 EquiPlus equine semen extenders (Minitube, Germany) with and without antibiotics were used. Each medium powder (67 g) was dissolved in 1 liter of sterile, bi-distilled water at 35 °C and equilibrated for 20 min to allow pH stabilization, then warmed at 37 °C for at least 10 min before semen dilution and evaluation.

2.3. Semen collection and processing

One week before the beginning of the study, clean-out semen collections were performed. Semen was then collected for two consecutive weeks for each stallion, in Study 1 for a total of 3 semen collections, from July to August. For Study 2, four semen collections were performed, from February to March. After collection, semen was filtered with a sterile gauze to remove dirt and gel fraction. The volume was then calculated, subjective motility was estimated, and the sperm concentration was evaluated using a spectrophotometer (Accucell, IMV Technologies, L'Aigle, France).

2.3.1. Study 1, semen cooling

During the first semen collection, the whole samples (including seminal plasma = SP) were analysed while, during the second and third collection, SP was removed.

- SP Group - For samples with SP, three 250×10^6 sperm aliquots were diluted with the pre-warmed (37 °C) INRA96 medium to obtain a final volume of 5 ml.
- NO SP Group - For samples without SP, one 750×10^6 sperm aliquot from each stallion was diluted 1:1 (v/v) in INRA96 and 2 ml cushion fluid (Maxifreeze, IMV, France) was added. Samples were centrifuged at 900 g x 15 min and then the cushion fluid and the supernatant were discarded, the semen pellet was gently mixed, divided into three aliquots, and extended in pre-warmed INRA96 to obtain a final volume of 5 ml.

In both groups, semen samples were then subjected to an identical ozonification treatment at a 1:1 (v/v) ratio of the diluent, as follows:

- Diluent + No ozonification (CTRL)
- Diluent + 5 μg/mL ozonification (O_3–5)
- Diluent + 15 μg/mL ozonification (O_3–15)

2.3.2. Study 1, frozen semen

One-and-a-half billion total spermatozoa from each sample were diluted 1:1 (v/v) in INRA96, 2 ml cushion fluid was added, and samples were centrifuged as described above. After removing the cushion fluid and supernatant, the pellet was gently mixed, divided into three aliquots extended in INRA Freeze to obtain a final concentration of 150×10^6/ml, and kept at room temperature. The semen was then ozonified at the same concentrations described above for the chilled semen and then equilibrated at 4 °C for 1 hour. Semen was loaded into 0.5 mL French straws before freezing with a programmable freezer (MicroDigit Cool, IMV, France), using a cooling rate of 60 °C/min from +4 °C to -140 °C. The straws were then plunged directly into liquid nitrogen, and placed inside a nitrogen tank for storage until use.

2.3.3. Study 2

Two billion sperm aliquots were diluted 1:1 (v/v) in the pre-warmed (37 °C) Equiplus medium without antibiotics. After dilution and first subjective evaluation, semen samples were transported in a polystyrene box at room temperature to the laboratory where semen was analyzed (T0) and processed within 1 hour of collection. Semen was centrifuged at 900 g x 15 min using 2 ml of cushion fluid (Minitube, Germany) to remove the medium and the SP. The semen pellet was then gently mixed and divided into four aliquots of 500×10^6 total sperm and re-suspended in Equiplus, obtaining a 5 ml final volume as follows:

- Diluent with antibiotics (ATB)
- Diluent without antibiotics (NO ATB)
- Diluent without antibiotics + 5 ml O_3 5 μg/mL (O_3–5)
- Diluent without antibiotics + 5 ml O_3 10 μg/mL (O_3–10)

2.4. Semen ozonification procedure

Semen ozonification was performed using a commercial ozone generator (Herrmann Medozon compact - Herrmann Apparatebau GMBH, Germany). In both studies, 1:1 (v/v) O_2–O_3 gas mixture at different concentrations (0–5–15 μg/mL O_3 in Study 1 vs 0–5–10 μg/mL O_3 in Study 2) was gently added to and mixed with the diluted semen sample in a sterile syringe closed with a plug (i.e. 5 ml diluent + 5 ml of air with 5 μg/mL O_3). After 30 s of co-incubation, the air was expelled, and the plug was replaced.

2.5. Semen in vitro evaluation

Chilled semen was evaluated to assess the quality of the semen samples over time. A total of 1 ml of sample was placed into a pre-warmed vial and incubated at 37 °C for five minutes in a water bath. The semen was then gently mixed, and motility was evaluated with a CASA system CEROS II (IMV Technologies, L'Aigle France) for Study 1, and Androvision (Minitube, Germany) for Study 2. Briefly, two 5-microliter drops were placed onto a glass slide under an 18 × 18 mm cover glass. Eight fields were analyzed for each sample with a minimum of 500 spermatozoa analyzed.

For chilled semen, sperm motility parameters were analyzed immediately after dilution and ozonification in Study 1 (T0-AD), and before centrifugation and dilution in Study 2 (T0-BD); and at 24 and 48 h after dilution with different extenders for both Studies (T24 and T48, respectively). For the frozen semen samples, at least two straws per sample were thawed in a 37 °C water bath for 30 s, dried with clean paper, and opened in a sterile prewarmed vial. Samples were diluted 1:4 (v/v) in prewarmed INRA96 and a thermoresistance test was performed, analyzing samples 5, 15, and 60 min after thawing (T5, T15, and T60, respectively), according to the same procedures previously described for chilled semen. Semen kinematic parameters were defined according to Boyers et al. (1989), and the manufacturer's Stallion setup was used for both CASA systems.

2.6. Bacteriological evaluation

For Study 2, immediately after dilution with different extenders, one ml of the semen samples was collected in sterile vials, packed in a cooled polystyrene box, and transported to the laboratory for bacteriology evaluation. With all the samples, a quantitative diagnostic of the bacterial load was carried out and expressed in colony-forming units per milliliter (CFU/ml). For the colony-forming unit (CFU) count, 100 μL of undiluted and 1:10 diluted seminal samples were plated on blood agar base (Liofilchem, Italy) with 7 % sterile defibrinated sheep blood, plate count agar (Liofilchem Italy), and Sabouraud dextrose agar (Liofilchem, Italy) with chloramphenicol. The inoculum was spread rapidly over the entire agar surface using a thin, bent glass rod. Blood agar plates and plate count agar plates were incubated at 37 °C for 48–72 h, while the Sabouraud dextrose agar plate was incubated at about +30 °C from 2 to 10 days. The colonies were counted and put in relation to the initial aliquot of 1 ml.

2.7. Statistical analyses

Statistical analyses were performed using the Jamovi Software Version 2.4.7. The distribution normality of the studied populations was assessed by the Shapiro-Wilk test.

For Study 1, populations resulted normally distributed ($P > 0.05$) and differences of each kinematic parameter (VAP, VSL, VCL, STR, LIN, ALH, BCF, WOB) were analyzed with ANOVA GLM for each time-point (T0-AD, T24, and T48 for chilled semen and T5, T15 and T60 for frozen-thawed semen), including in the model: the presence of seminal plasma (SP or NO SP – only for chilled semen) and O_3 concentrations (CTRL, O_3–5, O_3–15 ng/mL) as main factors and stallion and ejaculate were considered as random factors. Data were described as mean ± standard deviation.

For Study 2, populations resulted not normally distributed ($P < 0.05$) and differences among diluent (CTRL, O_3–5, O_3–10) for each time-point (T0-BD, T24, and T48) for each kinematic parameter (MOT, PRO, CIRC, FAST, SLOW, LOC, IMM, VCL, VSL, VAP, ALH, BCF, WOB, LIN; STR) and for bacterial concentration after 48 h of culture (CFU/mL) were calculated by Kruskall-Wallis Test and Dwass-Steel-Critchlow-Fligner post hoc test. Data were described as median and interquartile range (IQR). Differences were considered statistically different when $P < 0.05$.

3. Results

3.1. Study 1: semen cooling

Semen motility parameters of the samples with seminal plasma (SP, $N = 5$) or without (NO SP, $N = 10$) for the control group and different ozone treatments are presented before cooling at +4 °C and after 24, and 48 h of refrigeration are presented in Table 1.

Considering all the studied kinematic parameters: total motility (TOT); progressive motility (PRO); average path velocity (VAP); linear velocity (VSL); curvilinear velocity (VCL); straightness (STR), linearity (LIN); lateral head displacement (ALH); beat cross frequency (BCF); wobble (WOB = VAP/VCL) no statistical difference was observed ($P > 0.05$) at any observed time-point (after dilution, before cooling – after 24 h and after 48 h of refrigeration at 4 °C) for all the treatments showing that low-dosage O_3 supplementation affected motility parameters neither negatively nor positively ($P > 0.05$).

3.2. Study 1: semen freezing

Semen was frozen, and after thawing samples were evaluated after 5, 15, and 60 min (T5, T15, and T60, respectively) for the control group, and for the different ozone treatments. Total and progressive motility (%) are presented in Table 2. Considering all three time points no difference was found between groups ($P > 0.05$).

3.3. Study 2: semen cooling

Semen motility parameters with SP (T0-BD, before dilution, Table 3, $N = 24$) and without SP (T24 and T48, $N = 24$) after the different treatments and cooling at +4 °C for 24, and 48 h are presented in Table 3 (T0 before dilution), Table 4 (24 and 48 h). No difference was found between groups ($P > 0.05$) for all the considered parameters.

Table 5 reports the bacterial concentration expressed in colony-forming units (CFU)/ml of semen samples after treatments, according to the previously described procedures. Samples were analyzed after 48 h of refrigeration. It is possible to highlight that when commercial media containing antibiotics was used, bacterial populations (CFU/ml) were significantly lower ($P < 0.05$) than when a antibiotic-free commercial media supplemented with low-dose ozone was used.

4. Discussion

In the present study, the effect of low-dose O_3 supplementation on the cryopreservation diluent of equine semen kinetics and its potential effects on bacterial overgrowth in chilled semen was investigated.

Our results showed that low-dose O_3 supplementation had no negative effects on motility parameters in either chilled or frozen semen. To date, the literature on O_3 supplementation in semen is scarce, and results are still very contrasting (Gradil et al., 1995; Macêdo et al., 2021; Naserzadeh, Shahi, & Izadi, 2021; Pereira et al., 2022; Reis et al., 2023). Pereira et colleagues (Pereira et al., 2022) reported that 15 μg/mL O_3 supplementation resulted in a higher percentage of total and progressive motility compared to the control (60.3 ± 3 and 40.7 ± 3.4 vs.54.9 ± 4 and 35±4.4, respectively, after 24 h of refrigeration $P < 0.05$).

Table 1
Seminal parameters (MEAN±SD) of the equine semen at T0-AD (after dilution, before cooling), after 24 h (T24) and after 48 h (T48) of the control group (CTRL) and different concentrations of O_3 (5 and 15 μg/mL), ($P > 0.05$).

			CTRL	O_3–5	O_3–15				CTRL	O_3–5	O_3–15
TOT (%)	NO SP	T0-AD	63.1 ± 24.2	63.8 ± 27	67.8 ± 22.5	STR (%)	NO SP	T0-AD	60.6 ± 13.4	59.9 ± 16.7	63.5 ± 10.9
		T24	56 ± 21	48 ± 25.6	54.4 ± 22.1			T24	58 ± 13.1	52 ± 15.8	57.4 ± 16.4
		T48	55 ± 18.8	42.3 ± 27.5	50 ± 26.4			T48	57.9 ± 12.5	51.2 ± 14.4	55.1 ± 20.6
	SP	T0-AD	70.3 ± 16	68.8 ± 17.6	66.8 ± 17		SP	T0-AD	58 ± 10.2	56.2 ± 12	56 ± 14.4
		T24	67 ± 19.4	51.7 ± 25.3	54.5 ± 17			T24	64.7 ± 8.6	57.3 ± 14.5	59.2 ± 16.4
		T48	42.1 ± 24.6	46 ± 30.4	35.7 ± 24.8			T48	50.8 ± 16.6	54.3 ± 18.2	48.7 ± 18.4
PRO (%)	NO SP	T0-AD	19 ± 17	20 ± 20.5	20.8 ± 17	LIN (%)	NO SP	T0-AD	32.1 ± 8.5	31.7 ± 10.1	33.1 ± 7
		T24	17.5 ± 17	14.3 ± 12.4	17.5 ± 14.6			T24	31±9.6	27.6 ± 8	31 ± 11.3
		T48	14.2 ± 12.8	10.8 ± 10.7	17.4 ± 16.9			T48	31.3 ± 8.5	28.7 ± 10.2	31 ± 13.1
	SP	T0-AD	20.8 ± 15.3	21.5 ± 14	18.2 ± 12.1		SP	T0-AD	30.8 ± 8.2	28.7 ± 8.3	28.5 ± 10.2
		T24	24 ± 17.4	21.6 ± 18.5	20±18.4			T24	35.8 ± 6.2	32.7 ± 9	32.5 ± 11.5
		T48	15.1 ± 15.5	20 ± 19.7	14.6 ± 18.7			T48	50.8 ± 16.6	54.3 ± 18.2	48.7 ± 18.4
VAP (μm/s)	NO SP	T0-AD	57.4 ± 41	58.9 ± 44.4	61.8 ± 39.7	ALH (μm/s)	NO SP	T0-AD	5.4 ± 3.5	5.4 ± 3.5	5.8 ± 3.5
		T24	38.4 ± 29.8	39.8 ± 29.1	43.6 ± 26.7			T24	4.6 ± 2.6	4 ± 2.8	4.2 ± 2.3
		T48	47.1 ± 32.8	46.5 ± 40.8	45.1 ± 32.6			T48	4.8 ± 3.1	4.1 ± 3.2	4.4 ± 3
	SP	T0-AD	81±47.4	82.7 ± 54.3	70.3 ± 42.8		SP	T0-AD	7.1 ± 3.6	7.4 ± 4	6.5 ± 3.6
		T24	47.5 ± 33.3	46.2 ± 28.4	45.2 ± 22.2			T24	5.7 ± 2.3	3.8 ± 1.9	4 ± 1.3
		T48	42.2 ± 29.3	45.1 ± 33.8	34.4 ± 26.5			T48	4 ± 2.5	4 ± 2.5	3 ± 2
VSL (μm/s)	NO SP	T0-AD	43.3 ± 27.8	44 ± 30.7	46.6 ± 27.1	BCF (Hz)	NO SP	T0-AD	23.7 ± 7.1	23.2 ± 7.7	25.8 ± 6
		T24	37.5 ± 25.6	30.5 ± 21	34.6 ± 20.1			T24	22.4 ± 6.1	20 ± 7	22.3 ± 7
		T48	35±20.8	28.2 ± 20.9	35.2 ± 25			T48	22.5 ± 5.4	19 ± 6.8	20.5 ± 8.5
	SP	T0-AD	56.6 ± 30.1	56.5 ± 29	48.8 ± 25.1		SP	T0-AD	26.8 ± 4.7	27.2 ± 5.3	25.8 ± 5
		T24	51 ± 23.3	38 ± 24.3	36.7 ± 19.9			T24	29.3 ± 4.9	25.1 ± 8.4	25.8 ± 7.3
		T48	33.1 ± 24.4	37.3 ± 28.7	28.7 ± 22.9			T48	19.9 ± 6.8	22.2 ± 9.3	18.6 ± 8.1
VCL (μm/s)	NO SP	T0-AD	115.4 ± 83.3	117.5 ± 88.2	124.7 ± 80.9	WOB (%)	NO SP	T0-AD	40.1 ± 9.7	40 ± 11.5	41.4 ± 8.7
		T24	95.9 ± 64	83 ± 62	88.7 ± 54.2			T24	37.5 ± 10.7	33.5 ± 9.7	37 ± 12.2
		T48	98.5 ± 68.4	81.5 ± 69.8	91.6 ± 67.1			T48	38.5 ± 10.4	34.8 ± 11.4	37 ± 15
	SP	T0-AD	162.6 ± 90.6	167.3 ± 101.8	146.1 ± 89.6		SP	T0-AD	40 ± 10.3	39 ± 10.3	37.7 ± 10.2
		T24	131.6 ± 58.4	87.7 ± 47.5	88.6 ± 34.3			T24	44.2 ± 7.3	38.3 ± 10	38.3 ± 11.8
		T48	84.1 ± 53.5	84.8 ± 58.4	66.4 ± 45.7			T48	34.2 ± 11.5	37 ± 13.4	33.6 ± 12.7

TOT, total motility; PRO, progressive motility; VAP, average path velocity; VSL, linear velocity; VCL, curvilinear velocity; STR, straightness, LIN, linearity; ALH, lateral head displacement; BCF, beat cross frequency; WOB, wobble (VAP/VCL).

Table 2
Seminal parameters (MEAN ± SD) of the frozen/thawed equine semen during thermoresistance test after 5 (T5), 15 (T15), and 60 (T60) minutes at 37 °C from thawing in the control group (CTRL) and in different concentrations of O_3 (5 and 15 μg/mL). ($P > 0.05$).

		CTRL	O_3–5	O_3–15
T5	TOT (%)	22 ± 10.2	23 ± 11	21.1 ± 11.6
	PRO (%)	9.1 ± 7	9.2 ± 7	9.1 ± 8
T15	TOT (%)	17.7 ± 9.3	20.1 ± 11	18.7 ± 11.6
	PRO (%)	6.6 ± 5.7	7.2 ± 6.6	7.4 ± 7.5
T60	TOT (%)	11.5 ± 9.1	12.2 ± 10.2	11.8 ± 11
	PRO (%)	4 ± 5.3	4.6 ± 6.2	4.4 ± 6

TOT, total motility; PRO, progressive motility.

Interestingly, when considering the results from the same treatment category (15 μg/mL O_3 vs control) after 48 h of refrigeration, this improvement in the kinematic parameters was no longer observed. The same authors concluded that the effect of O_3 was dose-dependent: low ozone concentrations induced a pro-oxidative effect by activating cellular-specific antioxidant pathways. Pro-oxidation, however, has limited effectiveness, which is why, when used in higher concentrations, O_3 leads to an increase in lipid peroxidation by-products leading to a loss in sperm kinematic parameters (Pereira et al., 2022).

In our Study 1 the same procedure showed no differences, either at 24 or at 48 h of refrigeration, considering the addition of both 5 and 15 μg/mL O_3, compared to the control group. This observation was confirmed by the results of Study 2, where a comparison of different mediums with and without antibiotics, and two low-dosage O_3 supplementation treatments, no differences were shown between treatment and control groups on sperm kinematic parameters at the same time points.

The frozen-thawed evaluation of semen samples after incubation at 37 °C for one hour (T60) offers great reliability as an indicator of in vitro fertility parameters (Foster et al., 2011), simulating the presence of the semen in the female's genitalia (de Arruda, Barnabe, de Alencar & Barnabe, 1992). Once again, the scientific literature provides very little information on O_3 supplementation treatments and their effects on frozen semen samples.

It is well-known that semen quality deteriorates over time. However, when considering low-dosage O_3 supplementation treatments before the

Table 3
Seminal parameters expressed as median and interquartile range (IQR) of the equine semen at T0-BD (before dilution), before centrifugation and treatment.

T0-BD								
	MOT (%)	PRO (%)	CIRC (%)	FAST (%)	SLOW (%)	LOC (%)	IMM (%)	
MEDIAN	72.5	67.1	5.6	41.3	15.6	5.15	27.5	
IQR	12.1	11.3	5.65	10.9	10.4	4.52	12.2	
	VCL	VSL	VAP	ALH	BCF	WOB	LIN	STR
MEDIAN	119	50.1	57.9	1.2	16.6	0.5	0.4	0.85
IQR	34.1	13.7	16.1	0.25	2.73	0	0	0.1

MOT, total motility; PRO, progressive motility; CIRC, circular motility; FAST, fast motile sperm; SLOW, slow motile sperm; LOC, local motility; IMM, immotile; VCL, curvilinear velocity; VSL, linear velocity; VAP, average path velocity; ALH, lateral head displacement; BCF, beat cross frequency; WOB, wobble (VAP/VCL); LIN, linearity; STR, straightness.

Table 4
Seminal parameters (median and interquartile range) of the chilled equine semen after 24 (T24) and 48 (T48) hours of refrigeration in different treatment groups treated with media with antibiotics (ATB) or without (NO ATB) or without antibiotics + an ozone (O_3) supplementation of 5 and 10 μg/mL (O_3_5 and O_3_10, respectively). ($P > 0.05$).

	T		ATB	NO ATB	O_3–5	O_3–10		T		ATB	NO ATB	O_3–5	O_3–10
MOT (%)	T24	MEDIAN	69.7	72.1	69	67	VCL	T24	MEDIAN	83.5	85.6	85.5	75.8
		IQR	18.1	24.2	13.4	21.6			IQR	46.1	43.7	42.3	48
	T48	MEDIAN	64.6	62.6	67.3	60.3		T48	MEDIAN	72	75.5	74.5	70.3
		IQR	24.5	26.7	32.1	20.3			IQR	42.9	35.6	41.5	26.7
PRO (%)	T24	MEDIAN	63.9	65.9	62.3	60.3	VSL	T24	MEDIAN	29	31.9	31.5	27.1
		IQR	27.9	25.3	16.8	24.8			IQR	18.4	16.9	14.7	17.3
	T48	MEDIAN	55.8	56	61	53.9		T48	MEDIAN	22.8	25.6	25.3	22.1
		IQR	26.6	25.8	29.3	21.8			IQR	14.2	12.4	15.8	11.3
CIRC (%)	T24	MEDIAN	0.25	0.2	0.2	0.1	VAP	T24	MEDIAN	35.3	37.9	37	32
		IQR	1.23	0.925	0.95	0.625			IQR	22.8	17.7	19.4	21.2
	T48	MEDIAN	0.05	0.1	0.05	0.05		T48	MEDIAN	28.5	32	30.5	30.9
		IQR	0.525	0.225	0.225	0.2			IQR	17.4	17.6	18.8	12.2
FAST (%)	T24	MEDIAN	31.5	33.8	32.3	28.6	ALH	T24	MEDIAN	1.1	1.1	1.1	1.05
		IQR	26.2	27.4	26.8	29.4			IQR	0.6	0.575	0.7	0.7
	T48	MEDIAN	25.8	28.1	25	23.4		T48	MEDIAN	1	1	1.05	0.95
		IQR	20.8	20.5	25.1	23.5			IQR	0.5	0.425	0.525	0.425
SLOW (%)	T24	MEDIAN	24.9	22.8	24.9	25.1	BCF	T24	MEDIAN	11.4	11.4	12	10.9
		IQR	11.8	11	12	15.3			IQR	5.88	2.52	3.03	2.55
	T48	MEDIAN	22.7	25	26.1	23.1		T48	MEDIAN	9.7	9.95	9.6	8.3
		IQR	11.8	12.9	12	15.9			IQR	5.55	5.52	4.72	3.48
LOC (%)	T24	MEDIAN	5.05	5.75	6.1	4.95	WOB	T24	MEDIAN	0.4	0.4	0.4	0.4
		IQR	2.75	3.38	3	5.05			IQR	0	0.025	0	0.1
	T48	MEDIAN	6.1	5.95	6.55	5.8		T48	MEDIAN	0.4	0.4	0.4	0.4
		IQR	4.07	2.95	4.2	3.3			IQR	0	0	0	0
IMM (%)	T24	MEDIAN	30.4	27.9	31	33	LIN	T24	MEDIAN	0.35	0.4	0.4	0.35
		IQR	24.2	18.1	13.4	21.5			IQR	0.1	0.1	0.1	0.1
	T48	MEDIAN	35.5	37.4	32.7	39.8		T48	MEDIAN	0.3	0.3	0.3	0.3
		IQR	24.5	26.7	29.9	20.3			IQR	0.025	0.1	0.025	0
							STR	T24	MEDIAN	0.8	0.8	0.8	0.8
									IQR	0.1	0	0.1	0.1
								T48	MEDIAN	0.8	0.8	0.8	0.8
									IQR	0	0	0	0.1

MOT, total motility; PRO, progressive motility; CIRC, circular motility; FAST, fast motile sperm; SLOW, slow motile sperm; LOC, local motility; IMM, immotile; VCL, curvilinear velocity; VSL, linear velocity; VAP, average path velocity; ALH, lateral head displacement; BCF, beat cross frequency; WOB, wobble (VAP/VCL); LIN, linearity; STR, straightness.

Table 5
Bacterial concentration (CFU/ml) of semen samples after treatments and 48 h (T48) of refrigeration in different treatment groups treated with media with antibiotics (ATB) or without (NO ATB) or without antibiotics + an ozone (O_3) supplementation of 5 and 10 μg/mL (O_3_5 and O_3_10, respectively).

		T48			
		ATB	NO ATB	O_3–5	O_3–10
CFU/ml	MEDIAN	450^{a}	5150^{b}	$4850^{b,c}$	$4300^{b,c}$
	IQR	1575	4300	4400	5675

Values with a different superscript at the same row differ significantly: $^{a\approx b}$:$P = 0.028$; $^{a\approx c}$:$P = 0.003$, $^{a\approx d}$:$P = 0.011$.

freezing procedure, no differences were found between any of the treatments in Study 1 (control vs O_3–5 vs O_3–15) at any of the time points (5, 15, and 60 min after thawing, kept at 37 °C). Our finding is in line with a study by Macêdo and colleagues (Macêdo et al., 2021), who reported similar results after a thermoresistance test of frozen-thawed semen comparing the control group and treatments (6, 8, and 12 μg/mL O_3) in all the evaluated time points (0, 30 and 60 min after thawing). Similar tests were also performed in the aforementioned study by Peireira (Pereira et al., 2022), in which motility did not change after thawing over time between groups (also in this case, the O_3 supplementation was ≤ 15 μg/mL O_3).

In the present study, the low-dosage O_3 diluent supplementation did not affect the bacterial growth in chilled horse semen. To the best of our knowledge, this is the first report describing the effect of O_3 supplementation on bacterial growth in chilled horse semen. In a succinct study on bovine semen, Gradil et al. (1995) investigated the effectiveness of O_3 treatment in an experimentally contaminated sample (Gradil et al., 1995). The authors found that O_3 concentrations of 15–20 μg/mL reduced the bacteria population compared to the control within an 18-hour timeframe. On the other hand, these O_3 concentration levels added to the sample had a negative effect on semen motility parameters (Gradil et al., 1995). It is possible that a combination of factors influenced Gradil's study, starting with the semen used that was derived from a single bull. In addition, the diluent used did not contain cryoprotectant agents, and the procedure involved bubbling O_3 directly into the diluted semen samples for a long period (from 3 to 5 min according to the treatment group) which may have exerted a detrimental effect on semen motility.

The reduced number of animals as well as the lack of specific evaluations of the sperm membrane integrity can be considered as a limit of the study.

These two preliminary studies suggest that, to date, antibiotic-diluent is still the best option leading to the lowest colony-forming units without damaging the motility of equine and bovine semen ($P < 0.05$).

5. Conclusions

The semen extender low-dosage O_3 supplementation used in this study did not harm the cinematic parameters of cryopreserved equine semen. Our results thus confirm that to date, the addition of antibiotics to the diluent is the only method that can reduce the risk of disease transmission when shipping equine semen for AI. The applicability of low-dosage O_3 supplementation into semen extenders could also be of interest to other species like the bovine industry to control pathogens or contaminants of concern.

Other studies are necessary to test whether higher O_3 dosages may

affect the bacteria population growth in semen samples, with a minimal effect on motility parameters. In the case of positive results, an in vivo test should be carried out to investigate further the impact of O_3 supplementation of semen diluent on fertility.

Statement of ethics

The study was approved by the Ethics Committee of Pisa University (number 43/2022).

Written informed consent was obtained from client-owned animals.

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Data availability

Data is available upon reasonable request.

CRediT authorship contribution statement

Diana Fanelli: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Rebecca Moroni:** Writing – review & editing, Formal analysis. **Giulia Sala:** Writing – review & editing, Writing – original draft, Data curation. **Pierre Melanie:** Writing – review & editing, Resources, Methodology, Conceptualization. **Ilaria Tarabella:** Writing – review & editing, Formal analysis. **Nicole Telleschi:** Writing – review & editing, Formal analysis. **Saverio Maltinti:** Writing – original draft, Methodology, Formal analysis. **Mario Giorgi:** Writing – review & editing, Funding acquisition, Conceptualization. **Giovanni Barsotti:** Writing – review & editing, Funding acquisition, Conceptualization. **Fabrizio Passamonti:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Paola Marmorini:** Writing – review & editing, Resources, Investigation, Conceptualization. **Alessandra Rota:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Francesco Camillo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Duccio Panzani:** Writing – review & editing, Writing – original draft, Software, Resources, Funding acquisition, Data curation, Conceptualization.

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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