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Preliminary results on the antioxidant capacity of the *Coffea arabica* grounds extract on semen parameters of Fleckvieh cattle in the Amazonas region

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Abstract: Livestock farming is vital to a country's economy, and technological innovations in animal genetics and reproductive biotechnologies are key for environmental and socioeconomic development. This study aimed to evaluate the antioxidant capacity of *Coffea arabica* grounds (CAG) extract on the semen parameters of Fleckvieh cattle. CAG was processed, and its antioxidant capacity was assessed using DPPH, FRAP, and total phenols assays. Semen was collected from a pedigree bull and analysed macroscopically and microscopically. Different concentrations of CAG (0, 1, 1.5, and 2 mg/ml) were tested at 4, 12, 24, and 36 h post-refrigeration. The study used a completely randomised design with ten replications, and variables such as motility, kinetic parameters, membrane functionality, and acrosomal integrity were analysed. The normal distribution of the variables – motility, kinetic parameters, membrane functionality, and acrosomal integrity – was analysed using the Shapiro-Wilk statistical test ($P > 0.05$). An analysis of variance was then performed with a significance level of $P < 0.05$ to compare the means, using InfoStat software. Results showed that 1 mg of CAG significantly improved total motility and progressive motility at 4 h, along with kinetic parameters and membrane integrity. Overall, CAG exhibits antioxidant properties that enhance sperm characteristics, particularly at 1 mg/ml concentration after 4 h of refrigeration. However, further studies are needed to understand better the mechanisms of action and the overall efficacy of CAG as an antioxidant agent in this specific context.

Keywords: acrosomal integrity; bovine; kinetic parameters; membrane functionality

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The production and trade of genetic material through the use of biotechnological tools help to select and accelerate the reproduction of superior genetic lines and preserve native breeds (Rosete et al. 2021). Reproductive efficiency plays a fundamental role in increasing productivity, genetic improvement and livestock economics; through research and development of new methodologies, the reproductive performance of bulls can be analysed and optimised (Redivo et al. 2017)

One of the functions and objectives of reproductive biotechnology is to maintain and improve the quality of semen (Redivo et al. 2017). Factors such as semen volume, concentration, vitality, morphology, and motility can vary between individuals and may affect the male's fertilizing capacity (Hidalgo et al. 2015).

It is important to properly handle semen for its subsequent cryopreservation and use in reproductive biotechnologies, so the storage conditions, such as the type of extender, temperature and duration of refrigeration, can affect semen parameters (Bahmid et al. 2023).

Oxidative stress affects semen quality (sperm motility, kinetic parameters, acrosome integrity, and membrane functionality) (Izquierdo et al. 2020). To counteract these effects, synthetic and natural plant-derived antioxidants have been used, demonstrating that natural antioxidants are a treatment option to improve semen quality. However, further research is required to determine their specific intervention in the improvement of semen parameters (Puertaet al. 2019).

Coffee contains high levels of antioxidants such as caffeine, phenolic acids, polyphenols, and alkaloids (Andrea et al. 2015) In addition to the coffee bean, the by-products such as the mucilage, exocarp, and coffee grounds can also be utilised. These coffee components have beneficial properties, and their use has been the subject of research in various fields (Franca and Oliveira 2019).

According to Mussato et al. (2011), coffee grounds contain high levels of antioxidants, which could be beneficial to health and have potential applications in various areas of research.

In recent years, the use of fixed-time artificial insemination (FTAI) has significantly increased on livestock farms, driven by protocols that effectively synchronize ovulation, thereby improving pregnancy rates (Bo et al. 2018; Martinez et al. 2024). However, semen quality remains a crucial factor in the success of FTAI. Although cryopreservation is routine, it reduces sperm viability by approximately 50% due to structural and biochemical damage during freezing and thawing, negatively affecting the quantity and quality of sperm cells (Dias et al. 2018; Sharafi et al. 2022; Hai et al. 2024). On the other hand, refrigerated semen, by avoiding these processes, maintains higher viability and fertilising capacity, optimising the use of valuable breeders in FTAI programs (Verberckmoes et al. 2005; Bucher et al. 2009; Crespilho et al. 2013; Borges-Silva et al. 2016). Therefore, the objective of the research was to evaluate the antioxidant capacity of the *Coffea arabica* coffee grounds extract on semen parameters of Fleckvieh cattle in the Amazonas region.

MATERIAL AND METHODS

Sample collection and geographic location

The study was carried out with a 3-year-old Fleckvieh reproductive bull named Hans TE RG 1070 [Figures S1–S3 in Electronic supplementary material (ESM)]. The bull was housed in an individual pen and fed a diet based on alfalfa hay, corn silage and concentrate, at a daily amount of 10% of its live weight, dispensed twice a day in the morning (8:00 a.m.) and afternoon (4:00 p.m.) schedules. A total of 10 ejaculates were collected over a four-month period, using an artificial vagina. Macroscopic evaluations were performed through direct observations: volume, colour, odour, and pH; and microscopic evaluations: mass motility (MM) (0–5) – subjective assessment of the massive movement of spermatozoa according to Evans and Maxwell (Segura et al. 2023), concentration quantified using a photometer (SDM6 Printer; Minitube,Tiefenbach, Germany; kinetic parameters, membrane functionality, and acrosomal integrity). The collection of semen samples was conducted in accordance with the Peruvian National Law (No. 30407: Animal Protection and Welfare). The semen collection was conducted at the Centre for Semen Collection of the Chachapoyas Experimental Station, and semen evaluation and processing were carried out in the laboratories of the National University Toribio Rodríguez de Mendoza de Amazonas (UNTRM) (Figure 1).

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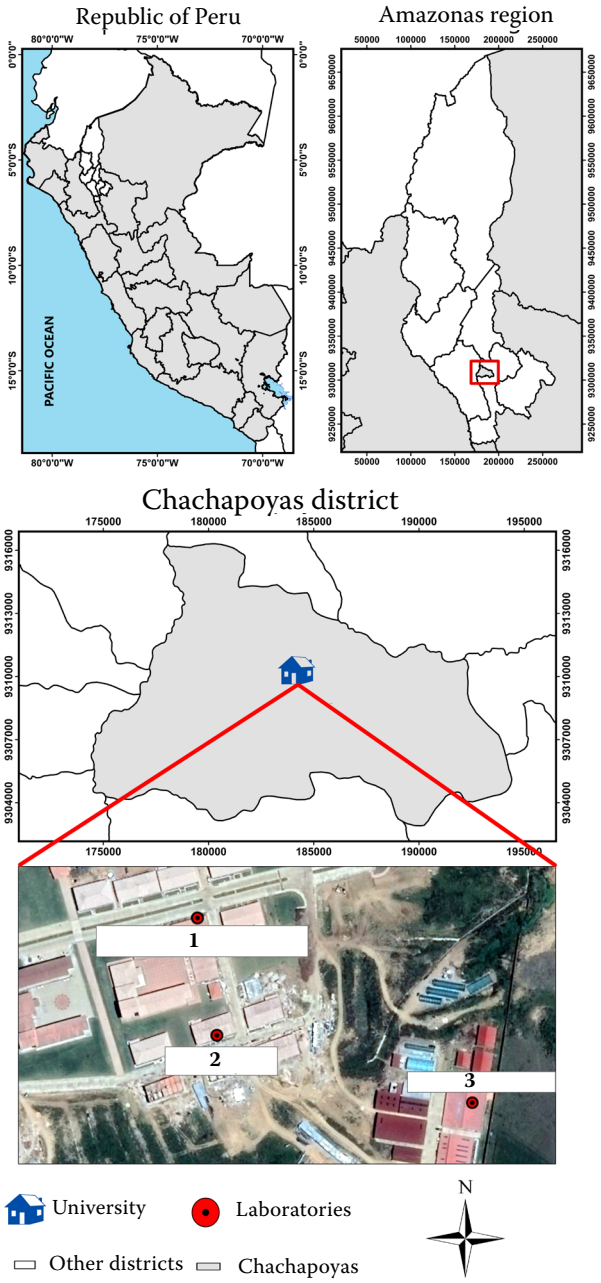

Figure 1. Geographic location of the National University Toribio Rodríguez de Mendoza of Amazonas, Chachapoyas, Peru

1 = Laboratory of Animal Biotechnology, Reproduction and Genetic Improvement; 2 = laboratory of coffee quality; 3 = centre for semen collection

Preparation of *Coffea arabica* grounds (CAG) extract

The CAG were obtained from the Bourbon variety provided by the Laboratorio de Calidad de Café. The CAG were dried in an oven (Venticell, ECOline, Germany) at 60 °C for 72 hours. They were then ground (SM6A013B; Bosh, Slovenia) to obtain particles of uniform size, and a 100 µm sieve was used to obtain a homogeneous final product. For the extraction of bioactive compounds from the CAG, 100 ml of Milli-Q water (Milli-Q® IQ 7000 Water System, Tiefenbach, Netherlands) was used as the extraction solvent with 4.5 g of dried CAG in a beaker. It was thoroughly mixed with a stirrer (Benchmark, Darmstadt, Germany) for 5 min and subjected to sonication (CPX5800H-E; Bransonic, Mexico City, Mexico) for 10 min to better extract the bioactive compounds from the CAG. The resulting extract was centrifuged (Pro-analitical CR4000; Centurion Scientific, Chichester, UK) at 4 800 rpm for 10 min and filtered with Whatman N° 40 paper. The filtrate was transferred to 50 ml centrifuge tubes and frozen (VF 360-86; Evo Safe-SERIES™, Tilburg, Netherlands) at –86 °C for 72 hours. After 3 days, the obtained product was lyophilized (Terroni, LC 1500 with acrylic chamber), which was then evaluated and used for the research.

Evaluation of CAG antioxidant capacity

The antioxidant capacity and phenolic content were determined using three independent assays: (*i*) 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH) (Singleton et al. 1968), (*ii*) Ferric reducing antioxidant power assay (FRAP) (Jeyendran et al. 1984), and (*iii*) Total phenolic content – Folin-Ciocalteu assay (Singleton et al. 1968) (Table S1).

Semen dilution

The semen sample was diluted using a commercial diluent (Optixcell®) following the manufacturer's instructions. The initial dilution ratio was 1 : 1 (semen/diluent), resulting in a concentration of 50 million spermatozoa/ml, quantified using the Scepter 3.0 Handheld Automated Cell Counter (Merck KGaA, Darmstadt, Germany). The temperature of the semen and diluent was continuously checked (68X001351; Digi Sense,Illinios, USA) to avoid thermal stress alterations to the spermatozoa until reaching the optimal refrigeration temperature (4 °C).

Each diluted ejaculate was divided into four parts and supplemented with different doses of CAG: treatment 0 (T0): control (without CAG), treatment 1 (T1):

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CAG 1 mg/ml, treatment 2 (T2): CAG 1.5 mg/ml, and treatment 3 (T3): CAG 2 mg/ml. These four treatments were evaluated at different time points after refrigeration: 4, 12, 24, and 36 hours.

Semen parameter analysis – Computerised SCA® System

The semen parameters were evaluated using the Sperm Class Analyzer-SCA® computerised system, (v.6.6.64, USA) (Table S2 in ESM), for which a 10-µl sample of the semen was loaded onto a pre-warmed slide and covered with a coverslip. Subsequently, the measurements were performed using a phase contrast microscope as part of the computerised SCA System. The following semen parameters were evaluated: total motility (*TM*; %), progressive motility (*MP*; %); kinematic parameters: curvilinear velocity (*VCL*; µm/s), average path velocity (*VAP*; µm/s), straight-line velocity (*VSL*; µm/s), straightness (*STR*; %), linearity (*LIN*; %), wobble index (*WOB*; %), amplitude of lateral head displacement (*ALH*; %), and beat-cross frequency (*BCF*; Hz). The analysis was based on the successive evaluation of digitised images obtained from five fields.

Membrane functionality – hypoosmotic swelling test (HOST)

The test HOST of Jeyendran et al. (1984) was used, with an adapted protocol. The hypoosmotic solution was prepared in 50 ml of double-distilled water, supplemented with 50 mOsm/l using 0.122 5 g of D-fructose and 0.225 g of sodium citrate. In a microtube preheated to 37 °C with a 100 µl aliquot of the hypoosmotic solution, 35 µl of the treated semen sample was added and incubated for 5 min in a water bath. After the incubation time, 31 µl of hypoosmotic solution with formaldehyde (1 ml of hypoosmotic solution + 3 µl of 40% formaldehyde) was added to stop the reaction. For the evaluation of the test, 5 µl of the sample was extracted and placed on a slide, which was then covered with a coverslip. The sample was observed under a phase contrast microscope using a 40× objective. A total of 200 spermatozoa per sample were evaluated. The classification of the spermatozoa was based on the osmotic function of the sperm membrane: with coiled tail (HOST+) – intact membrane and without coiled tail (HOST–) – damaged membrane (Figure 2).

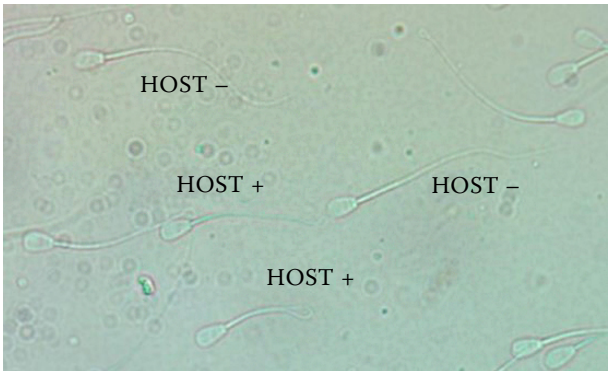

Figure 2. Microscopic field view of bovine spermatozoa subjected to the hypoosmotic swelling test (HOST) for the evaluation of membrane functionality (40 × magnification)

HOST+ = intact membrane and without coiled tail; HOST– = damaged membrane

Acrosomal integrity – Coomassie Brilliant Blue

It was evaluated using Coomassie Blue 0.22% staining following the methodology of Fumuso et al. (2014). To prepare the Coomassie Blue staining solution, the following components were mixed: 20 ml distilled water, 0.11 g Brilliant Blue for Coomassie, 25 ml methanol and 5 ml 99% acetic acid. Smears of the sperm samples were prepared (10 µl of sample from each treatment) and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min. After that, the smears were washed in 1X PBS with five continuous one-second immersions, and left to dry at room temperature. They were then submerged in the 0.22% Coomassie Blue staining solution for 5 minutes. Five continuous one-second immersions in distilled water were performed to remove the excess staining, and then they were left to dry at room temperature. Finally, the smears were observed under the microscope, classifying them into two categories: Blue^{+} (acrosome stained blue^{-} intact) and Blue– (acrosome unstained – damaged). For this, at least 200 spermatozoa per sample were evaluated (Figure 3).

Data analysis

This research was developed in a completely randomised design (CRD), with the treatments [T0: control (without CAG), T1: CAG 1 mg/ml, T2: CAG

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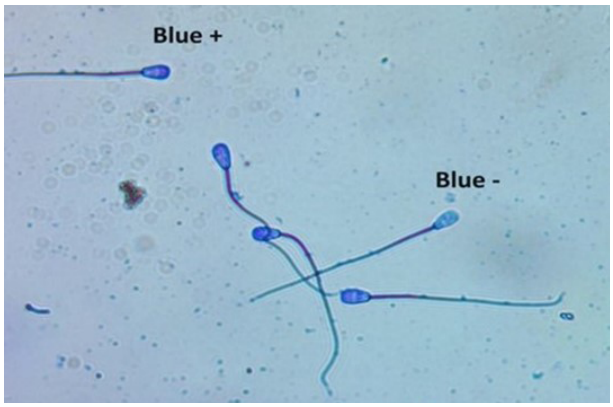

Figure 3. Microscopic field capture of bovine spermatozoa stained with 0.22% Coomassie Blue for acrosomal integrity evaluation (100× magnification)

1.5 mg/ml, T3: CAG 2 mg/ml] at 4 different times (4, 12, 24 and 36 hours) post-refrigeration, to evaluate the effect of the antioxidant capacity of CAG on semen parameters with 10 replications; it was carried out under a 4 × 4 factorial arrangement, with 10 replications and a total of 160 experimental units. The normal distribution of the variables – *MT*, kinetic parameters, membrane functionality, and acrosomal integrity – was analysed using the Shapiro-Wilk statistical test ($P > 0.05$). Then, an analysis of variance (ANOVA) was performed with a significance level of $P < 0.05$ to compare the means, using the InfoStat software, (v.2018p) (Balzarini et al. 2008).

RESULTS

Antioxidant capacity

The antioxidant capacity and phenolic content of CAG were verified according to the three evaluated independent assays (Table 1).

The results of the macroscopic and microscopic evaluations of fresh bovine semen are shown in Table 2.

Sperm motility

The addition of CAG had an effect on the motility parameters of refrigerated bovine semen. Table 3 shows the interaction of factors (dose × time) concerning the evaluation of *TM* of spermatozoa, highlighting that T1 at 4 h post-refrigeration

Table 1. Values of the assays for the determination of antioxidants and phenolic content

Assays	Mean	SD	Min	Max
DPPH (mg T Eq/100 ml CAG)	0.83	0.01	0.82	0.84
FRAP (mg AG/100 ml CAG)	40.64	14.52	25.89	54.92
Total phenols (mg AG/100 ml CAG)	603.48	41.34	559.64	641.76

AG = acid gallic; CAG = *Coffea arabica* grounds; DPPH = radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (mg T Eq/100 ml CAG); FRAP = ferric reducing antioxidant power (mg AEq/100 ml CAG); SD = standard deviation (%); T Eq = trolox equivalent

Table 2. Seminal characteristics of bovine semen after collection

Characteristics	Mean	SD
Volume (ml)	5.85	2.16
pH	6.85	0.15
Mass motility (%)	4.00	0.53
Sperm concentration (10^6 spz/ml)	985.3	406.6

SD = standard deviation; Spz = spermatozoa

(99.71 ± 0.27%) represents the best condition for development in this study. Additionally, the interaction of factors was evaluated regarding *PM*, where an improvement was observed in three CAG treatments at 4 h (T1: 93.89 ± 7.48%, T2: 93.89 ± 6.97%, and T3: 93.70 ± 5.03%) and in T1 at 12 h (93.83 ± 5.21%) post-refrigeration.

Kinetic parameters

In Table 4, the interaction of factors (dose × time) regarding the evaluation of kinetic parameters is shown. Improvements were observed in the *VAP* (μm/s) and *VSL* (μm/s) variables with T1 at 4 h (104.88 ± 18.61 μm/s and 84.97 ± 14.88 μm/s, respectively). However, it was observed that T0 at 36 h showed a kinetic decrease for both variables. The *BCF* variable improved with T1 (14.17 ± 2.83 Hz), T2 (13.99 ± 2.38 Hz), and T3 (14.36 ± 2.97 Hz) at 4 h compared to T0. Likewise, a significant decrease was observed at 36 h with T0, T1, T2, and T3. In the evaluation of the *VCL*, *STR*, *LIN*, *WOB*, and *ALH* variables, there were

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Table 3. Interaction of factors (dose × time) regarding the effect of bovine semen treatment on sperm motility related to post-dilution time

Dosage (mg CAG)	Time (hours)	Total motility (%)	Progressive motility (%)
0	4	98.27 ± 1.90$^{a–d}$	91.05 ± 7.87^{ab}
1		99.71 ± 0.27^{a}	93.89 ± 7.48^{a}
1.5		99.38 ± 0.77^{ab}	93.89 ± 6.97^{a}
2		99.09 ± 0.99^{ab}	93.70 ± 5.03^{a}
0	12	97.55 ± 2.06$^{b–d}$	90.57 ± 9.20^{ab}
1		99.03 ± 1.14^{ab}	93.83 ± 5.21^{a}
1.5		98.71 ± 2.00^{a-c}	91.65 ± 4.08^{ab}
2		98.80 ± 1.08^{a-c}	91.08 ± 6.64^{ab}
0	24	97.17 ± 2.35^{cd}	89.10 ± 5.97^{ab}
1		98.48 ± 1.38$^{a–d}$	88.22 ± 6.74^{ab}
1.5		98.66 ± 1.07$^{a–c}$	89.83 ± 6.20^{ab}
2		98.54 ± 1.91$^{a–c}$	89.63 ± 6.63^{ab}
0	36	96.72 ± 2.64^{d}	86.00 ± 9.38^{b}
1		98.01 ± 2.62$^{a–d}$	87.36 ± 7.92^{ab}
1.5		98.38 ± 1.74$^{a–d}$	86.58 ± 10.58^{ab}
2		98.01 ± 2.23$^{a–d}$	86.38 ± 8.91^{ab}

$^{a–d}$Means with a common letter are not significantly different ($P > 0.05$); Results are presented as the mean + SEM

Table 4. Interaction of factors (dose × time) regarding the effect of bovine semen treatment on kinetic parameters related to post-dilution time

Dosage (mg CAG)	Time (h)	*VCL* (µm/s)	*VAP* (µm/s)	*VSL* (µm/s)	*STR* (%)	*LIN* (%)	*WOB* (%)	*ALH* (µm/s)	*BCF* (Hz)
0	4	179.84 ± 33.69^{a}	99.75 ± 14.63^{ab}	80.36 ± 11.35^{abc}	80.47 ± 2.81^{a}	46.68 ± 5.24^{a}	55.25 ± 4.99^{a}	5.01 ± 1.90^{a}	13.75 ± 2.57^{ab}
1		189.40 ± 32.16^{a}	104.88 ± 18.61^{a}	84.97 ± 14.88^{a}	80.97 ± 2.37^{a}	47.62 ± 7.26^{a}	56.87 ± 4.82^{a}	4.61 ± 0.80^{a}	14.17 ± 2.83^{a}
1.5		181.94 ± 32.62^{a}	101.45 ± 15.49^{ab}	81.97 ± 11.80^{ab}	80.81 ± 2.46^{a}	48.50 ± 8.92^{a}	56.43 ± 5.59^{a}	4.42 ± 0.84^{a}	13.99 ± 2.38^{a}
2		182.44 ± 38.56^{a}	101.01 ± 17.79^{ab}	81.34 ± 13.21^{ab}	80.69 ± 2.47^{a}	47.70 ± 4.51^{a}	57.11 ± 2.61^{a}	4.44 ± 1.04^{a}	14.36 ± 2.97^{a}
0	12	175.95 ± 33.96^{a}	96.00 ± 17.06^{ab}	76.02 ± 12.79^{abc}	79.79 ± 2.96^{a}	46.51 ± 6.08^{a}	56.09 ± 2.56^{a}	4.44 ± 0.99^{a}	11.82 ± 2.56^{bc}
1		173.10 ± 50.68^{a}	96.29 ± 21.85^{ab}	77.48 ± 16.64^{abc}	80.13 ± 2.96^{a}	46.52 ± 3.83^{a}	57.71 ± 4.05^{a}	4.21 ± 1.19^{a}	12.42 ± 2.12^{abc}
1.5		163.01 ± 38.48^{a}	92.80 ± 17.26^{ab}	74.35 ± 12.30^{abc}	80.38 ± 3.63^{a}	47.29 ± 7.21^{a}	57.66 ± 4.25^{a}	4.28 ± 1.18^{a}	11.98 ± 1.35^{bc}
2		171.27 ± 49.28^{a}	95.06 ± 21.86^{ab}	76.18 ± 16.69^{abc}	80.30 ± 2.44^{a}	46.98 ± 5.04^{a}	57.45 ± 5.50^{a}	4.21 ± 1.21^{a}	12.94 ± 3.35^{abc}
0	24	175.80 ± 31.03^{a}	93.56 ± 15.36^{ab}	74.11 ± 11.44^{abc}	79.45 ± 2.02^{a}	44.75 ± 2.66^{a}	57.39 ± 5.48^{a}	4.40 ± 0.79^{a}	11.49 ± 1.53^{c}
1		165.42 ± 36.13^{a}	92.94 ± 16.08^{ab}	73.67 ± 12.22^{abc}	79.36 ± 2.05^{a}	46.37 ± 5.13^{a}	58.31 ± 4.34^{a}	4.11 ± 0.89^{a}	11.83 ± 1.46^{bc}
1.5		163.12 ± 40.25^{a}	91.59 ± 17.16^{ab}	72.52 ± 18.59^{abc}	79.78 ± 2.22^{a}	46.90 ± 4.72^{a}	58.90 ± 6.00^{a}	3.99 ± 1.00^{a}	11.87 ± 1.90^{bc}
2		161.79 ± 35.34^{a}	89.87 ± 19.02^{ab}	71.30 ± 13.58^{abc}	80.02 ± 3.54^{a}	46.68 ± 5.83^{a}	58.17 ± 5.20^{a}	4.03 ± 0.80^{a}	11.82 ± 1.67^{bc}
0	36	153.01 ± 44.93^{a}	84.38 ± 19.22^{b}	66.86 ± 14.26^{c}	79.35 ± 2.45^{a}	44.09 ± 4.12^{a}	58.16 ± 5.02^{a}	3.84 ± 1.10^{a}	11.27 ± 0.81^{c}
1		159.06 ± 34.73^{a}	88.15 ± 16.54^{ab}	69.72 ± 12.05^{bc}	79.39 ± 2.53^{a}	46.01 ± 3.69^{a}	58.63 ± 7.83^{a}	4.10 ± 0.95^{a}	11.11 ± 0.99^{c}
1.5		159.52 ± 51.77^{a}	90.87 ± 23.95^{ab}	72.40 ± 11.82^{abc}	79.57 ± 3.47^{a}	45.23 ± 4.58^{a}	59.78 ± 8.10^{a}	3.99 ± 0.94^{a}	11.50 ± 0.99^{c}
2		157.96 ± 39.32^{a}	89.35 ± 18.91^{ab}	70.72 ± 14.54^{abc}	79.43 ± 2.50^{a}	45.59 ± 2.73^{a}	59.18 ± 3.67^{a}	3.89 ± 0.90^{a}	11.31 ± 0.91^{c}

$^{a–c}$Means with a common letter are not significantly different ($P > 0.05$); Results are presented as mean + SEM

ALH = amplitude of lateral head displacement; *BCF* = tail beat frequency; CAG = *Coffea arabica* grounds; *LIN* = linearity index; *STR* = straightness ratio; *VAP* = average velocity; *VCL* = curvilinear velocity; *VSL* = linear velocity; *WOB* = wobble index

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no significant differences between T0 and the CAG treatments. Additionally, no adverse effects were observed with the addition of CAG over time.

Membrane functionality

The percentage of spermatozoa reactive to the HOST test is plotted and represented in Figure 4. Based on the results obtained, it can be inferred that the percentage of spermatozoa reactive to the HOST test shows a significant difference in T1 at 4 h (70.68 ± 2.10%) post-refrigeration compared to the control treatment. The spermatozoa with the lowest percentage in terms of membrane functionality were observed in the T0 treatment at 36 h (65.17 ± 0.48%) post-refrigeration.

Acrosomal integrity

The acrosomal integrity of the spermatozoa is shown in Figure 5. The results show a significant difference in the percentage of intact acrosomes for the T1 treatment at 4 h (89.78 ± 1.51%) post-refrigeration. The spermatozoa with the lowest percentage of acrosomal integrity were observed in the T0 at 36 h (85.80 ± 1.44%) post-refrigeration. The percentage of intact acrosomes was significant for T1 at 4 hours (89.78 ± 1.51%).

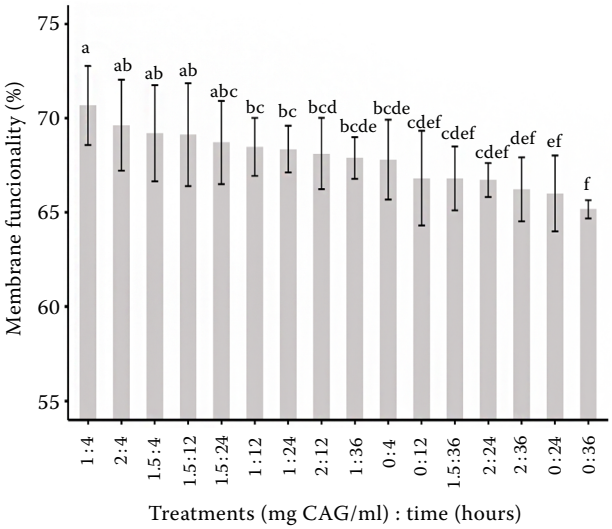

Figure 4. Effect of bovine semen treatment on the membrane functionality over time post-dilution

[a–e]Means with a letter are significantly different ($P < 0.05$)

CAG = *Coffea arabica* grounds

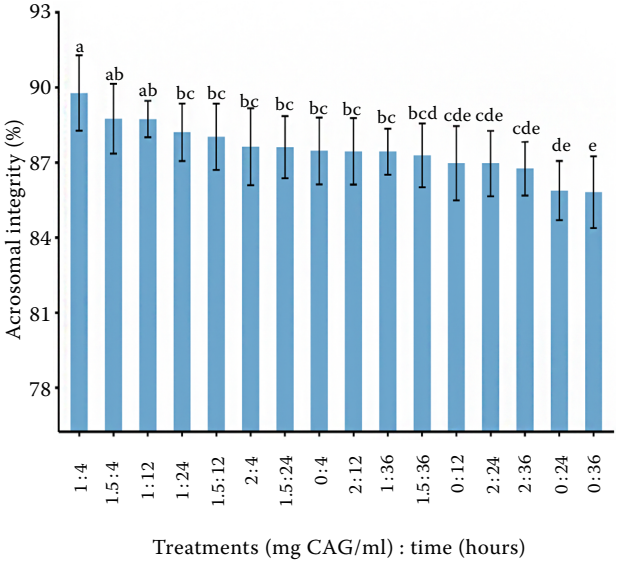

Figure 5. Effect of bovine semen treatment on acrosomal integrity at post-dilution time

[a–e]Means with a letter are significantly different ($P < 0.05$)

CAG = *Coffea arabica* grounds

DISCUSSION

The use of refrigerated semen in artificial insemination, *in vitro* fertilization, and other biotechnologies is relatively uncommon and is mainly used in specific contexts (Adams et al. 2000). Generally, cryopreservation is preferred; however, this method significantly reduces the quality of the semen due to the thermal shock and formation of ice crystals during the process, resulting in a low fertilisation rate (Ozimic 2023). Despite this, refrigeration does not allow for long-term storage, as semen quality decreases considerably over time. The reactive oxygen species (ROS) are among the main factors contributing to such degradation. Therefore, it is necessary to conduct further research to counteract this negative impact and prolong the viability of refrigerated semen with minimal decrease in its quality.

Using antioxidants may decrease the negative impact of ROS on spermatozoa (Kaltsas 2023). This research evaluated the antioxidant capacity and the effect of different doses of CAG on seminal parameters at different post-refrigeration times. The addition of antioxidants to extenders provides a protective effect that favours sperm quality in bulls, rams, goats, pigs, dogs, and humans (Shakouri et al. 2021; Zhang et al. 2022). This is supported by the antioxidant capacity and phenolic content of coffee grounds determined

https://doi.org/10.17221/115/2024-CJAS

by performing the DPPH, FRAP and total phenols of CAG assays and its effect on certain seminal parameters (Varela et al. 2020; Andrade et al. 2022).

Malo et al. (2012) reported that the addition of natural antioxidants to the dilution medium improves the preservation of sperm motility. They corroborated this by evaluating the semen of Bali bulls, adding a green tea extract to the diluent before cryopreservation (Prastiya et al. 2023), and also by evaluating boar spermatozoa, to which they added a fennel extract; it is worth mentioning that both substances have a natural antioxidant capacity. Likewise, Abdramanov et al. (2017) reported that the addition of elderberry (*Sambucus nigra*) extract had antioxidant properties that improved sperm motility in Holstein bulls when evaluated post-refrigeration; results that are shared with our research in the improvement of sperm motility by adding 1 mg/ml of CAG to bovine semen.

It was demonstrated that CAG improves *VAP*, *VSL* and *BCF* with T1 (1 mg/ml) at 4 h post-refrigeration, corroborating that the addition of antioxidants to semen samples has a beneficial impact on kinetic parameters. Similar results were obtained by Li et al. (2021), who found significant improvements in *VAP* and *VCL* in donkey semen samples by adding L-proline, an amino acid with strong antioxidant properties. However, this differs from the results of Ramazani et al. (2023), who observed a decrease in *VCL*, *VSL* and *VAP* when adding L-proline to buffalo bull semen samples. On the other hand, Khalil et al. (2017) stated that different reactions of semen to antioxidants could vary between species, which is related to the inherent physiology of each species and the specific context of each research. The high content of unsaturated fatty acids in the cell membrane causes the membrane functionality of spermatozoa to be negatively affected due to ROS, which is why the addition of antioxidants is crucial to reduce such damage, as mentioned by Walke et al. (2023). According to our results, 1 mg/ml of CAG significantly improved membrane functionality from 67.8% to 70.68% at 4 h post-refrigeration compared to the control, giving better results than those reported by (Bahmid et al. 2023), who obtained an average of 63.49% when evaluating different ejaculate volumes in Bali bulls at 4 h post-refrigeration without adding antioxidants. On the other hand, regarding the evaluations carried out at the different post-refrigeration times, a significant decrease was observed starting at 24 h with the addition of 1 mg/ml of CAG, while the control treatment showed a decrease in this characteristic starting at 4 h, corroborating that CAG maintains the membrane functionality. However, the research by Akhter et al. (2023) did not show a significant decrease in membrane functionality when evaluated at 0, 24, 48, and 72 h post-refrigeration with the addition of vitamin C, vitamin E, vitamin C + E, and control in semen samples (RAM). Similarly, Sangwan et al. (2012) did not show any significant differences when adding soy between 0 and 24 h post-refrigeration when they evaluated in buffalo semen samples.

The acrosomal integrity is fundamental for fertilisation due to its content of enzymes that facilitate the penetration of the sperm into the egg. Likewise, the intact acrosomal integrity and the ability to undergo the acrosome reaction are crucial for sperm fertility (Xu et al. 2018). It was corroborated by the molecular analyses of Talluri et al. (2022), who found that the genes in low-fertility bulls had a negative correlation with acrosomal integrity. Given the importance of the acrosome, our study found that the addition of 1 mg/ml of CAG significantly improved acrosomal integrity at 4 h, showing the benefit of antioxidants. Likewise, improvements were observed at all post-cooling hours compared to the control group, similar benefits to those reported by Rahman et al. (2019), who added bisphenol, an ROS-causing endocrine disruptor, to mouse seminal samples to see the effect of antioxidants (vitamin C and E) on the negative effects of bisphenol A on acrosomal integrity, concluding that the addition of these antioxidants prevents the decrease in sperm acrosomal integrity and reaction. In contrast, Losano et al. (2018) reported that the addition of antioxidants (glutathione peroxidase and superoxide dismutase) along with docosahexaenoic acid (DHA) to seminal samples minimally decreased acrosomal integrity, which could be due to the fact that they worked with samples extracted from the epididymis of post-mortem bulls.

It is worth emphasizing that the effects of CAG on bovine seminal samples have not been the subject of study before, making this the initial evaluation of the protective effect on seminal parameters in cattle, which is being provided by anatural

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by-product obtained from one of the most economically important crops in the world, coffee (*Coffea arabica*).

CONCLUSION

It was determined that CAG presents antioxidant capacity and its addition to seminal samples allows for the improvement of characteristics in motility (total motility and progressive motility), kinetic parameters [*VAP* (μm/s), *VSL* (μm/s), and *BCF* (Hz)], as well as membrane functionality and acrosomal integrity with T1 (1 mg/ml CAG) at 4 h post-refrigeration. This suggests a reduction in the oxidative stress of spermatozoa. However, additional studies are needed to better understand the mechanisms of action and the overall efficacy of CAG as an antioxidant agent in this specific context.

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Conflict of interest

Authors declare no conflict of interest.

REFERENCES

Abdramanov A, Massanyi P, Sarsembayeva N, Usenbayev A, Alimov J, Tvrda E. The in vitro effect of elderberry (Sambucus nigra) extract on the activity and oxidative profile of bovine spermatozoa. J Microbiol Biotechnol Food Sci. 2017;6(6):1319-22.

Adams M, Zhang G, Zhao Q, Zheng L, Zheng X, Zhong F, Zhong W, Zhou X, Woodage T, Worley K, Wu D, Yang S, Yao Q, Ye J, Yeh R, Zaveri J, Wang A, Wang X, Wang Z, Wassarman D, Weinstock G, Weissenbach J, Spier A, Spradling A, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Scheeler E, Shen H, Shue B, Siden-KiamosI, Simpson M, Skupski M, Smith T. The genome sequence of Drosophila melanogaster. Science. 2000; 287(5461):2185-95.

Akhter S, Zubair M, Mahmood M, Andrabi SMH, Hameed N, Ahmad E. Effects of vitamins C and E in tris citric acid glucose extender on chilled semen quality of Kail ram during different storage times. Sci Rep. 2023;13(1):1-8.

Andrade C, Perestrelo R, Camara JS. Bioactive compounds and antioxidant activity from spent coffee grounds as a powerful approach for its valorization. Molecules. 2022 Nov 3;27(21):7504.

Andrea M, Gabriela M, Fenoles CDE, Capacidad CY, Granos ADE, Elidai LS. Contenido de fenoles, cafeina y antioxidantes de granos de cafe verdes y totado en diferentes estados Mexico. [Phenol content, caffeine, and antioxidant capacity of green coffee beans and roasted in various states of mexico] Rev Iberoam Tecnol Potscosecha. 2015;16:293-8. (Spanish)

Bahmid NA, Jamil NI, Yusuf ODP, Farida S, Gustina S. Plasma membrane integrity and acrosomal integrity of fresh and frozen Bali bull semen based on different ejaculate volume. IOP Conf Ser Earth Environ Sci. 2023; May,1174(1): 012034.

Balzarini MG, Gonzalez LA, Tablada EM, Casanoves F, Di Rienzo JA, Robledo CW. InfoStat Manual Del Usuario [InfoStat User Manual]. 2008, 334. Available at https://www.researchgate.net/profile/Fernando-Casanoves/publication/319875343_Manual_del_usuario/links/5e2ee26992851c9af7280cfa/Manual-del-usuario.pdf

Bo GA, Huguenine E, de la Mata JJ, Nunez-Olivera R, Baruselli PS, Menchaca A. Programs for fixed-time artificial insemination in South American beef cattle. Anim Reprod. 2018 Aug 3;15(Suppl 1):952-62.

Borges-Silva JC, Silva MR, Marinho DB, Nogueira E, Sampaio DC, Oliveira LOF, Abreu UGP, Mourao GB, Sartori R. Cooled semen for fixed-time artificial insemination in beef cattle. Reprod Fertil Dev. 2016;28(7):1004-8.

Bucher A, Kasimanickam R, Hall JB, de Jarnette JM, Whittier WD, Kahn W, Xu Z. Fixed-time AI pregnancy rate following insemination with frozen-thawed or fresh-extended semen in progesterone supplemented CO-Synch protocol in beef cows. Theriogenology. 2009;71(7):1180-5.

Crespilho AM, Papa FO, Santos M de P, Filho MF de S. Use of cooled bull semen as a strategy to increase the pregnancy rate in fixed-time artificial insemination programs-case report. Am J Anim Vet Sci. 2013;7(4):175-9.

Dias EAR, Campanholi SP, Rossi GF, Freitas Dell'Aqua CP, Dell'Aqua JA, Papa FO, Zorzetto MF, de Paz CCP, Oliveira LZ, Mercadante MEZ, Monteiro FM. Evaluation

https://doi.org/10.17221/115/2024-CJAS

of cooling and freezing systems of bovine semen. Anim Rep Sci. 2018;195:102-11.

Franca AS, Oliveira LS. Coffee. Integrated processing technologies for food and agricultural by-products. Davis, USA: Elsevier Inc.; 2019. p. 413-38.

Fumuso FG, Gimenez ML, Neild DM, Giuliano SM, Chaves MG, Carretero MI. Comparison of washing methods and smear conservation periods for llama sperm acrosome assessment using the Coomassie Blue stain. Spermova. 2014;4(1):50-3.

Hai E, Li B, Zhang J, Zhang J. Sperm freezing damage: The role of regulated cell death. Cell Death Discov. 2024;10(1):1-13.

Hidalgo O, Tamargo C, Monforte M. Analisis del semen bovino [Analysis of bovine semen]. Boletín Inf del SERIDA. 2015;39-3.

Izquierdo A, Iglesias AE, Guerra JE, Huerta R, Sanchez R. El estres oxidativo en la fertilidad y desempeno reproductivo de mamiferos hembras y machos. [Oxidative stress in the fertility and reproductive performance of female and male mammals]. Rev Vet. 2020;31(1):97.

Jeyendran RS, Ven HH Van Der, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil. 1984; 70(1):219-28.

Kaltsas A. Oxidative stress and male infertility: The protective role of antioxidants. Med. 2023 Oct 4;59(10):1769.

Khalil K, Elayat M, Khalifa E, Daghash S, Elaswad A, Miller M. Generation of myostatin gene-edited channel catfish (Ictalurus punctatus) via zygote injection of CRISPR/Cas9 system. Sci Rep. 2017 Jul 5;7:7301.

Li N, Yu J, Yang F, Shao Y, Wu S, Liu B. L-proline: An effective agent for frozen and post-thawed donkey semen storage. J Equine Vet Sci. 2021 Jun;101:103393.

Losano JDA, Angrimani DSR, Rui BR, Bicudo LC, Dalmazzo A, Silva BCS, Nascimento J, Goncalves P. The addition of docosahexaenoic acid (DHA) and antioxidants (glutathione peroxidase and superoxide dismutase) in extenders to epididymal sperm cryopreservation in bulls. Zygote. 2018;26(3):199-206.

Malo C, Gil L, Cano R, Gonzalez N, Luno V. Fennel (Foeniculum vulgare) provides antioxidant protection for boar semen cryopreservation. Andrologia. 2012;44(SUPPL.1): 710-5.

Martinez IYH, Alvarez NTS, Duque JFS, Castro LMM, Pinzon FMC, Rodriguez AR. Fixed-time artificial insemination versus artificial insemination at stalk detected in cows and heifers. RGSA. 2024;18(4):e08016.

Mussatto SI, Ballesteros LF, Martins S, Teixeira JA. Extraction of antioxidant phenolic compounds from spent coffee grounds. Sep Purif Technol. 2011;83(1):173-9.

Ozimic S, Ban-Frangez H, Stimpfel M. Sperm cryopreservation today: Approaches, efficiency, and pitfalls. Curr Issues Mol Biol. 2023;45(6):4716-34.

Prastiya RA, Debora AE, Wijayanti A, Agustono B, Saputro AL, Amaliya A, Supriyadi D. Sperm kinematics and morphology of Bali bull (Bos javanicus) after freezing and thawing treated with green tea extract in extender. Trop Anim Sci J. 2023;46(4):418-27.

Puerta Suarez J, Carvajal A, Cardona W. Relacion entre los antioxidantes y la calidad seminal [Relationship Between antioxidants and seminal quality]. Rev Cuba Obstet Ginecol. 2019;45(2):1-13.

Rahman MS, Kang KH, Arifuzzaman S, Pang WK, Ryu DY, Song WH, Hossain MD. Effect of antioxidants on BPA-induced stress on sperm function in a mouse model. Sci Rep. 2019;Jul 22;29(1):1-10.

Ramazani N, Mahd Gharebagh F, Soleimanzadeh A, Arslan HO, Keles E, Gradinarska-Yanakieva DG, Gholami M. The influence of L-proline and fulvic acid on oxidative stress and semen quality of buffalo bull semen following cryopreservation. Vet Med Sci. 2023;9(4):1791-802.

Redivo N, Mendes M, Dutra C, Fonseca EA, De Oliveira D, Gasparin G, Ferreira A. Aspectos da reproducao em touros bovinos com uso de congelacao de semen e termografia infravermelha resumo [Aspects of reproduction in bulls concerning the use of semen freezing and infrared thermography]. Vet Zoot. 2017 Sep;24(Suppl1): 75-81.

Rosete J, Gallardo H, Duarte D, Islas A, Pelayo A, Utrera A, Gonzalez J. Reproductive biotechnologies in beef cattle: Five decades of research in Mexico. Rev Mex Ciencias Pecu. 2021 Nov;12(2):39-78.

Sangwan N, Lata P, Dwivedi V, Singh A, Niharika N, Kaur J, Kumar S. Comparative metagenomic analysis of soil microbial communities across three hexachlorocyclohexane contamination levels. PLoS One. 2012;7(9):e46219.

Segura GT, Portocarrero HA, Quispe-Ccasa JA, Saucedo-Uriarte JA, Rojas PAY, Valderrama MNL, Polanco CJV. Effect of two dilution temperatures on the quality of semen in cattle from zebu in the Peruvian tropic. Rev Vet. 2023;34(1):33-9.

Shakouri N, Soleimanzadeh A, Rakhshanpour A, Bucak MN. Antioxidant effects of supplementation of 3,4-dihydroxyphenyl glycol on sperm parameters and oxidative markers following cryopreservation in canine semen. Reprod Domest Anim. 2021;56(7):1004-14.

Sharafi M, Borghei-Rad SM, Hezavehei M, Shahverdi A, Benson JD. Cryopreservation of semen in domestic animals: A review of current challenges, applications, and prospective strategies. Animals (Basel). 2022 Nov 24; 12(23):3271.

https://doi.org/10.17221/115/2024-CJAS

Singleton L, Orthofer R, Lamuela-Raventos M. 3,5-di-Tert-butyl-4-hydroxytoluene (BHT) as an artifact from diethyl ether. Lipids. 1968;3(6):561.

Talluri TR, Kumaresan A, Sinha MK, Paul N, Ebenezer Samuel King JP, Datta TK. Integrated multi-omics analyses reveals molecules governing sperm metabolism potentially influence bull fertility. Sci Rep. 2022 Jun 23;12(1):10692.

Varela E, Rojas M, Restrepo G. Association between conventional and computerized sperm quality parameters with flow cytometric evaluation of frozen bovine semen. Rev Investig Vet del Peru. 2020;31(4):e19023.

Verberckmoes S, Van Soom A, Dewulf J, de Kruif A. Comparison of three diluents for the storage of fresh bovine semen. Theriogenology. 2005 Feb;63(3):912-22.

Walke G, Gaurkar SS, Prasad R, Lohakare T, Wanjari M. The impact of oxidative stress on male reproductive function: Exploring the role of antioxidant supplementation. Cureus. 2023 Jul 27;15(7):e42583.

Xu F, Guo G, Zhu W, Fan L. Human sperm acrosome function assays are predictive of fertilization rate in vitro: A retrospective cohort study and meta-analysis. Reprod Biol Endocrinol. 2018 Aug 24;16(1):81.

Zhang W, Min L, Li Y, Lang Y, Hoque SAM, Adetunji AO. Beneficial effect of proline supplementation on goat spermatozoa quality during cryopreservation. Animals (Basel). 2022 Sep 30;12(19):2626.

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