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RESEARCH ARTICLE

Evaluation of Bull Semen Frozen with Different Antioxidants

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Abstract

In this project, ejaculates (n=34) were collected from three Brown Swiss bulls using the artificial vagina method, then divided into seven equal parts and diluted to 60×10^6 spermatozoa/mL using the following extenders: Control, Control+1mM carnosic acid (CR1), Control+3mM carnosic acid (CR3), Control+2.5mM glutamine (G2.5), Control+7.5mM glutamine (G7.5), Control+CR1+G7.5, and Control+CR3+G2.5. All diluted aliquots were frozen after 3 hours of equilibration. Thawed samples were analyzed for motility, plasma membrane and acrosome integrity (PMAI), mitochondrial membrane potential (MMP), and DNA integrity. The Control, G7.5, and G2.5 groups showed the highest total and progressive motility. The highest PMAI value was observed in the Control group, while the lowest was in the CR3 group. The PMAI values of G2.5, G7.5, and G7.5+CR1 were significantly higher than those of CR1, CR3, and CR3+G2.5. There were no statistically significant differences in MMP among the groups. The lowest DNA damage was observed in the Control group and the highest in CR3+G2.5. Control, G2.5, G7.5, and CR1+G7.5 groups had statistically similar kinematic values (VAP, VSL, VCL) and outperformed the others. Although 2.5 mM and 7.5 mM glutamine showed a synergistic interaction, they did not significantly improve spermatological parameters. Carnosic acid appeared to act as an antagonistic antioxidant.

Introduction

Cryopreserved bull semen in artificial insemination (AI) represents the most widely implemented biotechnological approach for genetically improving livestock worldwide. In recent years, the frozen semen industry has evolved into a globally competitive market, with intercontinental trade that surpasses even the economic potential of the meat and dairy industries.

The foundation of this industry lies in the genetic transmission potential of the bull, whose semen is to be cryopreserved, and the ability to freeze the semen under optimal conditions. Numerous studies have investigated various combinations of semen extenders and antioxidants to enhance the viability and functional quality of frozen-thawed spermatozoa. Initially, semen extenders were developed using animal-derived proteins such as egg yolk or milk powder. However, components poses significant challenges, and extenders containing such bases have a relatively limited shelf life. Moreover, semen extenders containing animal-derived proteins carry the risk of contamination with pathogenic agents, including E. coli, Staphylococcus, Streptococcus, Pseudomonas, Haemophilus, Salmonella, Avian influenza, Campylobacter, Listeria, and Mycoplasma. Therefore, semen extenders developed in recent years for cryopreservation purposes and now widely used commercially are primarily based on plant-derived protein sources (Bousseau et al., 1998; Thun et al., 2002; Crespilho et al., 2012; Layek et al., 2016; Ansari et al., 2017; Murphy et al., 2018; Gavin-Plagne et al., 2019).

During the processes of semen collection from bulls via the artificial vagina, macroscopic and microscopic evaluation, equilibration, straw filling, freezing, and thawing, exposure to oxygen and lipid

phase transitions in spermatozoon membranes may lead to osmotic and mechanical stress. These stressors contribute to an increase in reactive oxygen species (ROS), which can negatively impact the viability of frozen-thawed sperm, ultimately leading to reduced pregnancy rates (Arı and Öztürkler, 2015; Gürler et al., 2016).

Under normal physiological conditions, ROS are generated as byproducts of intracellular signal transduction and enzymatic reactions. In mammalian spermatozoa, ROS species such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (•OH), and hypochlorite radical (OHCl) can be generated. During cryopreservation, spermatozoa are exposed to various fluctuations in temperature, pH, and osmolarity. At low temperatures, phospholipids, proteins, and lipid molecules—abundantly present in the plasma membrane—tend to form tighter interactions. The formation of extracellular ice crystals creates a hyperosmotic environment, during which water efflux from the cell occurs through membrane macromolecules, leading to cell shrinkage. These structural disruptions also lead to the loss of cytoplasm, which is rich in endogenous antioxidants. Consequently, spermatozoa remain highly susceptible to oxidative stress during the freezing and thawing processes (Arı and Öztürkler, 2015; Gürler et al., 2016).

A physiological level of ROS in the composition of semen has been reported to contribute positively to sperm function by participating in the tyrosine-dependent cyclic adenosine monophosphate (cAMP) signaling pathway, thereby promoting hyperactivation, capacitation, and the acrosome reaction. However, excessive levels of ROS induce oxidative stress within the spermatozoon, leading to impaired motility and viability. Moreover, it has been demonstrated that elevated oxidative stress causes lipid peroxidation and damages sperm DNA integrity (Luconi et al., 2006; O'Flaherty et al., 2006; Gürler et al., 2016; Ugur et al., 2019).

Rosemary (Rosmarinus officinalis) is an evergreen shrub characterized by its needle-like leaves and distinctive purple flowers. Carnosic acid, a bioactive compound found in rosemary and extracted from its leaves, is recognized as a potent agent that inhibits tumour development in cancer therapy. Several studies have reported that carnosic acid has been added as an antioxidant to semen extenders in various animal species, including buffalo, bull, ram, deer, and pig, demonstrating beneficial effects on spermatozoon quality parameters (Malo et al., 2010; Zanganeh et al., 2013; Daghigh-Kia et al., 2014; Luño et al., 2014; Motlagh et al., 2014; Yeni et al., 2018; Gungor et al., 2019). In their cryopreservation study (Yeni et al., 2018) using epididymal buffalo semen, it was reported that the addition of carnosic acid at doses of 12.5 and 25 µg/mL to an egg yolk-based extender had a positive effect on spermatozoon motility and viability parameters.

Glutamine, a tripeptide, plays a regulatory role in numerous metabolic processes, including cell integrity (apoptosis and cell proliferation), protein synthesis and degradation, redox potential, gene expression, and extracellular matrix synthesis. During the cryopreservation process of sperm, glutamine has been reported to contribute to the preservation of the cytoskeletal structure of the plasma membrane. The addition of glutamine to semen extenders has been reported to contribute to preserving the cytoskeletal structure of the spermatozoon plasma membrane during the cryopreservation process, thereby exerting a protective effect against freezing-induced damage. Studies conducted on semen from Angora bucks, rams, stallions, and bulls have demonstrated this beneficial role (Khlifaoui et al., 2005; Amirat-Briand et al., 2009; Bucak et al., 2009a; Bucak et al., 2009b; Tuncer et al., 2011). Studies involving the supplementation of egg yolk-based extenders with varying concentrations of glutamine have shown that concentrations exceeding 10 mM negatively impact sperm motility (Amirat-Briand et al., 2009).

A review of current literature reveals that cryopreservation studies involve the addition of carnosic acid and glutamine to egg yolk-based semen extenders. However, no studies have been identified that investigate the addition of these compounds to plant protein-based, egg-yolk-free extenders, particularly in the context of extender optimization and synergistic effects. This study aimed to enhance the quality of frozen-thawed semen by using Andromed, a plant-based (soy lecithin) semen extender, in combination with varying concentrations of the antioxidants carnosic acid and glutamine, either individually or in combination. Furthermore, the project sought to determine the optimal antioxidant supplementation strategy for semen cryopreservation using plant-derived extenders.

Materials and Methods

Animals and Semen Collection

Semen samples were collected from three Brown Swiss bulls, each at least two years old, housed at the Artificial Insemination Unit of the International Center for Livestock Research and Training (Ankara, Turkey). Ejaculates were obtained twice weekly from each bull using an artificial vagina. A total of 34 ejaculates were collected during the study. Only samples with ≥ 75% progressive motility were used. Motility was assessed under a phase-contrast microscope at ×100 magnification, and sperm concentration was measured with a photometric device (Accucell, IMV). Each ejaculate was divided into seven equal parts, resulting in 238 aliquots. Dilution was performed using Andromed (Minitüb

GmbH), a soybean lecithin-based extender, to a final concentration of approximately 60 × 10^6 motile spermatozoa/mL. The extender was used alone or supplemented with antioxidants, as described below.

Semen Cryopreservation

The dilution process was carried out via a one-step method in a 37°C water bath. Samples were cooled to +4°C within 45–60 minutes using a digitally controlled cold chamber, followed by a 3-hour equilibration period. The semen was loaded into 0.25 mL straws using an automatic filler-sealer (MX4, IMV Technologies), frozen to approximately −100°C with a programmable freezer (Digital Cool 5300ZB 250; IMV), and stored in liquid nitrogen at −196°C.

Experimental Design

The experimental groups were designed by adding specific antioxidants to the control extender. The control group included only the commercial extender (Andromed) (C); 1 mM carnosic acid (C + CR1); 3 mM carnosic acid (C + CR3); 2.5 mM glutamine (C + G2.5); 7.5 mM glutamine (C + G7.5); 1 mM carnosic acid and 7.5 mM glutamine (C + CR1 + G7.5); and 3 mM carnosic acid and 2.5 mM glutamine (C + CR3 + G2.5).

Semen Evaluation

The effects of carnosic acid and glutamine applied individually or in combination with a soybean lecithin-based extender were evaluated in terms of post-thaw sperm motility and kinematic parameters, viability assessed through mitochondrial membrane potential (MMP) and plasma membrane and acrosome integrity (PMAI), and DNA integrity as a separate indicator of nuclear damage.

Sperm Motility Parameters Assessment

The motility and kinematic characteristics of individual frozen-thawed spermatozoa were analyzed using a computer-assisted sperm analysis (CASA) system (IVOS I, Hamilton Thorne, Beverly, MA, USA). Frozen semen samples were thawed in a water bath at 37°C for 30 seconds before sperm analysis. 3 µL aliquot of each sample was placed onto a pre-warmed four-chamber slide with a depth of 20 µm (Leja, IMV, France). Analyses were performed automatically by a CCD camera (30 frames/s, 60 Hz) integrated with a phase-contrast microscope at 37°C and 10X magnification. Images from at least five microscopic fields were captured and evaluated for each sample.

The CASA system recorded total motility (TM, %) and progressive motility (PM, %) as well as kinetic parameters, including average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s). Spermatozoa were classified as progressively motile if VSL was ≥70% and VAP was ≥50 µm/s. Based on VAP values, motility subcategories were defined as follows: static, slow (>40 µm/s), medium (>70 µm/s), and rapid (>100 µm/s).

Viability Parameters Assessment

Sperm viability was assessed using a flow cytometer (CytoFLEX System B4-R0-V0, Beckman Coulter, USA) with a 488 nm laser. Evaluations included plasma membrane and acrosome integrity and mitochondrial membrane potential.

Plasma Membrane and Acrosome Integrity (PMAI)

Plasma membrane and acrosome integrity of spermatozoa were assessed by flow cytometry using dual fluorescent staining with propidium iodide (PI) and fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA). For this analysis, 5 µL of semen was diluted in 241 µL of Tyrode's solution and stained with 1.5 µL of PI and 2.5 µL of FITC-PNA. After 15 minutes of incubation at 37°C, samples were analyzed. Spermatozoa were classified into four subpopulations according to their plasma membrane and acrosome status: cells with intact plasma membrane and acrosome, considered viable (PMAI, %) (PI- and FITC-PNA-negative); cells with damaged plasma membrane but intact acrosome (PI-positive, FITC-PNA-negative); cells with intact plasma membrane but damaged acrosome (PI-negative, FITC-PNA-positive); and cells with both damaged plasma membrane and acrosome (PI- and FITC-PNA-positive).

Mitochondrial Membrane Potential (MMP)

The mitochondrial membrane potential of spermatozoa was assessed by flow cytometry (CytoFLEX System B4-R0-V0, Beckman Coulter, USA) following fluorescent staining. For this purpose, 5 µL of semen was diluted in 241 µL of Tyrode's solution and stained with 1.5 µL of propidium iodide (PI) and 2.5 µL of JC-1. After 15 minutes of incubation at 37°C, samples were analyzed. After the exclusion of PI-positive cells, two subpopulations were identified among live spermatozoa according to JC-1 fluorescence: cells emitting green fluorescence, representing spermatozoa with low mitochondrial membrane potential (LMMP), and cells emitting orange fluorescence, representing spermatozoa with high mitochondrial membrane potential (HMMP).

DNA Integrity Assessment

DNA integrity was evaluated using acridine orange staining. For this purpose, 20 µL of sperm suspension was smeared onto pre-cleaned glass slides and air-dried. The dried samples were fixed with Carnoy's fixative for 1 hour, stained with acridine orange for 5 minutes in the dark, rinsed with distilled water, air-dried again, and examined under a fluorescence microscope. Sperm with green fluorescence were

considered to have intact DNA, while those with yellow-orange fluorescence were classified as having fragmented DNA. A minimum of 100 sperm per sample was counted to calculate the percentage of DNA fragmentation.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Before statistical analysis, the data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. One-way analysis of variance (ANOVA) was used to compare more than two groups for spermatological parameters, and Tukey's multiple comparison test was applied to determine significant differences between groups. All statistical analyses were performed using SPSS version 22.0 for Windows (IBM, New York, USA), and differences were considered statistically significant at $P < 0.05$.

Results

The effects of carnosic acid and glutamine, used either individually or in various combinations, on sperm quality parameters are shown in Table 1. Total motility was highest in the Control, G7.5, G2.5, and CR1+G7.5 groups (50.6 ± 14.81; 48.79 ± 16.8; 48.23 ± 13.9; and 45.00 ± 13.28, respectively) ($P < 0.001$). Similarly, progressive motility was greatest in the Control (22.68 ± 6.00), G2.5 (21.02 ± 5.57), G7.5 (20.97 ± 6.40), and CR1+G7.5 (18.82 ± 5.47) groups ($P < 0.001$). The remaining groups, CR1 (17.13 ± 5.12), CR3 (16.30 ± 4.73), and CR3+G2.5 (13.48 ± 4.15), showed significantly lower values compared to the Control, G2.5, and G7.5 groups ($P < 0.001$). Although CR1+G7.5 differed significantly higher than CR3+G2.5 ($P < 0.001$), it did not differ statistically from the other groups ($P > 0.05$), indicating an intermediate pattern.

Linear kinematic parameters, including average path velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL), were evaluated to assess post-thaw sperm motion characteristics, and the results are presented in Table 2. These parameters were significantly elevated in the Control (68.59 ± 16.29; 56.25 ± 13.67; 96.15 ± 22.26 µm/s), G2.5 (66.26 ± 15.18; 53.77 ± 12.97; 92.74 ± 20.81 µm/s), G7.5 (66.37 ± 14.60; 53.44 ± 12.79; 94.53 ± 21.06 µm/s), and CR1+G7.5 (64.65 ± 14.55; 51.63 ± 12.55; 90.43 ± 19.60 µm/s) groups compared to the remaining groups *(P < 0.001)*.

Table 1. Total and progressive motility values of the experimental groups (Mean ± SD).

Group	n	Total Motility (%)	Progressive Motility (%)
Control (C)	34	50.6±14.81^{a}	22.68±6.28^{a}
C + Carnosic acid (CR) 1mM	34	35.35±11.9^{b}	17.08±5.68^{bc}
C + Carnosic acid (CR) 3 mM	34	31.29±8.79^{b}	15.88±4.47^{bc}
C + Glutamine 2.5 mM	34	48.23±13.9^{a}	21.02±5.57^{a}
C + CR 3 mM + Glutamine 2.5	34	30.58±8.29^{b}	15.20±3.58^{c}
C + Glutamine 7.5 mM mM	34	48.79±16.8^{a}	20.97±6.44^{a}
C + CR 1 mM + Glutamine 7.5 mM	34	45.00±13.28^{a}	19.5±4.73^{ab}

a,b,c: Different superscript letters within the same column indicate statistically significant differences *(P < 0.001).*

Table 2. CASA-based kinematic parameters of spermatozoa (Mean ± SD).

Group	n	VAP (µm/sn)	VSL (µm/sn)	VCL (µm/sn)
Control (C)	34	71.92±15.51^{a}	54.07±11.43^{a}	121.52±272.28^{a}
C + Carnosic acid (CR) 1mM	34	51.81±14.4^{b}	40.46±12.11^{b}	83.78±26.6^{b}
C + Carnosic acid (CR) 3 mM	34	45.94±15.8^{b}	34.92±13.06^{b}	73.77±30.42^{b}
C + Glutamine 2.5 mM	34	72.77±13.71^{a}	55.49±8,49^{a}	124.9±29.54^{a}
C + CR 3 mM + Glutamine 2.5	34	45.7±15.36^{b}	35.08±12.12^{b}	78.09±28.14^{b}
C + Glutamine 7.5 mM mM	34	71.72±20.56^{a}	53.99±14.09^{a}	123.39±34.45^{a}
C + CR 1 mM + Glutamine 7.5 mM	34	65.25±14.23^{a}	50.96±9.8^{a}	110.35±24.12^{a}

a,b,c: Different superscript letters within the same column indicate statistically significant differences ($P < 0.05$).

No significant differences were observed among these four groups.

Sperm viability was assessed by evaluating PMAI and MMP, with corresponding data summarized in Table 3. The Control group had the highest PMAI value (38.46 ± 12.97), which was significantly exceeding those of the CR1 (30.02 ± 8.91), CR3 (22.03 ± 8.71), and CR3+G2.5 (23.09 ± 7.60) groups (P < 0.001). However, the G2.5 (36.75 ± 11.86), G7.5 (35.75 ± 12.02), and CR1+G7.5 (32.32 ± 11.49) groups showed values statistically similar to those of the Control, as well as to CR1 (30.02 ± 8.91) (P > 0.05). Among all groups, the CR3 group (22.03 ± 8.71) exhibited the lowest PMAI value, which was significantly lower than those of all other groups except CR3+G2.5 (P < 0.001). No significant differences were found among groups for high mitochondrial membrane potential (P > 0.05).

DNA fragmentation results for the experimental groups are provided in Table 4. The Control group recorded the lowest level of DNA fragmentation (15.4 ± 2.14%) and differed significantly from all other groups (P < 0.001). The highest fragmentation rate was observed in the CR3+G2.5 group (19.31 ± 1.22%), which also showed significant differences from all other groups (P < 0.001). The G2.5 (16.25 ± 1.02%), CR3 (16.55 ± 0.5%), G7.5 (16.64 ± 0.58%), and CR1+G7.5 (16.91 ± 1.18%) groups shared statistically similar DNA fragmentation levels but were significantly different from both the Control and CR3+G2.5 groups (P > 0.05).

Table 3. Spermatozoon viability results (Mean ± SD).

Group	n	PMAI (%)	HMMP (%)
Control (C)	34	38.46±12.97^{a}	27.58±12.51
C + Carnosic acid (CR) 1mM	34	30.02±8.91b^{c}	21.79±11.19
C + Carnosic acid (CR) 3 mM	34	22.03±8.71^{d}	23.82±13.34
C + Glutamine 2.5 mM	34	36.75±11.86^{ab}	27.32±12.62
C + CR 3 mM + Glutamine 2.5	34	23.09±7.6^{cd}	22.03±13.73
C + Glutamine 7.5 mM mM	34	35.75±12.02^{ab}	27.38±11.77
C + CR 1 mM + Glutamine 7.5 mM	34	32.32±11.49^{ab}	22.0±11.78

a,b,c: Different superscript letters within the same column indicate statistically significant differences (P < 0.001).

Table 4. Spermatozoon DNA fragmentation results (Mean ± SD).

Group	n	DNA Damage (%)
Control (C)	34	15.4±2.14^{a}
C + Carnosic acid (CR) 1mM	34	17.13±0.4^{b}
C + Carnosic acid (CR) 3 mM	34	16.55±0.5^{c}
C + Glutamine 2.5 mM	34	16.25±1.02^{c}
C + CR 3 mM + Glutamine 2.5	34	19.31±1.22^{d}
C + Glutamine 7.5 mM mM	34	16.64±0.58^{c}
C + CR 1 mM + Glutamine 7.5 mM	34	16.91±1.18^{bc}

a,b,c: Different superscript letters within the same column indicate statistically

Discussion

Reproductive success in cattle is a multifaceted process influenced by various parameters, including animal care, nutrition, effective herd management, accurate estrus detection, the timing of artificial AI, the quality of frozen-thawed semen, and the expertise of inseminators (Gökçen, 2020). Among these factors, the production of high-quality frozen bull semen is the responsibility of bull stations and is regulated by national legal authorities. Frozen semen must be analyzed in authorized andrology laboratories as required by national legislation before its commercial use (HAYGEM, 2020).

In Türkiye, physical and morphological analyses of both imported and domestically produced frozen bull semen are conducted in laboratories authorized by the Ministry of Agriculture and Forestry for approval before use. According to national regulations, non-

sexed frozen semen must have at least 40% motility, a maximum of 30% abnormal spermatozoa, and a minimum concentration of 5 million motile spermatozoa per straw to be deemed acceptable for use. For sexed semen, the requirements include a minimum of 1 million motile spermatozoa per straw and ≤ 30% abnormal spermatozoa (HAYGEM, 2020). While these spermatological parameters may be adequate for intensive breeding, higher-quality semen may be more suitable for improving reproductive success in small-scale, family-operated farms that lack a structured reproductive management system.

Türkiye has approximately 14 million cattle, and the distribution of farms by herd size is as follows: 6.08% of farms have 200 or more animals, 16.45% have between 50 and 199 animals, and 77.47% have between 1 and 50 animals (Aytekin, 2011; TAGEM, 2019), as shown in Table 5.

Small-scale farms, also classified as family-based operations, are those that maintain between 1 and 50 head of cattle and collectively account for 77.47% of Türkiye's total cattle population. These farms generally operate under semi-intensive systems and are commonly populated with crossbreeds of native and dual-purpose cattle. Rather than aiming for high milk yields, the primary objective of these farms is often to produce animals for beef fattening. Most of these farms rely on services from private veterinarians to meet their herd health and AI needs. Estrus detection in such farms is typically performed through visual observation rather than systematic monitoring. In the absence of structured estrus detection systems, heat observation is usually carried out during morning and evening feeding or milking times, making AI procedures considerably more complicated (Önal and Özder, 2008; Roelofs et al., 2010; Aytekin, 2011; Aksoy and Yavuz, 2012; Pothmann et al., 2014; Tüzemen, 2015; Yener, 2017).

Due to the complex and multifactorial nature of reproductive success in family-type cattle farms, field veterinarians often demand high-quality frozen bull semen with superior motility and viability to be used in insemination procedures. Recently developed biotechnologies, such as microfluidic chip-based sperm separation and slow-release cryopreservation systems, have gained significant attention and support for this trend (Knowlton et al., 2015; Perteghella et al., 2017).

In line with these developments, various antioxidants or compounds with potential antioxidant properties have been added to semen extenders to reduce oxidative stress during both short-term and long-term storage, thereby improving post-thaw semen quality. Among these, glutamine and carnosic acid are commonly used in semen cryopreservation protocols (Arı and Öztürkler, 2015). The addition of 10 mM glutamine to a tris-egg yolk-based extender improved post-thaw sperm motility by approximately 5%, while higher doses (20, 30, 40, 80, 120 mM) adversely affected motility, as reported by (Amirat-Briand et al., 2009).

Similarly, a positive effect of 20 mM glutamine on boar sperm stored at 17°C for five days was also observed in a study by (Wang et al., 2018). Significant improvements in spermatological parameters were reported when 5 mM glutamine was added to a ram semen extender, according to (Bucak et al., 2009b). Furthermore, the addition of 60 mM glutamine was shown to have a beneficial effect on chimpanzee semen cryopreservation (Bottrel et al., 2018). In the present study, the addition of 2.5 mM, 7.5 mM glutamine or CR1+G7.5 to a soybean lecithin-based extender did not significantly affect total or progressive motility compared to the control group. This similarity was also reflected in linear kinematic parameters (VAP, VSL, VCL) and PMAI results. Although a positive correlation between intracellular glutamine concentration and sperm quality has been reported, the optimal exogenous glutamine dose may

Table 5. Number and percentage of cattle farms according to their scale

Farm Animal Capacity	Number of Farms (Units)	(%)	Number of Animals	Distribution by Animal Population (%)
01-09	1.155.958	75.77	4.251.049	31.35
10-49	339.306	22.24	6.252.878	46.12
50-199	28.580	1.87	2.231.149	16.45
≥200	1.757	0.11	823.466	6.08

vary by species and extender type, and remains unclear (Narud et al., 2020). Based on the present findings, neither 2.5 mM nor 7.5 mM glutamine appears to be ideal concentrations for soybean lecithin-based bull semen extenders.

Conversely, groups supplemented with 1 or 3 mM carnosic acid (CR1, CR3), or with 3 mM carnosic acid combined with 2.5 mM glutamine (CR3+G2.5), exhibited lower total and progressive motility, PMAI, and linear kinematic values. Similar adverse effects were observed in ram semen supplemented with 0.2 mM and 0.05 mM carnosic acid in tris-egg yolk extenders (Gungor et al., 2019). While positive impacts of 12.5 and 25 µg/mL carnosic acid on the short-term storage of Anatolian buffalo epididymal sperm at +4°C were reported (Yeni and Avdatek, 2017), another study found that rosmarinic acid containing 25–200 µg/mL of carnosic acid did not significantly improve motility or kinetic parameters in frozen bull semen (Yeni et al., 2018). Thus, the specified concentrations of carnosic acid are considered antagonistic when used in soybean lecithin-based bull semen extenders.

Mitochondrial membrane potential is an indicator of adenosine triphosphate production through oxidative phosphorylation in mitochondria and is associated with capacitation (Korkmaz and Çil, 2020). Due to the motile activity of spermatozoa, superoxide anions are generated within the mitochondria and may leak into the cytoplasm, potentially damaging both the plasma membrane and the DNA. (Aitken et al., 2014).

In this study, no significant differences in MMP were observed among groups. The lowest level of DNA damage was found in the control group; however, DNA damage in other groups did not correlate with other spermatological parameters. This may be explained by the fact that, despite the presence of oxidative stress during cryopreservation, the harmful effects of freezing itself on cells may be more severe than oxidative damage. Additionally, endogenous antioxidant mechanisms in semen may have mitigated oxidative damage during the process (Aitken and Baker, 2004; Arı and Öztürkler, 2015).

Although several studies have investigated the supplementation of semen extenders with various antioxidant compounds, there is no definitive evidence linking oxidative stress-related sperm damage to reduced fertilization or pregnancy rates. This may be due to the difficulty of optimizing experimental conditions in field settings (Arı and Öztürkler, 2015).

In conclusion, the addition of 2.5 mM and 7.5 mM glutamine to soybean lecithin-based extenders for the cryopreservation of Brown Swiss bull semen demonstrated a synergistic interaction; however, it did not result in significant improvements in motility, kinematic parameters, PMAI, HMMP, or DNA integrity compared to the control group. Moreover, the addition of 1 mM and 3 mM carnosic acid is considered to represent antagonistic doses when used in soybean lecithin-based extenders. The observation that some parameters in the CR1+G7.5 group were similar to those in the control may suggest that the antagonist effect of carnosic acid was partially compensated for by the presence of 7.5 mM glutamine.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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