Research Article

Effect of Dietary Supplementation of Nano-Selenium with Vitamin E on Semen Characteristics in Holstein Bulls

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Abstract | This study aimed to investigate the effect of different levels of nano-selenium (SeONPs) and Vitamin E supplementation on semen characteristics in Holstein-Friesian bulls. Twelve Holstein bulls, aged 5-7.5 years and weighing 500-950 kg, were used in this study. They were divided into three groups (n = 4): T1, a basic diet without additives; T2, a basic diet supplemented with 0.1 mg SeONPs and 300 IU Vitamin E/kg DM; and T3, a basic diet supplemented with 0.3 mg SeONPs and 300 IU Vitamin E/kg DM. The treatments were supplemented twice a week for three months. Semen samples were collected one month after the feeding period, which was conducted weekly for 4 weeks during the feeding period and continued for an additional 4 weeks afterward. Samples were diluted with a Tris-egg yolk extender and then evaluated at 37˚C, cooled at 5˚C, and -196 ˚C. The results showed no significant improvement between the treatments during and after feeding, and at the time intervals (37˚C, 5˚C, and -196˚C) in most semen traits like individual motility, percentage of live sperm, total abnormalities, plasma membrane integrity, and acrosome integrity. Additionally, semen plasma analysis showed no significant improvement in the levels of malondialdehyde (MDA), total antioxidant capacity (TAC), or DNA damage. In conclusion, the study indicated no significant effects of nano-selenium and vitamin E on semen traits during and after feeding at 37˚C, 5˚C, and -196˚C. Further studies are recommended to explore the synergistic effects of nano-selenium in combination with other vitamins on improving semen quality traits, particularly through their antioxidant properties.

Keywords | Antioxidants, Holstein bulls, Free radicals, Semen standards, Trace minerals, Vitamins

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INTRODUCTION

The processes of cooling, freezing, and liquefying sperm represent the most practical methods for long-term sperm storage in males (Bresm and Habeeb, 2022). However, these cryopreservation techniques expose sperm to oxidative stress, which can significantly impair their viability, motility, and fertilization capacity (Deng *et al*., 2017). This issue is particularly pronounced in bull sperm, which contains a high proportion of polyunsaturated fatty acids that are especially vulnerable to peroxidation by reactive oxygen species (Bresm and Habeeb, 2023; Habeeb *et al*., 2024). To counteract these detrimental effects, researchers have increasingly focused on the supplementation of antioxidants during cryopreservation. The inclusion of specific vitamins and minerals with antioxidant properties has been shown to play a critical role in protecting sperm from oxidative damage and improving post-thaw sperm quality (Habeeb *et al*., 2025; Zaini, 2012).

Vitamin E is a potent fat-soluble antioxidant known for its ability to protect cell membranes from lipid peroxidation and neutralize free radicals. It has been identified as a key component of the antioxidant defense system in sperma-

tozoa (Surai *et al*., 1998). A deficiency in vitamin E has been linked to increased testicular damage and a reduction in spermatocyte numbers (Yue *et al*., 2010). Several studies have reported that inadequate dietary intake of vitamin E adversely affects sperm quality, including reduced motility, concentration, and ejaculate volume, along with a higher proportion of dead and morphologically abnormal sperm (Losano *et al*., 2018). Conversely, dietary supplementation with vitamin E has been shown to improve semen characteristics by lowering mitochondrial-derived free radical production and mitigating oxidative stress (Yue *et al*., 2010).

Selenium is a trace mineral with a very narrow safety margin between its daily requirement and toxic dose (Khanal and Knight, 2010). Dietary supplementation with selenium at appropriate levels has been shown to enhance the efficiency of the antioxidant system (Shi et al., 2018) and improve semen quality parameters such as sperm motility, concentration, and morphology (Marai *et al*., 2009; Liu *et al*., 1982). However, excessive selenium intake can negatively impact sperm quality by reducing motility and increasing morphological abnormalities (Hawkes and Turek, 2001).

A significant synergistic relationship exists between vitamin E and selenium in protecting cells and tissues from oxidative stress (Bansal and Bilaspuri, 2009). Their combined antioxidant roles are particularly important in the reproductive system, contributing to improved semen characteristics, enhanced spermatogenesis, increased fertility, and the prevention of reproductive disorders (Chand *et al*., 2021).

Recent advances in nanotechnology have introduced nano-selenium as a promising alternative to traditional selenium forms, due to its superior antioxidant potential, higher bioavailability, and reduced toxicity (Hosnedlova *et al*., 2018). When combined with other compounds, nanomaterials such as nano-selenium may exhibit synergistic or antagonistic effects depending on the context (Abo-Shama *et al*., 2020).

To date, no studies have specifically investigated the synergistic effects of nano-selenium and vitamin E on the semen quality of Holstein-Friesian bulls. Therefore, the present study was designed to evaluate the impact of dietary supplementation with varying levels of nano-selenium and vitamin E on the semen characteristics of Holstein bulls.

MATERIALS AND METHODS

Experiment Design

All experimental ethical protocols were approved in accordance with the ethical clearance issued by the Department of Animal Production, Al-Qasim Green University (Approval No. 7486, 2023), Babylon, Iraq. The experiment was conducted at the Artificial Insemination Department, Abu Ghraib, Ministry of Agriculture, from November 13, 2023, to April 13, 2024. Twelve Holstein bulls were previously trained for semen collection by an artificial vagina. Bull ages ranged from 5 to 7.5 years, and their body weights ranged between 500 and 950 kg. The animals were housed in semi-open shelters, under veterinary supervision, and were in good general health, free from disease. Bulls were divided into three groups (n = 4): T1, a basic diet without additives; T2, a basic diet supplemented with 0.1 mg green-synthesized nano-selenium (SeONPs) and 300 IU Vitamin E (Kaesler, Germany) per kg DM; and T3, a basic diet supplemented with 0.3 mg SeONPs and 300 IU Vitamin E/kg DM. The treatments were supplemented twice a week for three months. Semen samples were collected one month after the feeding period, which was conducted weekly for 4 weeks during the feeding period and continued for an additional 4 weeks afterward. Following collection, semen samples were diluted with a Tris-egg yolk extender and then evaluated at 37°C, cooled at 5°C, and stored at -196 °C.

Semen Collection and Evaluation

The semen collection started at 7:00 a.m. by using an artificial vagina, which had a temperature ranging between 41–42°C at the time of collection, one ejaculate/bull/week. To increase sexual desire and prepare for ejaculation, the bulls were allowed to perform a false mount. The artificial vagina was equipped with a graduated glass tube in which the semen sample was collected. This tube was placed inside a special container, with its opening sealed using a thumb to prevent light exposure and maintain temperature, thus avoiding environmental shock. The samples were transferred to the laboratory and placed in a water bath at 37°C until evaluation and dilution. The fresh semen from each sample was then evaluated at 37 °C for volume, concentration, individual motility, percentage of live sperm, total abnormalities, plasma membrane integrity, and acrosome integrity.

Dilution and Cooling of Semen

After preparing the tris extender, it is placed in a water bath at 37°C. The semen is then gradually added to the extender using a graduated pipette. The tubes are then sealed tightly and kept in a water bath. After dilution, the samples are placed in plastic containers at 32°C using a glass thermometer and then transferred to a special refrigerator maintained at 5°C. The gradual decrease in the temperature of the samples is monitored for approximately 1.15 - 1.45 hours until it reaches 5°C. The diluted semen at 5°C is then evaluated for individual motility, percentage of live sperm, total abnormalities, plasma membrane integrity, and acrosome integrity.

Semen Cryopreservation

After the samples were stabilized in the refrigerator at 5°C, they were left for 4 hours to allow the glycerol to equilibrate. The cooled samples were automatically filled into artificial insemination straws (0.25 mL) using a filling machine. Following the filling process, the straws were marked with colors and labels for identification. They were then placed on a unique rack inside a liquid nitrogen tank at -120°C, 5 cm above the liquid nitrogen surface, for 9 minutes. Subsequently, the straws were fully submerged and stored in a liquid nitrogen tank at -196°C. They were later thawed and evaluated after 48 hours for individual motility, percentage of live sperm, total abnormalities, plasma membrane integrity, acrosome integrity, malondialdehyde (MDA), total antioxidant capacity (TAC), and DNA integrity.

Semen Analysis

Individual motility: Individual sperm motility was assessed by placing a drop of semen on a small drop of 2.9% sodium citrate solution on a clean glass slide at 37°C and covering it with a cover slide. The sample was then examined under a light microscope at 40x magnification, and motility was calculated based on the percentage of sperm showing forward progressive movement, according to the method described by Walton (1933).

Percentage of live sperm: The percentage of live sperm was determined by placing a drop of semen on a drop of eosin-nigrosin stain (5% eosin and 10% nigrosin) on a clean glass slide at 37°C. The mixture was then spread on a new slide at a 45° angle and allowed to dry at 37°C. The slide was examined under a microscope at 40x magnification, where live sperm appeared colorless, while dead sperm seemed to be pink due to the uptake of the dye. The percentage of live sperm was calculated by counting 200 sperm from different fields of the slides using the method described by Swanson and Bearden (1951).

Percentage of abnormal sperm: The percentage of abnormal sperm was calculated using Hancock's (1951) method, which involved using the same slide and magnification as for the live sperm. 200 sperm were counted from different microscopic fields. Abnormalities were classified according to the criteria described by Melrose and Laing (1970).

Plasma membrane integrity: The percentage of intact plasma membranes was measured using the method described by Jeyendran *et al.* (1984). A 20 µL semen sample was added to a tube containing 1 mL of hypoosmotic solution (HOST) (8.72 g/L fructose, 4.74 g/L sodium citrate, osmotic pressure 100 mOsm/L, and a pH of 8) in a water bath at 37°C. After 60 minutes, a drop of the sample was placed on a glass slide and smeared. 200 sperm were counted in different fields of view under 40x magnification. Sperm with swollen heads and coiled tails were considered to have intact plasma membranes.

Acrosome integrity: Acrosome integrity was measured according to the method described by Kovács and Foote (1992). A smear was made from the semen sample on a glass slide at 37°C and then allowed to dry. The slide was dipped in fixative solution (10 mL formalin and 90 mL potassium bichromate solution) for 45 minutes, followed by washing with distilled water. The slide was then stained for 1.5 hours with a Giemsa stain solution (9 mL Giemsa solution and 6 mL buffer solution prepared from NaCl, KCl, Na2HPO4, and KH2PO4). After washing with distilled water, the slides were stored in the dark for 48 hours before being examined under an oil immersion microscope at 100x magnification. Sperm with intact acrosomes were distinguished by their blue-violet head color, while damaged acrosomes appeared white. The percentage of sperm with intact acrosomes was calculated by counting 200 sperm from different microscopic fields.

Malondialdehyde (MDA) concentration in seminal plasma: MDA concentration in seminal plasma was estimated using the method described by Kumaresan *et al.* (2005), which involves measuring thiobarbituric acid (TBA) and trichloroacetic acid (TCA) levels. In brief, 150 µL of seminal plasma (after sperm separation by centrifugation at 3000 rpm for 10 minutes) was added to a test tube containing 1 mL of TBA and TCA solutions, then mixed gently. The mixture was incubated in a boiling water bath for 30 minutes and then allowed to cool for 20 minutes. Afterward, 1 mL of TCA solution was added. The mixture was centrifuged again at 3000 rpm for 10 minutes. Absorbance was measured at 532 nm using a spectrophotometer to determine MDA concentration.

Total antioxidant capacity in seminal plasma: Total antioxidant capacity (TAC) in seminal plasma was measured using a kit from Sigma (USA), as described by Brand-Williams *et al.* (1995). This test evaluates the activity of both enzymatic (glutathione peroxidase, superoxide dismutase, catalase, and glutathione reductase) and non-enzymatic (vitamins E and C, beta-carotene) antioxidants. The assay is based on the ability of antioxidants to inhibit or prevent the oxidation of the compound 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) to ABTs+. Absorbance of ABTs+ was measured at 517 nm using a spectrophotometer, and TAC was expressed as the concentration of ascorbic acid.

DNA damage in sperm: DNA damage in sperm was estimated using the Sperm Chromatin Structure Assay (SCSA) technique, as described by Tejada *et al.* (1984). A 0.1 mL semen sample was smeared on a glass slide, allowed to air dry, then fixed for 24 hours. After drying, a staining

solution was applied for 5 minutes in the dark, followed by washing with distilled water. The slides were examined under a fluorescent microscope at 40x magnification, with a wavelength of 490-530 nm. The percentage of sperm with DNA damage was assessed by counting 100 sperm, where green indicated intact DNA and yellow or red indicated damaged DNA.

Statistical Analysis

The Statistical Analysis System (Cary, 2012) software was used to analyze the data, examining the effect of treatment and time on the studied traits, following a Completely Randomized Design (CRD) in a row-column format. However, some traits, such as MDA, TCA, and DNA oxidation (without times), were studied based solely on the effect of treatment. Significant differences between means were compared using Duncan's multiple-range test (1955).

Table 1: Effect of treatment and time on individual sperm motility rate during and after dietary supplementation with green-synthesized nano-selenium and vitamin E.

Feeding	Treatments	Mean ± Standard Error			Level of Significance
		37°C (Fresh)	5°C Cooling	-196°C (Freezing)	
During feeding	T1	1.42± 28.44aA	23.44 ±1.42bA	17.81 ±1.51 cA	**
	T2	29.06± 1.53aA	24.06 ±1.53bA	18.44 ±1.42 cA	**
	T3	28.12± 1.93aA	22.19 ±1.93bA	15.94 ±1.72 cA	**
	Level of Significance	NS	NS	NS	---
After feeding	T1	35.93 ±2.00aA	30.31 ±2.11bA	20.93 ±1.23 cA	**
	T2	29.28 ±0.88aB	23.57 ±0.97bB	16.43 ±1.22 cB	**
	T3	29.37 ±2.08aB	23.75 ±2.06bB	15.63 ±1.64 cB	**
	Level of Significance	*	*	*	---

The means with different capital letters within a column and different lowercase letters within a row are significantly different from each other * (P≤0.05); ** (P≤0.01), NS: not significant.

RESULTS AND DISCUSSION

Individual Motility Rate

In the current study, there were no significant differences in individual motility during feeding for all treatments (Table 1). Regarding the effect of time, the results indicated a highly significant decrease (P ≤ 0.01) in the individual motility in the cooling and freezing periods in T1 (23.44 ± 1.42 and 17.81 ± 1.51), T2 (24.06 ± 1.53 and 18.44 ± 1.42), and T3 (22.19 ± 1.93 and 15.94 ± 1.72) compared to fresh (28.44 ± 1.42, 29.06 ± 1.53, and 28.12 ± 1.93), respectively. After feeding, the individual motility showed a significant decrease (P ≤ 0.05) in T2 and T3 compared to T1. However, there were no significant differences between T2 and T3 (Table 1). Regarding time, the data showed a significant decrease (P ≤ 0.01) in the cooling and freezing periods in T1 (30.31 ± 2.11 and 20.93 ± 1.23), T2 (23.57 ± 0.97 and 16.43 ± 1.22), and T3 (23.75 ± 2.06 and 15.63 ± 1.64) compared to fresh (35.93 ± 2.00, 29.28 ± 0.88, and 29.37 ± 2.08), respectively (Table 1).

The results of this study indicate that the control was more significant compared to both nano-selenium and vitamin E in individual motility. These results align with studies by Butt *et al.* (2019), who did not observe improvements in sperm motility with selenium and vitamin E supplementation. They mentioned that the amount of selenium required to trigger a selenium response in the blood is higher for bulls compared to cows or calves. The lack of effect in our study may be due to the type of animals, as previous studies have indicated that bulls require more selenium for a response. Additionally, the experimental conditions, which also affect animal response, in terms of dosage and treatment periods, may have contributed to these outcomes (Liu *et al.*, 2014). Similarly, Dorostkar *et al.* (2012) showed that doses of 4 and 8 µg/ml of selenium significantly reduced sperm vitality and motility compared to the control group in water buffaloes (*Bubalus bubalis*). In contrast to the previous results, many studies on farm animals have shown positive effects of nano-selenium and vitamin E on individual sperm motility in goats (Shi *et al.*, 2010), sheep (Nateq *et al.*, 2020), pigs (Paul *et al.*, 2024), and bulls (Khalil *et al.*, 2019; Losano *et al.*, 2018). This indicates that the synergistic effect of nano-selenium and vitamin E is breed and dose-dependent.

Live Sperm Percentage

In the current study, there were no significant differences in live sperm percentage during feeding for all treatments (Table 2). Regarding the effect of time, the results indicated a highly significant decrease (P ≤ 0.01) in the live sperm percentage in the cooling and freezing periods in T1 (72.29 ± 0.95 and 65.86 ± 1.1), T2 (73.09 ± 0.98 and 66.26 ± 1.09), and T3 (71.59±1.11 and 64.49±1.29) compared to fresh (77.32 ± 1.06, 77.92 ± 0.97, and 77.03±1.28), respectively (Table 2). After feeding, the live sperm percentage showed a significant decrease (P ≤ 0.05) in T2 and T3 compared to T1 in all periods. However, there were no significant differences between T2 and T3 (Table 2). Regarding time, the data showed a significant decrease (P ≤ 0.01) in the cooling and freezing periods in T1 (75.60±0.72 and 67.93±0.92), T2 (73.48 ± 0.46 and 64.35 ± 0.94), and T3 (72.27 ± 1.01

and 64.34 ± 1.17) compared to fresh (81.61±0.89, 78.64 ± 0.36, and 67.93 ± 0.92), respectively (Table 2).

Table 2: Effect of treatment and time on sperm viability percentage during and after feeding green-synthesized nano-selenium and vitamin E.

Feeding	Treatments	Mean ± Standard Error			Level of Significance
		37°C (Fresh)	5°C Cooling	-196°C (Freezing)	
During feeding	T1	77.32 ±1.06aA	72.29± 0.95bA	65.86±1.1 cA	**
	T2	77.92 ±0.97aA	73.09± 0.98b A	66.26± 1.09cA	**
	T3	77.03± 1.28aA	71.59± 1.11bA	64.49± 1.29cA	**
	Level of Significance	NS	NS	NS	---
After feeding	T1	81.61 ±0.89aA	75.60± 0.72bA	67.93± 0.92cA	**
	T2	78.64 ±0.36aB	73.48± 0.46bBA	64.35± 0.94cB	**
	T3	77.47 ±1.23aB	72.27± 1.01bB	64.34± 1.17cB	**
	Level of Significance	*	*	*	---

The means with different capital letters within a column and different lowercase letters within a row are significantly different from each other * (P≤0.05); ** (P≤0.01), NS: not significant.

From the results, it is clear that neither Vitamin E nor nano-selenium had a significant effect on sperm viability before and during feeding when compared to the control. Despite this, Vitamin E and selenium are essential for the growth of germ cells in the testes during sperm development, and they have positive effects on sperm motility, function, sperm formation, and survival (Moslemi and Tavanbakhsh, 2011). These results are consistent with a study conducted by Piagentini *et al.* (2017), who indicated that selenium supplementation did not affect sperm viability or mortality. These results can be explained by several reasons, including that the doses of Vitamin E and Nano-selenium used may not have been sufficient to achieve noticeable effects on sperm viability, as dosage plays a crucial role in determining biological effects. Additionally, the bulls used in the study may have been under low or moderate oxidative stress, reducing the need for additional effects from these supplements. In contrast, researchers have reported positive effects of both Vitamin E and selenium on increasing sperm viability and motility (Chand *et al.*, 2021; Butt *et al.*, 2019).

Total Abnormalities Percentage

In the current study, there were no significant differences in live sperm percentage during feeding for all treatments (Table 3). Regarding the effect of time, the results indicated a highly significant increase (P ≤ 0.01) in the total abnormalities percentage in the cooling and freezing periods in T1 (13.87±0.34 and 17.82±0.25), T2 (13.46±0.36 and 17.60±0.20), and T3 (14.48±0.52 and 17.83±0.40) compared to fresh (9.69±0.36, 9.82±0.45, and 10.37±0.65), respectively (Table 3). After feeding, the total abnormalities percentage showed a significant increase (P ≤ 0.05) in T3 compared to T1, but not T2 in all cooling and freezing periods (Table 3). Regarding time, the data showed a significant decrease (P ≤ 0.01) in the cooling and freezing periods in T1 (12.40 ± 0.41, 16.94 ± 0.26), T2 (13.75 ± 0.22, 17.93 ± 0.17), and T3 (13.93 ± 0.48, 17.96 ± 0.31) compared to fresh (8.32 ± 0.39, 9.19 ± 0.23, and 9.84 ± 0.52), respectively (Table 3).

Table 3: Effect of treatment and time on the percentage of total sperm abnormalities during and after feeding green-synthesized nano-selenium and vitamin E.

Feeding	Treatments	Mean ± Standard Error			Level of Significance
		37°C (Fresh)	5°C Cooling	-196°C (Freezing)	
During feeding	T1	9.69± 0.36aA	13.87± 0.34bA	17.82± 0.25cA	**
	T2	9.82± 0.45aA	13.46± 0.36bA	17.60± 0.20cA	**
	T3	10.37± 0.65aA	14.48± 0.52bA	17.83± 0.40cA	**
	Level of Significance	NS	NS	NS	---
After feeding	T1	8.32± 0.39aA	12.40± 0.41bA	16.94± 0.26cA	**
	T2	9.19± 0.23aAB	13.75± 0.22bB	17.93± 0.17cB	**
	T3	9.84± 0.52aB	13.93± 0.48bB	17.96± 0.31cB	**
	Level of Significance	*	*	*	---

The means with different capital letters within a column and different lowercase letters within a row are significantly different from each other * (P≤0.05); ** (P≤0.01), NS: not significant.

From the above, it is evident that supplementation with either nano-selenium at 0.1 or 0.3 mg or Vitamin E at 300 IU/kg of dry matter may cause an increase in total abnormalities percentage compared to the control group. Domosławska *et al.* (2015) found that using higher doses of Vitamin E and selenium could lead to increased sperm damage, even though both are beneficial dietary supplements for semen quality. Hawkes and Turek (2001) found that any increase in selenium could reduce sperm motility and increase abnormalities, and that very high doses of

Vitamin E (up to 20 times the natural dose) could have harmful effects on sperm.

Although Vitamin E and selenium are essential nutrients and key components of antioxidant systems responsible for tissue and cell defense, selenium, along with Vitamin E, helps protect cells as a biological antioxidant (Zubair *et al.*, 2015). These results suggest that high doses may induce oxidative stress by increasing antioxidants above the naturally required levels, resulting in an imbalance between oxidation and antioxidants in the semen, which can lead to an increase in abnormalities and deterioration of sperm quality. According to Schulte *et al.* (2010), a certain level of programmed cell death is necessary to prevent overproduction of sperm and remove damaged sperm that negatively affects semen quality. Dead sperm cells are primarily removed by Sertoli cells, and failure in this mechanism can lead to an increased number of abnormal sperm in the semen. Based on our results, we can infer that the doses of nano somal selenium and Vitamin E were insufficient to maintain higher selenium concentrations in the blood for an extended period, thereby increasing the total percentage of abnormalities.

In contrast, a study conducted by Khalil *et al.* (2019) who reported a decrease in the total abnormalities percentage in sperm supplemented with Vitamin E and nano -selenium, attributing the role of Vitamin E in protecting the plasma membrane from peroxidized unsaturated fatty acids and the role of selenium in reducing abnormalities in sperm tails, maintaining sperm straightness, motility, and metabolism (Zubair, 2017).

Plasma Membrane Integrity

The results of the current study, presented in Table 4, show no significant differences in the plasma membrane integrity of sperm during feeding for all treatments (T1, T2, and T3). However, with respect to the effect of time within a single treatment, the study revealed highly significant differences (P ≤ 0.01) in plasma membrane integrity across treatments T1, T2, and T3. Plasma membrane integrity decreased significantly after both cooling and freezing compared to the 37°C (Fresh) period. In treatment T1, plasma membrane integrity significantly decreased (P ≤ 0.01) after cooling and freezing (70.73 ± 0.97 and 66.71 ± 0.84) compared to the 37°C (Fresh) period (75.54 ± 1.21). In treatment T2, plasma membrane integrity significantly decreased (P ≤ 0.01) after cooling and freezing (71.72 ± 1.09 and 66.38 ± 0.67) compared to the 37°C (Fresh) period (76.03 ± 0.96). In treatment T3, plasma membrane integrity significantly decreased (P ≤ 0.01) after cooling and freezing (69.77 ± 1.06 and 60.51 ± 4.05) compared to the 37°C (Fresh) period (74.82 ± 1.18). Regarding plasma membrane integrity after feeding, the results showed significant differences (P≤0.05) across all treatments. Treatments T2 and T3 exhibited a significant decrease (P ≤ 0.05) in plasma membrane integrity (77.00 ± 0.36 and 75.80 ± 1.23, respectively) compared to the control treatment T1 (79.76 ± 0.78) at 37°C (Fresh). After cooling, treatments T2 and T3 showed a significant decrease (P ≤ 0.05) in plasma membrane integrity (71.73 ± 0.55 and 70.91 ± 1.04, respectively) compared to the control treatment T1, which recorded 74.32 ± 0.8. After freezing, plasma membrane integrity significantly decreased (P ≤ 0.05) for treatments T2 and T3 (65.43 ± 0.86 and 64.97 ± 1.01, respectively) compared to treatment T1, which recorded 68.50 ± 0.70 (Table 4).

Table 4: Effect of treatment and time on plasma membrane integrity during and after feeding green-synthesized nano-selenium and vitamin E.

Feeding	Treatments	Mean ± Standard Error			Level of Significance
		37°C (Fresh)	5°C (Cooling)	-196°C (Freezing)	
During feeding	T1	75.54± 1.21aA	70.73± 0.97bA	66.71± 0.84cA	**
	T2	76.03± 0.96aA	71.72± 1.09bA	66.38± 0.67cA	**
	T3	74.82± 1.18aA	69.77± 1.06aA	60.51± 4.05bA	**
	Level of Significance	NS	NS	NS	---
After feeding	T1	79.76± 0.78aA	74.32± 0.8b A	68.50± 0.70cA	**
	T2	77.00± 0.36aB	71.73± 0.55bB	65.43± 0.86cB	**
	T3	75.80± 1.23aB	70.91± 1.04bB	64.97± 1.01cB	**
	Level of Significance	*	*	*	---

The means with different capital letters within a column and different lowercase letters within a row are significantly different from each other * (P≤0.05); ** (P≤0.01), NS: not significant.

The current results indicate that both nano-selenium and Vitamin E caused a decrease in plasma membrane integrity compared to the control group. This finding contrasts with several previous studies (Hozyen *et al.*, 2019; El-Hawary *et al.*, 2018; Rezaeian *et al.*, 2016; Dorostkar *et al.*, 2012), which showed an improvement in plasma membrane integrity when using nano-selenium and Vitamin E. This discrepancy may be due to several factors, including differences in the doses used, the application method, or the type of samples studied. For example, the interaction between nano-selenium and Vitamin E at the doses used in the current study may have caused unexpected effects on

plasma membrane integrity due to excessive stimulation or inappropriate inhibition of certain biochemical processes. Additionally, differences in sample types or environmental conditions surrounding the study may have contributed to these results, suggesting the need for further investigation to understand the underlying mechanisms behind the effects of these substances. Furthermore, the timing or duration of exposure to the studied factors may have been a significant factor influencing these results. Previous studies used different experimental setups or focused on specific biological contexts, making direct comparisons more complex. Regarding the time factor, the results showed higher plasma membrane integrity after the 37°C (Fresh) period compared to the post-cooling and post-freezing periods, as both cooling and freezing processes increase oxidative stress on the plasma membrane, leading to higher levels of reactive oxygen species (ROS) produced by lipid peroxidation, resulting in sperm damage. This is because sperm plasma membranes are rich in polyunsaturated fatty acids (Bucak *et al.*, 2010). Chatterjee *et al.* (2001) also explained that significant metabolic changes occur during the freezing period due to increased ROS production and lipid peroxidation, which reduce sperm vitality, motility, and mitochondrial membrane integrity.

Acrosome Integrity

The results of the statistical analysis, presented in Table 5, showed no significant effect on acrosomal integrity between the treatments during feeding. However, the effect of time revealed highly significant differences (P≤0.01) within each treatment. In treatment T1, acrosomal integrity significantly decreased (P ≤ 0.01) in the post-cooling and post-freezing periods compared to the 37°C (Fresh) period (74.01 ± 0.95, 68.38 ± 0.94, and 79.06 ± 1.07, respectively). For the second treatment, T2, there was a significant decrease (P ≤ 0.01) in acrosomal integrity during the post-cooling and post-freezing periods (74.82 ± 0.99, 68.57 ± 0.88) compared to the 37°C (Fresh) period (79.69 ± 0.98). In treatment T3, acrosomal integrity also significantly decreased (P ≤ 0.01) during the post-cooling and post-freezing periods (73.20 ± 1.18, 67.08 ± 1.19, and 78.55 ± 1.24, respectively). Regarding acrosomal integrity after feeding, the results showed significant differences (P ≤ 0.05) in acrosomal integrity across all treatments. During the 37°C (Fresh) period, treatments T2 and T3 exhibited significant reductions (P ≤ 0.05) in acrosomal integrity, with values of 80.44 ± 0.41 and 78.88 ± 1.21, respectively. This was compared to the control group, T1, which recorded a value of 83.14 ± 0.84. No significant difference was observed between these treatments. In the post-cooling period, treatments T2 and T3 also showed a significant reduction (P ≤ 0.05) in acrosomal integrity (75.16 ± 0.40 and 74.11 ± 1.06, respectively) compared to the control group, T1, which recorded 77.64 ± 0.62. During the post-freezing period, acrosomal integrity significantly decreased (P ≤ 0.05) in both treatments, T2 and T3 (67.02 ± 0.87, 70.65 ± 0.95, respectively), compared to the control group, T1, which recorded 70.65 ± 0.95 (Table 5).

Table 5: Effect of treatment and time on acrosomal integrity during and after feeding green-synthesized nano-selenium and vitamin E.

Feeding	Treatments	Mean ± Standard Error			Level of Significance
		37°C (Fresh)	5°C (Cooling)	-196°C (Freezing)	
During feeding	T1	79.06± 1.07aA	74.01± 0.95bA	68.38± 0.94c A	**
	T2	79.69± 0.98aA	74.82± 0.99bA	68.57± 0.88cA	**
	T3	78.55± 1.24aA	73.20± 1.18bA	67.08± 1.19cA	**
	Level of Significance	NS	NS	NS	---
After feeding	T1	83.14± 0.84aA	77.64± 0.62bA	70.65± 0.95cA	**
	T2	80.44± 0.41aB	75.16± 0.40bB	67.02± 0.87cB	**
	T3	78.88± 1.21aB	74.11± 1.06bB	66.37± 1.08cB	**
	Level of Significance	*	*	*	---

The means with different capital letters within a column and different lowercase letters within a row are significantly different from each other * (P≤0.05); ** (P≤0.01), NS: not significant.

The acrosome reaction is a physiological process that sperm must undergo in order to successfully fertilize the egg (Atta *et al.*, 2017). However, the results of this study showed a decrease in acrosomal integrity after feeding with nano-selenium and vitamin E. These results are consistent with the study by Beheshti *et al.* (2011), which found no significant differences in acrosomal integrity between the vitamin E group and the control group for Azerbaijani buffalo bulls. The researchers attributed this to the differences in the types of sperm diluents used and interspecies variations. These results are also supported by the study of Chand *et al.* (2021), which was conducted on bulls and found no significant differences in acrosomal integrity between the group of bulls supplemented with vitamin E (50 mg) and selenium (15 mg as sodium selenite) and the control group. The reason for these results may be that the doses used or the nature of the interaction between nano-selenium and vitamin E were not sufficient to achieve a significant improvement in acrosomal integrity. In contrast, several studies have shown a significant improvement in acrosomal integrity when both nano-selenium and vitamin E were added (Hozyen *et al.*, 2019; Li *et al.*, 2023).

Malondialdehyde, Total Antioxidant Capacity and DNA Integrity

In the current study, the results showed a significant difference (P≤0.05) in the malondialdehyde (MDA), total antioxidants (TAC), and DNA integrity in seminal plasma. The MDA level was significantly (P ≤ 0.05) increased in T2 (70.47 ± 4.71) compared to T3 (55.49 ± 4.73) but it did not differ significantly from T1 (65.94 ± 9.18) (Table 6). In addition, regarding the total antioxidant capacity, the results showed a significant decrease (P ≤ 0.05) in the total antioxidant level in T2 compared to the T1, but not different from T3 (0.0049 ± 0.0001, 0.0060 ± 0.0002, and 0.0056 ± 0.0002), respectively (Table 6). Furthermore, DNA damage, as presented in Table 6, did not differ significantly among the treatment groups.

Table 6: Effect of treatment on malondialdehyde (MDA), total antioxidant capacity (TAC), and DNA damage.

Treatments	Mean ± Standard Error		
	MDA	TAC	DNA
T1	65.94 ±9.18A	0.0060 ±0.0002A	90.71 ±0.75
T2	70.47 ±4.71B	0.0049 ±0.0001B	90.20 ±0.91
T3	55.49 ±4.73B	0.0056 ±0.0002A	91.33 ±0.41
Level of Significance	*	*	NS

Means with different letters within the same column are significantly different from each other * (P≤0.05); NS: not significant.

The current results showed that T3 recorded a higher reduction in MDA than T2. MDA is a final product of lipid peroxidation, resulting from the breakdown of membrane phospholipids that occurs when reactive oxygen species oxidize unsaturated fats in cell membranes. MDA is widely used as an indicator of lipid peroxidation in various cell types, including sperm (Gawe *et al*., 2004). Sperm from ruminants are sensitive to ROS damage due to the relatively high content of unsaturated fatty acids in the phospholipids of sperm membranes (Bresm and Habeeb, 2023). Any increase in MDA is a sign of elevated lipid peroxidation, which negatively impacts sperm motility and fertility (Castiglioni *et al*., 2021). The current results align with several studies (Hozyen *et al*., 2019; Khalil *et al*., 2019; Safa *et al*., 2016). This is attributed to the synergistic role of Vitamin E and nano-selenium in protecting cells or organelles from the harmful effects of lipid oxidation. Vitamin E is present in various cell membranes and prevents oxidation, protecting polyunsaturated fatty acids from oxidative damage caused by free radicals (Shastak *et al*., 2023). The hydroxyl group on the chromanol ring in Vitamin E is responsible for donating hydrogen atoms to peroxyl lipid radicals, forming less reactive species and thereby preventing lipid peroxidation chain reactions (Liao *et al*., 2022). Meanwhile, selenium performs its function in the cell cytoplasm by breaking down peroxides. It activates the enzyme glutathione peroxidase, which is one of the strongest natural antioxidants against lipid peroxides. This enzyme plays a central role in cellular defense mechanisms against reactive oxygen species by reducing hydrogen peroxide (H2O2) and lipid hydroperoxides (LOOH) to their non-radical forms (H2O and LOH), simultaneously converting GSH to its active reduced form (Beytut *et al*., 2018). These results suggest that combining Vitamin E and nano-selenium at a concentration of 0.3 mg effectively protects sperm from oxidative stress, improving their efficiency and reducing the likelihood of infertility. This highlights the importance of this combination in therapeutic applications to improve fertility in bulls.

The observed decrease in total antioxidant capacity in T2 compared to T1 may be attributed to the dosage levels or timing of nano-selenium and vitamin E supplementation not aligning with the physiological requirements of the bulls. This mismatch could have reduced the efficacy of the supplements, thereby diminishing their antioxidant effects. Additionally, the supplements may have interacted with other trace minerals, such as zinc or copper, potentially disrupting the natural antioxidant balance and leading to unintended oxidative stress in the bulls. Conversely, the positive effect of selenium and Vitamin E supplementation on antioxidant levels was demonstrated previously. Vitamin E acts as an antioxidant, preventing membrane damage caused by free radicals (Yue *et al*., 2010). Ratnani *et al*. (2020) demonstrated that the protective effects of Vitamin E against oxidative damage to bull sperm cells are more evident under cryopreservation conditions. Additionally, Bansal and Bilaspuri (2009) showed that Vitamin E enhances sperm vitality and reduces lipid peroxidation when exposed to oxidative stressors. However, the bulls used in this study were apparently healthy and not exposed to oxidative stress, which may explain the adverse effects of Vitamin E and selenium. Regarding DNA damage, no significant differences were observed between the study groups. These results align with those found by Chuang ChengHung et al. (2005) but differ from those of Paul *et al*. (2024).

CONCLUSIONS AND RECOMMENDATIONS

This study is the first to investigate the effects of dietary supplementation with green-synthesized nano-selenium and vitamin E on semen characteristics in Holstein-Friesian artificial insemination bulls. The results revealed no significant effects of nano-selenium and vitamin E supplementation on semen traits assessed at various stages immediately after collection (37°C), after cooling (5°C),

and post-thawing following cryopreservation (-196°C). Based on these findings, we recommend further research to explore the potential synergistic interactions between nano-selenium and other vitamins, and to evaluate their antioxidant efficacy in enhancing semen quality.

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NOVELTY STATEMENT

This study represents the first evaluation of the impact of dietary supplementation with green-synthesized nano-selenium and vitamin E on semen characteristics in Holstein-Friesian bulls used for artificial insemination.

AUTHOR'S CONTRIBUTIONS

Malik Hamad Saad Al-Bukhati: Conceptualization, investigation, supervision, and validation.
Hayder Mohammed Hassan Habeeb: Formal analysis, methodology, investigation, writing – original draft.

Conflict of Interest

None.

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