Effect of caffeic acid phenyl ester (CAPE) on spermatological parameters in short-term storage of ram semen

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Abstract

This study was conducted to investigate the effects of caffeic acid phenyl ester (CAPE), one of the biologically active components of bee propolis, on spermatological parameters during short-term storage (up to 72 hours at +4 °C) of ram semen. Ejaculates from five Merino rams were mixed and divided into four groups: control, 10 µg/ml, 50 µg/ml, and 100 µg/ml CAPE treatment groups. Sperm samples were evaluated for motility, abnormal spermatozoon rate, and plasma membrane integrity (HOS test) at 0, 24, 48, and 72 hours. The results revealed that CAPE contributed to the preservation of semen quality, especially at the 100 µg/ml dose. According to the study findings, while no significant differences were found between the groups in terms of motility and abnormal spermatozoon rates at 0, 24, and 48 hours, increases in plasma membrane integrity (HOS test), particularly in the 100 µg/ml group, were found to be statistically significant. At 72 hours, the significant increases in motility values and decreases in abnormal spermatozoon rates in the 10 and 100 µg/ml groups indicate that CAPE supports sperm cell membrane stabilization during short-term storage. The lack of any studies using CAPE for short-term storage of ram semen makes this research a valuable contribution to the field. Evaluating CAPE at different doses, in relation to longer storage times and fertilization rates, will contribute to a better understanding of its potential applications in reproductive biotechnology.

Keywords: *CAPE, ram, short-term storage, sperm*

INTRODUCTION

Artificial insemination is the most important assisted reproductive technique performed by transferring fresh, diluted, chilled, or frozen-thawed semen collected from the male animal into the female reproductive tract. Artificial insemination is a useful technique for genetic improvement and reproductive management (Alvares et al., 2015). By applying short- or long-term semen storage techniques, it is possible to utilize genetically superior males more extensively in reproduction programs (Faigl et al., 2012). The detrimental effects of cryopreservation on ram spermatozoon morphology and function, combined with the high cost of cervical artificial insemination, make chilled short-term storage an effective option for semen preservation and cervical artificial insemination. Compared to frozen-thawed semen, chilled short-term stored semen possesses advantages such as ease of use, higher fertilization results, and cost-effectiveness (Yang et al., 2018). Therefore, performing artificial insemination with chilled short-term stored semen is a good alternative to frozen semen (Gibbons et al., 2019; Maxwell & Watson, 1996; Salamon & Maxwell, 2000).

Recently, there have been published studies regarding the chilled short-term storage of mammalian semen (Acharya et al., 2019; Dai et al., 2019; Falchi et al., 2018). Reducing the temperature of mammalian semen (5°C) extends the survival time of the spermatozoon by causing a decrease in cellular metabolism and reactive oxygen species (Vishwanath & Shannon, 2000). Furthermore, hypothermia destabilizes the activity of the sodium-potassium pump by causing intracellular sodium levels to rise to cytotoxic levels. Consequently, this leads to membrane damage (Murphy et al., 2016; Vishwanath & Shannon, 2000;). In particular, the high proportion of unsaturated fatty acids found in the plasma membrane and limited antioxidant defense mechanisms make spermatozoa vulnerable to oxidative stress (Agarwal et al., 2021). Therefore, the accumulation of reactive oxygen species (ROS) under short-term storage conditions leads to negative effects on motility, membrane integrity, acrosome structure, and DNA damage (Li et al., 2023).

During dilution, cooling, storage, and freezing-thawing processes, semen extenders protect spermatozoa metabolism in many ways, including stabilizing the plasma membrane and maintaining intracellular ion concentrations, thereby reducing cold shock damage and osmotic pressure shock (Bustani & Baiee, 2021; Gungor et al., 2018; Holt, 2000; Rizkallah et al., 2022). Various additives are added to semen extenders. In particular, antioxidants play a critical role in protecting semen from oxidative stress-induced damage. Although many antioxidants have been reported to exert beneficial effects in various animal species, their effectiveness differs among species, is highly dose-dependent, and may even lead to toxic effects under certain conditions (Agarwal & Majzoub, 2017; Amidi et al., 2016). Therefore, the use of more effective and safe new antioxidants has become an interesting research area in reproductive biotechnology.

Caffeic acid phenethyl ester (CAPE), is a hydroxyl derivative of cinnamic acid. CAPE is a diphenolic com-

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pound with the molecular formula C17H16O4 and a molecular weight of 284.3. The full chemical name of CAPE is (E)-3-(3,4-dihydroxyphenyl)-2-propionic acid, 2-phenylethyl 3-(3,4-dihydroxyphenyl)-2-propenoate. CAPE is a white, fine crystalline powder that is insoluble in water but freely soluble in acetone, dimethyl sulfoxide, ethanol, and methanol (Gündoğan et al., 2021). In addition to its anti-inflammatory, immunomodulatory, and anticancer properties, CAPE's strong antioxidant capacity also draws attention (Pérez et al., 2023). CAPE is effective in scavenging free radicals, inhibiting lipid peroxidation, and preserving mitochondrial functions (Balaha et al., 2021). In recent years, studies on the use of CAPE in semen storage processes have revealed promising results. In particular, in a study by Gündoğan et al., (2021) it was observed that among different doses of CAPE added to ram semen during cryopreservation, the 100 µg/ml group provided the best protection in terms of spermatozoon motility, abnormal spermatozoon rate, and HOS test rate compared to the control group and other groups. Additionally, Lan et al., (2022) reported that CAPE supplementation during the short-term storage of boar semen at 17 °C protected sperm motility, plasma membrane, and acrosome integrity, while also reducing lipid peroxidation by increasing total antioxidant capacity. The main objective of this research is to determine the effects of CAPE added to ram semen extender on spermatological characteristics during short-term storage. The lack of sufficient studies on this subject in the existing literature demonstrates the originality and importance of our research.

MATERIALS AND METHODS

Animal material

This study was approved by the Afyon Kocatepe University Animal Research Local Ethics Committee (Approval number and date: 35-18, 13.03.2018, AKÜHADYEK). Five Merino rams between 2 and 3 years of age were used in the study. The semen collection process was performed using the artificial vagina method. During semen collection, ewes determined to be in estrus were used as teasers for the rams to mount; when females in estrus were not available, non-estrous ewes were preferred. In this manner, semen was collected from the rams regularly twice a week during the non-breeding season. The ejaculates in each semen sample collected from the rams were mixed, and this procedure was replicated six times. Semen samples from each ram were first collected in separate tubes, then combined into a single tube, and a total of 60 semen samples were subjected to spermatological examination. Subsequently, the obtained pooled ejaculates were divided into four groups of equal volume. A Tris-based extender containing 15% egg yolk was used for the dilution process. One group was designated as the control, while the other three groups were formed by adding CAPE at different concentrations (10, 50, and 100 µg/ml). The highest concentration group of CAPE (100 µg/ml) was prepared by dissolving it in 1 ml of DMSO (Merck, 99%) to prepare the stock, from which the other groups were prepared. The diluted semen groups were evaluated in terms of spermatological parameters at 0, 24, 48, and 72 hours.

Spermatozoa motility

To determine sperm motility, 10 µl of semen from the sample groups was placed on a slide situated on a heating stage adjusted to 37°C, and a coverslip was placed over it. After locating the image using the 100x objective of the microscope (Olympus CX31), the sample was examined at 400x magnification. Motility values were determined as a percentage by counting spermatozoa exhibiting strong linear movement in any direction in three different fields under a phase-contrast microscope (Avdatek et al., 2023).

Abnormal spermatozoa rate

Abnormal spermatozoa rates in the semen samples were analyzed using Giemsa staining (ADR Group Cat No: 129024UBB). Four hundred (400) spermatozoa were counted under a phase-contrast microscope; structures not conforming to normal spermatozoon morphology were accepted as abnormal. Head, mid-piece, tail, and total anomalies of the spermatozoa were evaluated separately, and the results were recorded as percentages (Güngör et al., 2024).

Spermatozoa membrane integrity

The hypo-osmotic swelling test (HOST) was applied to determine membrane integrity in the semen samples. For this purpose, 1 mL of HOST solution (8.7 g fructose and 4.9 g sodium citrate dissolved in 1000 ml of bi-distilled water; 100 mOsm), equilibrated in a water bath at 37°C, and a 10 µL semen sample were placed into Eppendorf tubes. The mixture was then incubated at 37°C for 30 minutes. At the end of this period, 400 spermatozoa were examined under a phase-contrast microscope (Olympus CX31). Spermatozoa with preserved membrane integrity, identified by swollen (coiled) tails, were counted and the results were recorded (Gündoğan et al., 2021).

RESULTS

The mean spermatological parameter values obtained at 0 hours are presented in Table 1. Statistical analysis showed no significant differences among the experimental groups with respect to motility, head, mid-piece, tail,

Table 1. *Average of 0th hour spermatozoa parameters (± SEM, n:60)*

Group	Motility(%)	Head (%)	Mid-piece (%)	Tail (%)	Total (%)	HOST (%)
Control	83.33 ± 2.11	1.00 ± 0.13	0.67 ± 0.21	8.75 ± 0.77	10.42 ± 0.70	57.33 ± 0.56^{bc}
10 µg/ml	81.67 ± 1.67	2.58 ± 0.95	1.58 ± 0.55	10.75 ± 1.30	14.92 ± 2.38	59.00 ± 0.58^{b}
50 µg/ml	84.17 ± 1.54	2.08 ± 0.52	1.08 ± 0.27	8.75 ± 0.79	11.92 ± 0.66	57.00 ± 0.58^{c}
100 µg/ml	83.33 ± 2.11	1.58 ± 0.33	1.17 ± 0.42	8.67 ± 0.67	11.42 ± 0.77	61.33 ± 0.76^{a}
p	**0.815**	**0.269**	**0.441**	**0.327**	**0.134**	**0.000***

a-c: Differences between values with different letters in the same column are statistically significant (P<0.05).

or total abnormal spermatozoon rates at this time point ($p > 0.05$). However, evaluation of plasma membrane integrity using the hypo-osmotic swelling test (HOST) revealed a statistically significant increase in the 100 µg/ml group compared to the control group ($p < 0.05$).

The spermatological parameters assessed at 24 hours are summarized in Table 2. No statistically significant differences were observed among the groups in terms of motility, head, mid-piece, tail, or total abnormal spermatozoon rates at 24 hours ($p > 0.05$). In contrast, HOST results demonstrated a significant increase in the highest dose group (100 µg/ml) compared to both the control and the other experimental groups at this time point ($p < 0.05$).

The findings obtained at 48 hours are presented in Table 3. At this stage, no significant differences were detected among the groups regarding motility or abnormal spermatozoon rates ($p > 0.05$). Nevertheless, HOST evaluation indicated that the 100 µg/ml group exhibited a statistically significant increase in plasma membrane integrity compared to the control group at 48 hours ($p < 0.05$).

The spermatological parameter values recorded at 72 hours are shown in Table 4. At this time point, motility was significantly higher in the 10 µg/ml and 100 µg/ml groups compared to the control group, whereas head, tail, and total abnormal spermatozoon rates were significantly lower in these groups ($p < 0.05$). Furthermore, HOST results revealed a statistically significant increase in the 50 µg/ml and 100 µg/ml groups compared to the control group ($p < 0.05$). The group × time interaction effects for the evaluated spermatological parameters are illustrated in Figure 1.

DISCUSSION

In this research, the effects of adding different doses of CAPE, a biologically active component of honeybee propolis, to the extender during the short-term storage (+4°C, up to 72 hours) of ram semen on spermatological parameters were evaluated. The findings obtained in the study revealed that CAPE contributed to the preservation of semen quality, particularly when applied at a dose of 100 µg/ml. According to the study findings, although no significant difference was detected among the groups regarding motility and abnormal spermatozoon rates at hours 0, 24, and 48, increases in plasma membrane integrity (HOST), particularly in the 100 µg/ml group, were found to be statistically significant. At hour 72, the significant increase in motility values and the decrease in abnormal spermatozoon rates in the 10 and 100 µg/ml groups indicate that CAPE supports the stabilization of the spermatozoon cell membrane during the short-term storage process.

In our research, the fact that the group supplemented with 100 µg/ml CAPE consistently exhibited statistically higher values in HOST results compared to the control group throughout the storage period highlights the rapid and lasting protective effect of CAPE on spermatozoon membrane integrity. Cold shock and storage duration lead to lipid peroxidation (LPO) of unsaturated fatty acids in the cell membrane, negatively affecting spermato-

Table 2. *Average of 24th hour spermatozoa parameters (± SEM. n:60)*

Group	Motility(%)	Head (%)	Mid-piece (%)	Tail (%)	Total (%)	HOST (%)
Control	76.67 ± 1.05	2.17 ± 0.33	1.58 ± 0.20ab	10.42 ± 0.78	14.17 ± 0.75	41.33 ± 1.41b
10 µg/ml	79.17 ± 1.54	2.00 ± 0.29	1.42 ± 0.20ab	11.67 ± 0.56	15.08 ± 0.61	42.33 ± 0.88b
50 µg/ml	80.83 ± 1.54	2.08 ± 0.20	1.00 ± 0.29b	12.08 ± 0.66	15.17 ± 0.78	41.50 ± 0.76b
100 µg/ml	80.00 ± 2.24	1.33 ± 0.25	1.83 ± 0.28a	11.00 ± 0.63	14.17 ± 0.59	45.83 ± 0.95a
p	**0.336**	**0.152**	**0.142**	**0.326**	**0.592**	**0.019***

a-b: Differences between values with different letters in the same column are statistically significant (P<0.05).

Table 3. *Average of 48th hour spermatozoa parameters (± SEM. n:60)*

Group	Motility(%)	Head (%)	Mid-piece (%)	Tail (%)	Total (%)	HOST (%)
Control	69.17 ± 0.83	2.75 ± 0.44	2.92 ± 0.27^{ab}	12.50 ± 1.06	18.17 ± 1.47	34.50 ± 0.76^{b}
10 µg/ml	70.83 ± 0.83	2.17 ± 0.80	1.67 ± 0.49^{b}	13.00 ± 1.51	16.83 ± 2.11	36.50 ± 0.76^{ab}
50 µg/ml	69.17 ± 1.54	2.17 ± 0.17	4.08 ± 1.24^{a}	13.25 ± 0.62	19.50 ± 1.81	35.67 ± 0.76^{b}
100 µg/ml	73.33 ± 2.47	1.92 ± 0.37	2.83 ± 0.60^{ab}	11.33 ± 0.42	16.58 ± 1.28	38.67 ± 0.76^{a}
p	**0.227**	**0.689**	**0.188**	**0.543**	**0.604**	**0.007***

a-b: Differences between values with different letters in the same column are statistically significant (P<0.05).

Table 4. *Average of 72th hour spermatozoa parameters (± SEM. n:60)*

Group	Motility(%)	Head (%)	Mid-piece (%)	Tail (%)	Total (%)	HOST (%)
Control	58.33 ± 1.05^{c}	4.83 ± 0.21^{a}	4.42 ± 0.20	18.33 ± 0.49^{b}	27.75 ± 0.75^{ab}	24.33 ± 1.05^{b}
10 µg/ml	63.33 ± 1.67^{ab}	3.42 ± 0.20^{b}	4.00 ± 0.77	17.83 ± 0.87^{b}	25.42 ± 1.65^{b}	27.00 ± 1.29^{ab}
50 µg/ml	59.17 ± 2.01^{bc}	3.83 ± 0.56^{ab}	3.75 ± 0.36	21.67 ± 0.79^{a}	29.25 ± 1.09^{a}	29.33 ± 1.45^{a}
100 µg/ml	66.67 ± 1.05^{a}	3.17 ± 0.31^{b}	3.83 ± 0.40	18.17 ± 0.48^{b}	25.17 ± 0.65^{b}	31.00 ± 1.39^{a}
p	**0.003***	**0.016***	**0.770**	**0.002***	**0.049***	**0.010***

a-c: Differences between values with different letters in the same column are statistically significant (P<0.05).

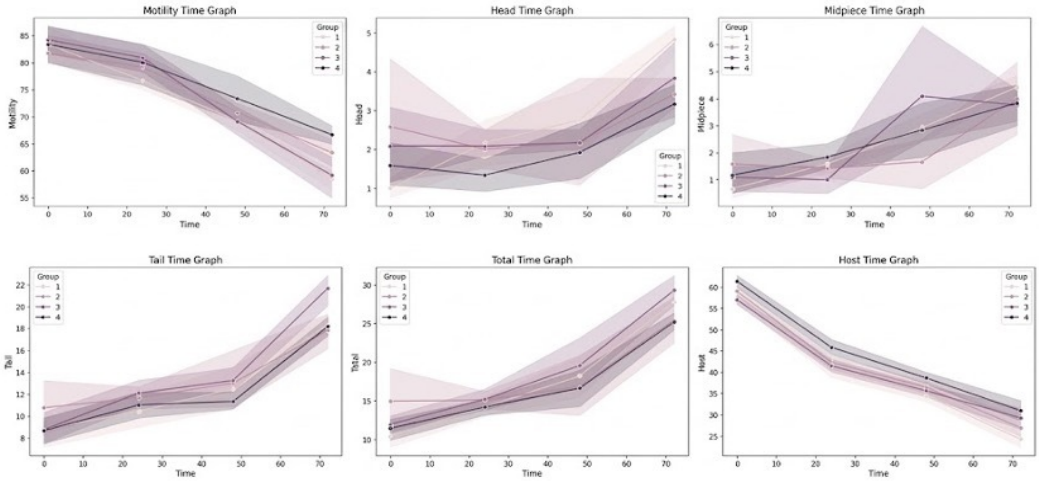

Figure 1. *Group x time change graphs of spermatological parameters*

zoon viability and function. The phenyl ester structure and strong antioxidant property of CAPE suggest that the marked improvement in plasma membrane integrity observed in our study is due to CAPE preserving membrane stability by preventing lipid peroxidation. The most significant effects of CAPE on spermatological parameters emerged at the 72nd hour, when stress reached its maximum level. The fact that 10 µg/ml and 100 µg/ml CAPE concentrations significantly increased motility and significantly reduced the abnormal spermatozoon rate demonstrates that CAPE plays a positive role not only in providing structural protection but also in the energy metabolism and functional integrity of the spermatozoon. This is in alignment with literature demonstrating the positive effects of CAPE or structurally similar compounds on spermatozoon membranes of different species.

Lan et al., (2022) reported that the addition of 210 µmol/L CAPE to the extender during short-term storage of boar semen showed significantly improving effects on motility, membrane integrity, and abnormal spermatozoon rate. It was reported that while spermatological values decreased in all groups as time extended, this decrease occurred at the lowest level in the CAPE-containing group. Our findings indicate that similar protective effects of CAPE can be observed in ram semen. Avdatek et al., (2020) reported that the use of proanthocyanidin, a polyphenol compound, in the short-term storage of ram semen improved motility and HOST values at hours 24, 48, and 72. Similarly, CAPE is a polyphenolic compound, and it was determined that it protects spermatozoon cells from oxidative stress caused by short-term storage through its antioxidant effect. In a study by Gündoğan et al., (2021) it was observed that among different doses of CAPE added to ram semen during cryopreservation, the 100 µg/ml group provided the best protection in terms of spermatozoon motility, abnormal spermatozoon rate, and HOST rate compared to the control and other groups. In a study by Yılmaz et al., (2025) it was stated that adding 150 µM caffeic acid to the extender during ram semen freezing showed a protective effect on post-thaw spermatozoon motility, mitochondrial membrane potential, and general semen quality. Furthermore, Soleimanzadeh et al., (2020) detected significant increases in total motility, viability, and plasma membrane integrity in the group treated with 100 µM caffeic acid after freezing buffalo semen, and reported a decrease in the abnormal spermatozoon rate. Similarly, in a study conducted by Namula et al., (2018) with the addition of 100 µM caffeic acid in boar semen, positive effects on spermatozoon motility, viability, and plasma membrane integrity were determined at 0 and 3 hours post-thaw. The results of these studies support that caffeic acid derivative compounds show a similar protective effect on semen quality in different species. The positive effects of CAPE observed in ram spermatozoa in our study overlap with findings obtained with other antioxidant polyphenols. These findings suggest that CAPE, being a phenethyl ester derivative of caffeic acid, can preserve spermatological parameters through similar biochemical mechanisms.

The antioxidant effects of CAPE have been described in detail not only in semen storage studies but also in testicular damage and toxicity models. Abdallah and El-Refaei, (2021) demonstrated the protective role of CAPE against the negative effects of cadmium on male fertility; Ceylan et al., (2020) reported that CAPE produced corrective effects on spermatological parameters against cisplatin-induced testicular damage. Abdallah et al., (2012) Gülhan et al., (2023) and Huyut et al., (2020) reported that CAPE administration increased spermatozoon motility and viability and reduced the abnormal spermatozoon rate in testicular toxicity models induced by carbon tetrachloride, doxorubicin, and lambda-cyhalothrin, respectively. Similarly, Eşrefoğlu et al., (2004) stated that CAPE administration provided effective protection in a myocardial ischemia/reperfusion-induced testicular damage model. These findings support CAPE's potential for protection against oxidative stress, spermatogenesis, spermatozoon functions, and spermatological parameters.

CONCLUSION

In conclusion, this study reveals that CAPE possesses the potential to preserve quality in spermatological parameters during the short-term storage of ram semen. In particular, the positive effects of the 100 µg/ml application dose of CAPE on spermatozoon plasma memb-

rane integrity and motility were determined; this effect is thought to be associated with CAPE's strong antioxidant capacity. The fact that no study regarding the use of CAPE in the short-term preservation of ram semen has been encountered in the current literature makes this research a novel contribution to the field. Evaluating CAPE at different doses in association with longer storage times and fertilization rates will contribute to a better understanding of its potential application areas in reproductive biotechnology.

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Conflict of interest

There is no conflict of interest among the authors. The authors declare that there are no conflicts of interest that could be perceived as prejudicial to the impartiality of the reported research.

Ethical statement

This study was approved by the Afyon Kocatepe University Local Ethics Committee for Animal Experiments (AKÜHADYEK) at the meeting dated 13.03.2018 with decision reference number 35-18.

Author Contributions

MG, DY, FA, and ÖH contributed to the conception, design, and execution of the study. DY and FA contributed to data collection. MK, DY, and FA analyzed the data. MK, DY, and FA drafted and wrote the manuscript. MK, DY, and FA critically reviewed the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The data and materials of this study are available from the corresponding author upon reasonable request.

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