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Investigation of the effects of *Nepeta italica* subsp. *cadmea* extract on frozen-thawed Ankara buck spermatozoa

S. Boztas,[1*] N. Keskin,[2] M. N. Bucak,[3*] B. Er Urgancı,[4] M. Bodu,[3] F. Taspinar[5]

[1]*Department of Histology and Embryology, Graduate School of Health Sciences, Pamukkale University, Denizli, Turkey*

[2]*Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Turkey*

[3]*Department of Reproduction and Artificial Insemination, Faculty of Veterinary Science, Selcuk University, Konya, Turkey*

[4]*Department of Medical Biology, Faculty of Medicine, Pamukkale University, Denizli, Turkey*

[5]*Department of Physiology, Faculty of Medicine, Aksaray University, Aksaray, Turkey*

ABSTRACT: Semen cryopreservation causes adverse effects on the sperm structure especially on plasmalemma, acrosomes and DNA which results in lowered fertilization capacity. Hereby, cryoprotective antioxidant molecules have been extensively investigated to overcome the deleterious effects of oxidative stress caused by cryopreservation in spermatozoa. In recent years, natural antioxidants rich in flavonoids and phenolic compounds have gained increasing attention to develop as novel cryoprotectants in spermatozoa cryopreservation. *Nepeta italica* subsp. *cadmea* (Boiss.) rich in terpenoids and flavonoids is endemic to Turkey. In this study, the effects of three different doses of *Nepeta italica* subsp. *cadmea* extract (0.75 µL/10 mL, 1.5 µL/10 mL and 6 µL/10 mL) on motility, acrosome integrity, mitochondrial activity and DNA fragmentation in frozen-thawed Ankara buck spermatozoa were investigated. The plants were harvested during the flowering stage on Honaz Mountain (Denizli, Turkey) and air dried in the shade at room temperature. The extract of the powdered plant was extracted by hydrodistillation using a Clevenger apparatus system, then stored at 4 °C in an amber colored glass bottle. The ejaculates taken from the Ankara bucks during the breeding season with an electroejaculator were combined and used in the diluting and freezing of semen. The experomental groups were formed by adding three different doses of *Nepeta italica* subsp. *cadmea* extract (0.75 µL/10 mL, 1.5 µL/10 mL and 6 µL/10 mL) to the control extender. The semen was diluted with the different extenders at 35–37 °C and loaded into straws. Sperm samples frozen in liquid nitrogen vapors, following equilibration, were stored in liquid nitrogen. The plant extract at the dose of 0.75 µL/10 mL improved motility, acrosome integrity, mitochondrial activity and DNA fragmentation in post-thawed Ankara buck spermatozoa ($p<0.05$). As a result, it can be suggested that the most effective dose of 0.75 µL/10 mL of *Nepeta italica* subsp. *cadmea* (Boiss.) extract can be used as an effective additive for freezing Ankara buck spermatozoa.

Keyword: Ankara buck; *Nepeta italica* subsp. *cadmea*; sperm cryopreservation.

Correspondence author:
S. Boztas, M. N. Bucak,
Department of Histology and Embryology, Graduate School of Health Sciences, Pamukkale University, Denizli, Turkey
Department of Reproduction and Artificial Insemination, Faculty of Veterinary Science, Selcuk University, Konya, Turkey
E-mail address: s.boztas79@gmail.com, mnumanbucak@gmail.com

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INTRODUCTION

Cryopreservation and long-term storage of mammalian sperm is a valuable process for preservation of spermatozoa survival and fertilizing ability. On the other hand, cryopreservation of spermatozoa causes structural and molecular alterations because of oxidative, temperature and osmotic stresses, resulting in deteriorating of sperm parameters (motility, viability and DNA integrity etc.) (Ozimič et al., 2023). However, the addition of effective additives to the cryopreservation medium provide a cryoprotective effect on sperm survival (Karaşör et al., 2022).

Recently, herbal natural products with high antioxidant potential have fostered interest as a cheap and natural source of additives for the cryopreservation of spermatozoa (Ros-Santaella and Pintus, 2021; Rahim et al., 2023). It has been determined that the addition of some antioxidant substances such as glutathione (an essential metabolite for plants in the control of reactive oxygen species-ROS) (Gadea et al., 2011; Dorion et al., 2021; Zou et al., 2021), resveratrol (a bioactive compound in many foods) (Brito et al., 2020; Meng et al., 2020), quercetin (a natural flavonoid compound present in the plant kingdom) (Seifi-Jamadi et al., 2016; Wang et al., 2022), Coenzyme Q_{10} (CoQ_{10}; mitochondrial electron transport chain cofactor, also a lipophilic antioxidant) (Yousefian et al., 2018), curcumin (a constituent of the traditional medicine known as turmeric) (Bucak et al., 2010; Nelson et al., 2017), arbutin (a glycosylated hydroquinone found in high amounts in the leaves of the plant) (Aboagla and Maeda, 2011) to the freezing medium during cryopreservation reduce oxidative stress (OS) caused cryodamages in spermatozoa.

Flavonoids are polyphenolic plant compounds which have antiviral, antiallergic, antiplatelet, anti-inflammatory, antitumor, antidiabetic, immunostimulating, antiapoptotic and antioxidant effects (Tanwar and Modgil, 2012; Wang et al., 2019; Ye et al., 2020). They also play a role in the treatment of male reproductive system dysfunction, such as testicular structural disruption, spermatogenesis disturbance and sperm quality decline (Ye et al., 2020). Hence, many studies have attempted to use herbs such as *Rosmarinus officinalis (*Malo et al., 2010, 2011), *Foeniculum vulgare* (Malo et al., 2012), *Melissa officinalis, Ilex paraguensys* (Luno et al., 2015), *Rhodiola rosea* L. (Zhao et al., 2009), *Camellia sinensis* (green tea) (Mustofa et al., 2021), *Marsilea crenata* (Wahjuningsih et al., 2021a), *Mesona palustris* B. (Wahjuningsih et al., 2021b), *Turraea fischeri* (Hassan et al., 2021), *Nigella sativa* (Maidin et al., 2018), *Tribulus terrestris*, *Cinnamomum zeylanicum* (Ariyan et al., 2021), *Thymus vulgaris* (Vahedi et al., 2018), *Silybum marianum* (Eskandari and Momeni, 2016) and *Opuntia ficus-indica* (Allai et al., 2016) as natural cryoprotectants for sperm cryopreservation.

Researches have found that natural plant components improve sperm quality by protecting the sperm plasma membrane from OS-related damage (Maidin et al., 2018), reducing sperm lipid membrane peroxidation (Luno et al., 2015), and decreasing DNA fragmentation (Allai et al., 2016). They also enhance sperm motility (Baiee et al., 2018), preserve plasma membrane and acrosome integrity (Eskandari and Momeni, 2016), and maintain normal sperm mitochondrial function (Yan et al., 2020).

The genus *Nepeta* (Lamiaceae) is represented by 39 species, in total 50 taxa, 19 of which are endemic in Turkey (Yılmaz et al., 2020). They have been extensively used as traditional medicine, particularly in India, Pakistan, Nepal, China, Iran and Turkey (Sharma et al., 2021). Their major component is nepetolactones (Shafaghat and Oji, 2010) and they are also rich in flavonoids (epicatechin, quercetin, and rutin), phenolic acids (gallic acid, hydroxybenzoic acid, chlorogenic acid, p-coumaric acid, ferulic acid and cinnamic acid), tannin content and mineral composition (P, Mg, K, Fe and Cu) (Kaska et al., 2019). They have several activities such as an antioxidant, anti-inflammatory, cytotoxic, genotoxic, anticonvulsant, analgesic, antidepressant (Süntar et al., 2018), antibacterial, antifungal, antispasmodic and anti-asthmatic (Kaska et al., 2019). *Nepeta italica* L. subsp. *cadmea* (Boiss.) A. L. Budantsev is one of the endemic species of the genus *Nepeta* and is extensively distributed in the Western, Southwest and Southern Anatolia of Turkey (Dirmenci, 2003). In this study, we aimed to investigate the possible cryoprotective dose-related effects of *Nepeta italica* subsp. *cadmea* extract on motility, acrosome integrity, mitochondrial activity and DNA fragmentation in Ankara buck spermatozoa.

MATERIALS AND METHODS

Preparation of essential oil

Nepeta italica subsp. *cadmea* was collected from Honaz Mountain, Denizli, Turkey, in the months of July and August. They were air dried in a shaded and moisture-free room at room temperature and then stored in polyethylene bags until used. The flowers,

leaves, seeds and stems of the dried plants were cut into small pieces and pulverized in a shredder. The essential oil was extracted by hydrodistillation using the Clevenger apparatus. The mixture (1:8-plant and water) inside of the distillation flask in the Clevenger apparatus was boiled for 4 hr and the vapor was condensed. The condensate was collected in a small burette and then the top oil layer was removed. The oil was dried over anhydrous sodium sulfate and stored in an amber colored glass bottle at 4 °C (Sarikurkcu et al., 2016).

Determination of phytochemical compounds of the plant

Analysis of the content of *Nepeta italica* subsp. *cadmea* extract was performed by gas chromatography using two types of detectors: flame ionization and mass spectrometric at the Bezmialem Vakıf University Phytotheraphy Education Research and Application Center, Istanbul, Turkey (Table 1).

Table 1. The percentage composition of *Nepeta italica* subsp. *cadmea* extract detected by GC-MS and GC-FID analysis.

Compounds	(%)
α-Pinene	0,522
Sabinene	0,440
α-Terpinene	0,261
1,8-Cineole	1,155
γ-Terpinene	0,509
Linalool	3,965
Caryophyllene	6,889
4-Terpineol	1,423
α-Copaene	0,349
α-Humulene	0,330
Lavandulol	0,930
α-Terpineol	0,349
Germacrene	0,863
γ-Cadinene	0,518
Calamenene	1,326
Caryophyllene oxide	2,293
Nepetalactone (Total isomers)	74,008
Others	3,870
Total	100

Animals and semen collection

Ejaculates from 5 Ankara bucks aged 3-4 years, which are known to be healthy and fertile, were collected by an electroejaculator three times a week for six weeks during the breeding season. Only ejaculates with spermatozoa motility of ≥80%, concentration of spermatozoa ≥2×10^9/mL were pooled and used in the freezing process of semen (Bucak et al., 2024).

Semen extending, freezing and thawing

The Tris-based extender solution (500 mL) consisting of 297.58 mM Tris, 96.32 mM citric acid, 82.66 mM fructose, 0.5 mL of the penicillin-streptomycin-amphotericin B mixture, 15% egg yolk, and 5% glycerol (pH value: 6.90; osmotic pressure: 304 mOsm/kg) was used for semen freezing (Bucak et al., 2024).

The pooled ejaculate was divided into four aliquots, and each aliquot was diluted at 37 °C with one of the freezing extenders containing *Nepeta italica* subsp. *cadmea* extract (0.75 µL/10 mL, 1.5 µL/10 mL and 6 µL/10 mL), and no additives (control) for a total of four experimental groups to a final concentration of approximately 100 million spermatozoa per one straw (0.25 mL). Groups were as follows: T0: Control (base extenders), T1: (base extenders+ 0.75 µL/10 mL extract), T2: (base extenders+1.5 µL/10 mL extract), T3: (base extenders+6 µL/10 mL extract).

The process took place in one step using a 10 mL glass centrifuge tube. Diluted sperm groups were drawn into straws (0.25 mL) at room temperature, the open ends were sealed with polyvinyl, and they were equilibrated at 4 °C for 2.5-3 hr. After equilibration, the straws were frozen by keeping them in liquid nitrogen vapor for 15 min at approximately -120 °C at a height of 5 cm above the liquid nitrogen level. Afterwards, semen frozen in liquid nitrogen vapor was stored in liquid nitrogen at ~-196 °C at least 1 week and then thawed in a 37 ºC water bath for 30 seconds. The research was carried out in five replications (Karaşör et al., 2022).

Spermatozoa motility test

After taking a drop from the spermatozoa sample on a slide and covering it with a coverslip, the test was performed under a phase-contrast microscope with a 37 °C heating table at 400x magnification by considering the spermatozoa that move forward smoothly in at least 3-5 different microscope fields. The average of the motility values in the fields was taken and recorded as the % motility ratio (Evans and Maxwell, 1987).

Assessment of spermatozoa acrosome integrity

The method of Nagy et al. (2003) was modified to determine acrosome integrity in spermatozoa. Acrosome-specific Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA)/propidium iodine (PI) fluorescent staining was applied. The spermatozoa in 0.25 mL straws dissolved at 37 °C was taken into an ependorf tube and diluted by adding 300 µL of phosphate buffered saline (PBS). 60 µL of this sample was taken and 10 µL of FITC-PNA and 3 µL of PI solutions were added and then incubated at 37 °C for 10 min. Thereafter, the reaction was terminated by adding 1 µL of Hancock liquid. After the 2.5 µL sample was taken on a slide, it was covered with a coverslip and evaluated for acrosome integrity under a phase-contrast microscope with fluorescent attachment (Leica DM3000, Germany) at a wavelength of 450-490 nm. Those who stained the acrosome green showed sperm with damaged acrosomes and those who did not stain the acrosome with green showed sperm with intact acrosomes. At least 200 cells were counted for each sample, and sperm acrosome integrity was expressed as a percentage.

Spermatozoa mitochondrial activity

Garner et al. (1997) method was modified to determine mitochondrial activity in spermatozoa. JC-1/PI fluorescent staining was applied. The sperm sample in 0.25 mL straws thawed at 37 °C was taken into eppendorf tube and 300 µL of PBS was added. 3 µL of JC-1 and 3 µL of PI stock liquids were added to 300 µL of diluted spermatozoa and incubated at 37 °C for 10 min. Then, the reaction was terminated by adding 1 µL of Hancock liquid. 2.5 µL of samples were taken on slides and covered with coverslips. The midpiece of spermatozoa was stained orange to bright green showed the presence of mitochondrial activity, while those with a matte color midpiece showed no mitochondrial activity. 200 cells were counted for each sample and the presence of spermatozoa mitochondrial activity was expressed as a percentage.

TUNEL assay

The TUNEL (TdT-mediated dUTP nick end labeling) assay is a method that shows DNA breaks (~180-200 base pairs) caused by the activation of endonucleases in the apoptotic process of cells. For this method, spermatozoa in straws stored in liquid nitrogen were thawed by keeping them in a water bath at 37 °C for 30 sec, transferred to eppendorf tubes, and then centrifuged at 2500 rpm for 5 min to remove diluents. Then, 100 µL of PBS was added to the spermatozoa, which were then washed three times with PBS, 10 µL of the sample was spread on poly-L-lysine coated slides as smears and dried at room temperature for 30 min. Then, the TUNEL kit (Roche, InSitu Cell Death Detection Kit, POD, Roche-11 684 817 910) procedure was applied according to the manufacturer's recommendations. Slides were fixed in 4% paraformaldehyde for 1 hr at room temperature and washed three times with PBS. They were left for 10 min in a blocking solution (3% H_2O_2 in methanol) and then washed with PBS. Next, the slides were incubated and washed on ice for 10 min with permabilization solution (0.1% Triton X 100 and 0.1% Sodium Citrate). The coverslips were covered by adding 50 µL of the label solution (450 µL) and enzyme (50 µL) mixture. They were all incubated for 60 min at 37 °C in a dark and humid environment and then washed three times with PBS. For signal conversion to slides, 50 µL of converter-POD was added to each slides and covered them with coverslips. The slides were kept in the dark for 30 min by adding 100 µL of DAB substrate per sample for color rendering, and after washing, they were counterstained with 0.5% methyl green and then washed. After the slides were closed, statistical analysis was performed by counting TUNEL-positive cells at 1000x magnification from 6 different regions of each slide.

Statistical Analysis

In statistical analysis, the averages of the data obtained from five different replications were used. The overall data were defined as a mean ± SE. The means of microscopic sperm parameters were analyzed with Duncan's post-hoc test and variance analysis to adjust for considerable differences. Statistical analysis were verified through IBM SPSS (Version 22), and statistical relevance was adjusted at $p<0.05$.

RESULTS

The effects of the doses of 0.75 µL/10 mL (T1), 1.5 µL/10 mL (T2), 6 µL/10 mL (T3) of *Nepeta italica* subsp. *cadmea* extract (NE) on motility, acrosome integrity, mitochondrial activity and DNA fragmentation were evaluated in post-thaw Ankara buck spermatozoa.

Spermatozoa motility in post-thawing semen was significantly different between groups ($p<0.05$). The sperm motility was highest in the $NE_{0.75\ \mu L/10\ mL}$ (T1) group (63%) compared to the other groups [control (T0): 46.25%, T2: 58.75%, and T3: 41.67%] (Figure 1).

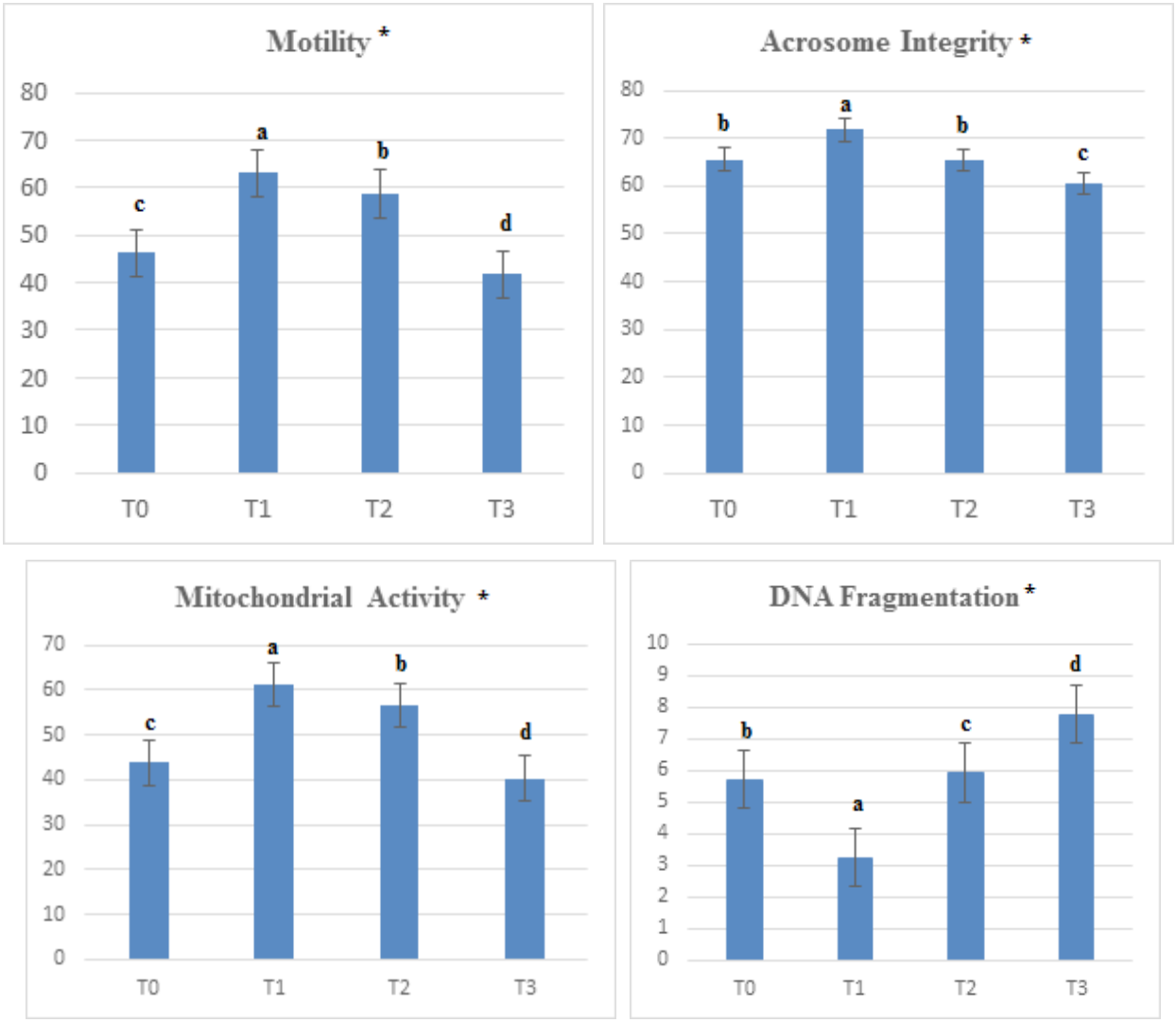

Figure 1. Post-thawed motility, acrosome integrity, mitochondrial activity and DNA fragmentation of Ankara buck spermatozoa. Control (T0), $NE_{0.75\ \mu L/10\ mL}$ (T1), $NE_{1.5\ \mu L/10\ mL}$ (T2), $NE_{6\ \mu L/10\ mL}$ (T3). (*): $p<0.05$, the different letters denote significant differences between groups.

Acrosome integrity of spermatozoa after thawing was 71.71% in the $NE_{0.75\ \mu L/10\ mL}$ (T1) group, whereas it was 65.34% in the dose of $NE_{1.5\ \mu L/10\ mL}$ (T2) and 60.64% in the dose of $NE_{6\ \mu L/10\ mL}$ (T3) compared with the T0 (65.58%) ($p<0.05$) (Figure 1). In the acrosome integrity analysis, green covered cap on the sperm heads showed damaged acrosomes, on the other hand, without a green cap on the sperm heads showed intact acrosomes (Figure 2).

According to the results obtained for sperm mitochondrial activity, the T1 group (61.21%) showed a higher mitochondrial activity rate than the control (T0) (43.84%), $NE_{1.5\ \mu L/10\ mL}$ (T2) (56.51%) and $NE_{6\ \mu L/10mL}$ (T3) (40.26%) groups ($p<0.05$). Therefore, $NE_{0.75\ \mu L/10\ mL}$ (T1) supplementation appeared to be the optimum dose for mitochondrial activity (Figure 1). The stained bright green in color in the spermatozoa midpiece was considered to have mitochondrial activity, whereas the spermatozoa midpiece with a matte color indicated no mitochondrial activity (Figure 3).

Looking at the results of the TUNEL assay (Figure 1), the group with $NE_{0.75\ \mu L/10\ mL}$ (T1) has the lowest DNA fragmentation as 3.23% compared to the other groups (T0: 5.70%, T2: 5.90%, T3: 7.77%). DNA fragmentation was observed at the highest level in $NE_{6\ \mu L/10\ mL}$ (T3) group. Tunnel-positive DNA fragmentation was observed as dark brown fragments in the sperm head (Figure 4).

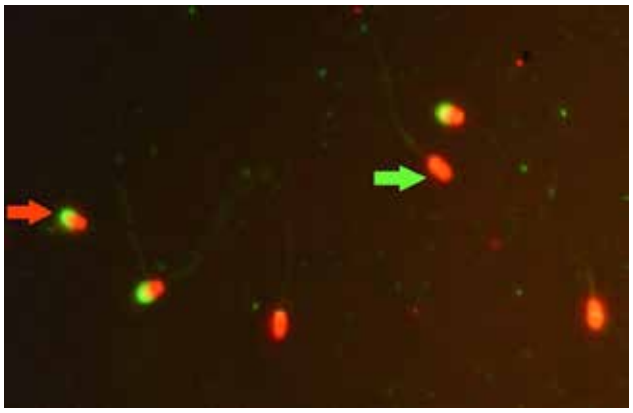

Figure 2. The green cap (red arrow) on the sperm heads demonstrates damaged acrosomes, without a green cap (green arrow) on the sperm heads demonstrates intact acrosomes. FITC-PNA/PI staining.

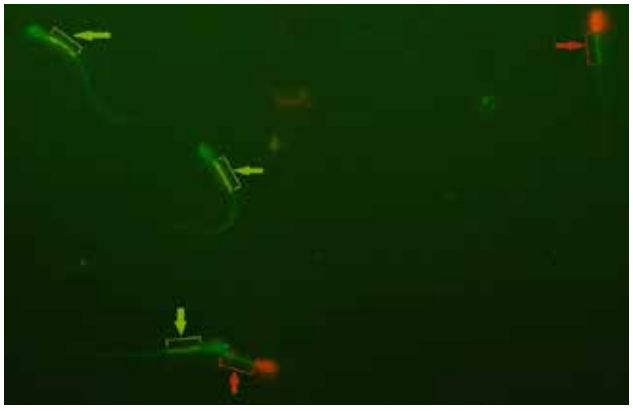

Figure 3. The stained bright green fluorescence of the sperm midpiece (green arrows) was considered to indicate mitochondrial activity, whereas the sperm midpiece with matte color (red arrows) indicated no mitochondrial activity. JC-1/PI staining.

DISCUSSION

Recent studies have shown that different antioxidants can effectively improve spermatozoa quality during cryopreservation. In this regard, many herbs with antioxidant properties have gained the special attention of researchers. Due to their bioactive compounds such as flavonoids and phenols, several plants have antioxidant properties (Ariyan et al., 2021). It has been also revealed that flavonoids and phenolic acids have antioxidative activities as therapeutic agents (Saxena et al., 2012). Flavonoids scavenge reactive oxygen species (Baskar et al., 2018) to display their antioxidant properties (Moretti et al., 2012). Considering the findings in the previous studies, we investigated the effects of extract obtained from *Nepeta italica* subsp. *cadmea*, which contains flavonoids, on motility, acrosome integrity, mitochondrial activity and DNA fragmentation parameters in cryopreserved Ankara buck spermatozoa.

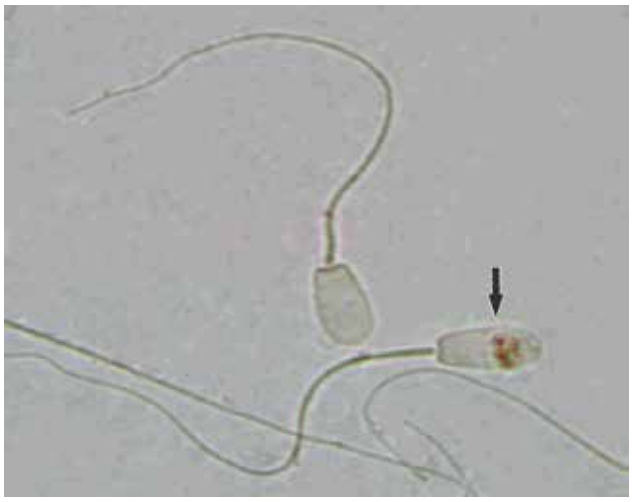

Figure 4. TUNEL-positive DNA fragmentation (arrow), 1000x.

Sperm motility is an important parameter of semen quality that should be considered before artificial insemination. The reduction in the number and motility of spermatozoa is highly correlated with an increased risk of infertility (Dcunha et al., 2022). Cryopreservation of spermatozoa causes a decrease in motility. Therefore, many studies have been performed to diminish cryodamages in spermatozoa adding antioxidant agents such as herbal antioxidants. For instance, according to post-thawing assessments, the sperm motilities in semen extended with Tris-citrate-glucose extender supplemented with 0.64 mg/mL *Moringa oleifera* leaf methanolic extract were found as 55.22 ± 0.98% in ram spermatozoa (El-Seadawy et al., 2022). In addition, adding 0.1 mg of green tea extract/100 mL extender in bull semen showed the highest percentages of sperm motility as 69.17 ± 1.47% (Susilowati et al., 2021). Also, 3% *Marsilea crenata* extract (contains flavonoid bioactive components) in egg yolk skim milk extender was the best concentration to improve motility as 58.5 ± 4.74% in post-thawing buck semen (Wahjuningsih et al., 2019). In this study, we added different doses of *Nepeta italica* subsp. *cadmea* extract (0.75, 1.5 and 6 µL/10 mL) to the extender (tris+citric acid+fructose+egg yolk). Then, post-thaw motility of Ankara buck semen was investigated. The highest sperm motility 63 ± 1.23% was

detected in the dose of $NE_{0.75\ \mu L/10\ mL.}$ Therefore, it has been evaluated that the dose of 0.75 µL/10 mL is the best concentration/dose to preserve sperm motility. The motility findings in this study also showed that *Nepeta italica* subsp. *cadmea* extract improved the motility of spermatozoa in a dose-dependent manner as shown in previous studies.

Semen cryopreservation negatively affects acrosome integrity (Sun et al., 2020). When there is an acrosome dysfunction, fertility can be compromised (Esteves and Verza Jr, 2011). Herbal agents were used as cryoprotectants, for instance, adding 50 µg/mL thymoquinone, which is the most essential active compound of *Nigella sativa*, to the ram semen increased the acrosome integrity to 59.56 ± 5.92% (İnanç et al., 2022). The concentration of 1.0 mmol genistein, a polyphenol from the flavonoid group of compounds that is abundant in many plants, gave the highest mean percentage of bull spermatozoa acrosome integrity (49.58 ± 0.98 %) after freezing (Prihantoko et al., 2020). In our study, consistent with the same concentration/dose of *Nepeta italica* subsp. *cadmea* extract in motility results, acrosome integrity values as 71.71 ± 0.33% were preserved at the highest value obtained at $NE_{0.75\ \mu L/10\ mL}$ compared to the control and other groups ($p<0.05$). The findings of the acrosome integrity after thawing in this research are generally consistent with the herbal agents dose-depented effects on acrosome integrity revealed in previous studies.

Mitochondria produce adenosine triphosphate through oxidative phosphorylation to provide energy to spermatozoa. They are also one of the main source of ROS generation in spermatozoa (Park and Pang, 2021). The increase in ROS during cryopreservation causes changes in mitochondrial membrane permeability (Gualtieri et al., 2021). Changes in mitochondrial integrity and functionality have been associated with a loss of sperm quality parameters (Costa et al., 2023). Because of the association between mitochondrial activity and sperm motility, researchers have been tested the effects of herbal antioxidants on mitochondrial integrity as cryoprotectants in cryopreserved spermatozoa. Mao et al. (2018) showed that under OS induced by cadmium (Cd^{+2}), quercetin (a plant pigment /flavonoid) protected the mitochondrial function of buck spermatozoa. Besides, 50 µM taxifolin (an antioxidant, natural flavonoid) significantly improved motility, mitochondrial membrane potential and the ratios of spermatozoa with acrosomal damage compared to the control group in rabbit semen after freezing-thawing processes (Yılmaz et al., 2023). In addition, Bucak et al. (2020) reported that co-supplementation of tris-based extender having 3% glycerol with 10 µM taxifolin hydrate resulted in the highest post-thawed viability and mitochondrial activity in ram spermatozoa. In our study, a dose of $NE_{0.75\ \mu L/10\ mL}$ had cryoprotective effects on mitochondrial activity compared to other groups which is similar to the herbal extracts dose-response related previous studies.

Sperm DNA integrity is very sensitive to the freezing-thawing process (Alcay et al. 2016). It has been suggested that ROS and cryopreservation lead to DNA fragmentation in equine spermatozoa (Baumber et al., 2003). OS induced sperm DNA damage might cause a detrimental effect on fertilization (Hosen et al., 2015). The DNA damage level provides very important clues about the quality and reproductive potential of the spermatozoa. Therefore, determining DNA damage using the TUNEL technique is of great importance for assessment of quality of spermatozoa. Studies are being conducted showing the effects of many different cryoprotectants on DNA damage in spermatozoa. Anderson et al. (2000) reported that flavonoids transfer electrons to radical sites on DNA and thereby repair some oxidative damage caused by hydroxyl radical attack. Besides, addition of 50 µg/mL *Tribulus terrestris* ethanol extract to the buck spermatozoa extender showed the lowest DNA fragmentation (Ariyan et al., 2021). Azimi et al. (2020) showed that the supplementation of 50 µg/mL purslane methanolic extract to the buck spermatozoa extender reduced DNA fragmentation. Also, it has been stated that adding 10 µM taxifolin hydrate along with 5% glycerol to the ram sperm extender prevents DNA fragmentation (Bucak et al., 2020). Additionaly, 10 mM raffinose and 5 mM raffinose + 2.5 mM hypotaurine groups resulted in lower sperm with TUNEL-positive cell (1.5 ± 1.2% and 2.1 ± 0.9%) than that of control (4.9 ± 2.5%) (Bucak et al., 2013). In the present study, the dose of $NE_{0.75\ \mu L/10\ mL}$ reduced DNA fragmentation (3.23 ± 0.19%) compared to the other groups ($p<0.05$). This findings revealed that the dose of $NE_{0.75\ \mu L/10\ mL}$ showed a positive effect on DNA fragmentation as for motility, acrosome and mitochondrial integrity parameters. Our findings are in agreement with the findings of some similar studies as mentioned above.

CONCLUSION

As a result, our study has shown that the best dose of 0.75 µL/10 mL of *Nepeta italica* subsp. *cadmea*

extract containing antioxidants improve motility, DNA fragmentation, acrosome and mitochondrial integrity as a cryoprotectant in post thaw Ankara buck spermatozoa. However, it can be suggested that the dose of $NE_{0.75\ \mu L/10\ mL}$ extract should be tested using further molecular analysis on the post-thaw quality of spermatozoa.

Declaration of interest

The authors declare no conflicts of interest associated with this manuscript.

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Ethical statement

Our study was approved by Pamukkale University Animal Ethics Committee (No: PAUHDEK-2021/01).

Author contribution

Sıddıka Boztas: Material preparation, Data collection, Methodology; **Mustafa Numan Bucak, Filiz Taspınar and Mustafa Bodu**: Cryopreservation, Methodology and Statistical Data Analysis; **Buket Er Urgancı**: Methodology; **Nazan Keskin**: The Study Design and Writing with input from all other authors.

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