

Effect of extender supplementation with low-molecular-weight antioxidants on selected quality parameters of cryopreserved canine spermatozoa

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Abstract

Introduction: The addition of low-molecular-weight antioxidants during the freezing process improves post-thaw sperm quality. The high antioxidant potential of cryopreserved semen could have a positive effect on the motility, viability, and energy status of sperm cells and their ability to bind to the *zona pellucida* of oocytes. The aim of the study was to determine the effects of different concentrations and combinations of vitamins E and C in a semen extender on selected quality parameters of frozen-thawed canine spermatozoa. **Material and Methods:** The experimental material was the semen of four mixed-breed dogs. Sperm viability (motility, plasma membrane integrity, and mitochondrial function) was examined at 0, 60, and 120 min in semen samples supplemented with the extender and in the controls. **Results:** Combined supplementation with vitamins C + E at a concentration of 200 + 200 $\mu\text{M} / 1 \times 10^9$ spermatozoa had the most profound effect on total sperm motility, linear motility, and the percentage of spermatozoa with intact plasma membrane and active mitochondria. **Conclusion:** The synergistic activity of vitamins E and C had a more beneficial influence on the quality of frozen–thawed sperm than these non-enzymatic antioxidants applied separately.

Keywords: canine sperm, cryopreservation, spermatozoa, antioxidants, viability.

Introduction

Innovative methods for the cryopreservation of canine semen facilitate the transfer of desirable genetic material from the most valuable stud dogs, contribute to the preservation of endangered canine breeds, and enhance the utilisation of specific genetic traits in male dogs (32). Recent research has contributed valuable information about the role of genes encoding behavioural and personality traits in dogs, including assistance dogs (22).

Cryopreservation influences sperm quality. Each step of the cryopreservation process, including dilution, chilling, freezing, and thawing, decreases the fertilising capability of sperm for artificial insemination (AI) (3, 26). The fertilising capability of mammalian sperm cells is determined mainly by the content of polyunsaturated fatty acids (PUFAs) which conditions their susceptibility to peroxidative damage (11).

Certain amounts of free radicals produced in the respiratory chain are required for normal sperm function. However, cryopreservation induces lipid peroxidation and increases the levels of reactive oxygen species (ROS), which disrupt the balance between free radicals and antioxidant system of frozen-thawed spermatozoa, leading to oxidative stress (10). Prolonged exposure of spermatozoa to ROS compromises plasma membrane integrity causes a rapid loss of intracellular ATP and axonemal damage, decreases sperm motility, leads to the loss of intracellular enzymes and DNA fragmentation, impairs the fertilisation ability of sperm cells, and decreases pregnancy rates after IVF (1, 6, 25).

ROS can be neutralised by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). In sperm cells, the defence mechanism against lipid peroxidation also involves non-enzymatic molecules such as thioredoxin

and glutathione, thiol-containing molecules, and vitamins E, C, and D (42). For human and several mammalian species sperm, the freezing medium has been supplemented with low-molecular-weight antioxidants to reduce oxidative stress (16, 17, 23, 28).

The aim of this study was to determine the effects of different concentrations and combinations of Trolox (water-soluble vitamin E) and L-ascorbic acid (vitamin C) in a semen extender on selected quality parameters of frozen-thawed canine semen.

Material and Methods

Semen collection. The experimental material was the semen from four mixed-breed dogs. Sperm-rich fractions of ejaculates were collected once weekly over a period of eight weeks. The dogs were housed in individual pens and fed commercial canine food. Water was available *ad libitum*.

During a preliminary assessment of the collected ejaculates, sperm motility was evaluated by the computer-aided sperm analysis (CASA) method and sperm cell concentrations were determined by the cytometric method in a haemocytometer. Semen samples with total motility higher than 80% and sperm counts higher than 200×10^6 sperm cells per 1 cm^3 of semen were qualified for further analyses.

Freezing-thawing procedure. The procedure of cryopreservation was conducted according to Nizanski *et al.* (20) with some modifications (36). After preliminary assessment, the ejaculates were pooled and centrifuged ($700 \times g$, 5 min). Seminal plasma was removed and semen sediments were diluted with standard Tris-citrate-fructose (TCF) extender (26) to a concentration of 2×10^8 spermatozoa/ cm^3 and left to stand for 30 min at room temperature (20°C). The samples were then cooled to 5°C for 60 min. After chilling, the second dilution (1:1) was performed with an extender containing 5.0 cm^3 of TCF, 4.0 cm^3 of hen egg yolk, 0.8 cm^3 of glycerol, and 0.2 cm^3 of Orvus Es Paste (Equex STM, Minitube, Germany) to obtain a concentration of 1×10^8 spermatozoa/ cm^3 . The final concentrations of hen egg yolk, glycerol, and Orvus Es Paste in diluted semen samples were determined at 20%, 4%, and 1%, respectively. Equilibration time at 5°C was 15 min. Then the semen was placed in 0.25 cm^3 plastic straws (Minitube, Germany) and frozen in a closed expanded polystyrene box by placing filled straws on a rack suspended 4 cm above the surface of liquid nitrogen and allotting 10 min freezing time. Frozen straws and tubes were placed in a container with liquid nitrogen. Straws were thawed in a water bath at 70°C for 5 s (21).

Antioxidant treatment. During the second supplementation, the extender was enhanced with water-soluble vitamin E (Trolox, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma Chemical Co., USA) and vitamin C (Sigma Chemical

Co., USA). The following extender variants were used in the study: 1) extender without additives as the control sample; 2) extender with vitamin E at $400 \mu\text{M}/1 \times 10^8$ spermatozoa; 3) extender with vitamin C at $200 \mu\text{M}/1 \times 10^8$ spermatozoa; 4) extender with vitamins C + E at $200 + 400 \mu\text{M}/1 \times 10^8$ spermatozoa; and 5) extender with vitamins C + E at $200 + 200 \mu\text{M}/1 \times 10^8$ spermatozoa.

Post-thaw sperm viability (motility, plasma membrane integrity, and mitochondrial function) was examined at 0, 60, and 120 min in semen samples supplemented with the extender and in the controls.

Motility. Sperm motility was evaluated using the Hamilton-Thorne IVOS sperm analyser version 12.3 (Hamilton-Thorne Biosciences, USA). Software settings recommended by the manufacturer were used in analyses of canine sperm: 30 frames acquired, 60 Hz frame acquisition rate, 75 minimum cell contrast, 6 pixel minimum cell size, 75% straightness threshold, $100 \mu\text{m}/\text{s}$ path velocity threshold, 9.9 m/s low average path velocity (VAP) cut-off, $20 \mu\text{m}/\text{s}$ low straight line velocity (VSL) cut-off, 0.80–4.93 static size gates, 0.49–1.68 static intensity gates, and 22–84 static elongation gates. Total motility (%) and progressive motility (%) were determined in the IVOS analyser.

Plasma membrane integrity. Sperm plasma membrane integrity was assessed by dual fluorescent staining with SYBR-14 and propidium iodide (PI) (Live/Dead Sperm Viability Kit, Thermo Fisher Scientific, USA) according to the method described by Garner and Johnson (8) with minor modifications. Briefly, aliquots of sperm samples (20×10^6 spermatozoa/ cm^3) were incubated with SYBR-14 (1 mM SYBR-14 in dimethyl sulphoxide (DMSO)) and PI solutions ($2.4 \mu\text{M}$ PI in Tyrode's salt solution) for 10 min at 37°C . After incubation, stained sperm cells were placed on microscopic slides and examined at $600\times$ magnification under a fluorescence microscope (Olympus CH 30 RF-200, Japan). Sperm cells displaying only bright green fluorescence were regarded as viable spermatozoa with an undamaged plasma membrane. A minimum of 200 cells per slide were examined in random fields of each aliquot.

Evaluation of mitochondrial function by fluorescent microscopy. Sperm mitochondrial function was assessed by dual staining with the fluorescent probes 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, JC-1 (Molecular Probes, USA) and PI (Sigma Chemical Co., USA) according to previously described methods (9), with certain modifications (7). Aliquots of sperm samples (20×10^6 spermatozoa/ cm^3) were incubated with JC-1 solution (1 mg JC-1/ cm^3 dimethyl sulphoxide, DMSO) for 15 min at 37°C . After incubation, sperm samples were stained with PI (10 μL of PI solution in $0.5 \text{ mg}/\text{cm}^3$ of phosphate buffer solution) for 10 min at 37°C , washed (5 min at room temperature), and sperm pellets were re-suspended in a HEPES buffer solution (10 mM

HEPES, 0.85% NaCl, 0.1% bovine serum albumin, pH 7.4).

Statistical analysis. Values were expressed as the mean \pm standard deviation (SD). The data were analysed by ANOVA, followed by the Duncan multiple comparison. Differences between means were considered significant at $P \leq 0.05$.

Results

The effect of semen extender supplementation with different concentrations and combinations of vitamins E and C on the total motility of post-thaw semen with different incubation times is presented in Fig. 1.

Regardless of incubation time, a significantly higher percentage ($P \leq 0.05$) of frozen motile spermatozoa was preserved in the extender supplemented with vitamins C + E ($200 + 200 \mu\text{M}/1 \times 10^9$ spermatozoa) than in the control. Similar results were observed after 60 and 120 min of incubation in semen cryopreserved with vitamins C and E (the latter in the higher concentration) ($200 + 400 \mu\text{M}/1 \times 10^9$ spermatozoa). Rapidly thawed semen was characterised by a significantly higher percentage ($P \leq 0.05$) of total motile spermatozoa in samples supplemented with vitamin C at $200 \mu\text{M}/1 \times 10^9$ spermatozoa compared with the control.

A significantly higher ($P \leq 0.05$) percentage of spermatozoa exhibiting linear motility was observed in samples supplemented with vitamins C + E at concentrations of $200 + 200 \mu\text{M}/1 \times 10^9$ spermatozoa and $200 + 400 \mu\text{M}/1 \times 10^9$ spermatozoa than in the controls (Fig. 2), also regardless of incubation time.

In an effect similarly undifferentiated by incubation time, the percentage of cryopreserved motile spermatozoa and sperm cells exhibiting linear motility was somewhat higher in samples supplemented with vitamin E at $400 \mu\text{M}/1 \times 10^9$ spermatozoa than in the controls (Figs 1 and 2); however, this difference was not statistically significant.

The effect of semen extender supplementation with different concentrations and combinations of vitamins E and C on the mitochondrial function and plasma membrane integrity of post-thaw semen with different incubation times is presented in Figs 3 and 4.

After 60 and 120 min incubation periods, the percentage of spermatozoa with active mitochondria and intact plasma membranes in post-thawed semen was significantly higher ($P < 0.05$) in semen supplemented with vitamins C + E ($200 + 200 \mu\text{M}/1 \times 10^9$ spermatozoa) than in the controls. Regardless of incubation time, supplementation with other concentrations of Trolox, vitamin C, or their combination did not exert a significant influence on the percentage of spermatozoa with active mitochondria and integral plasma membranes.

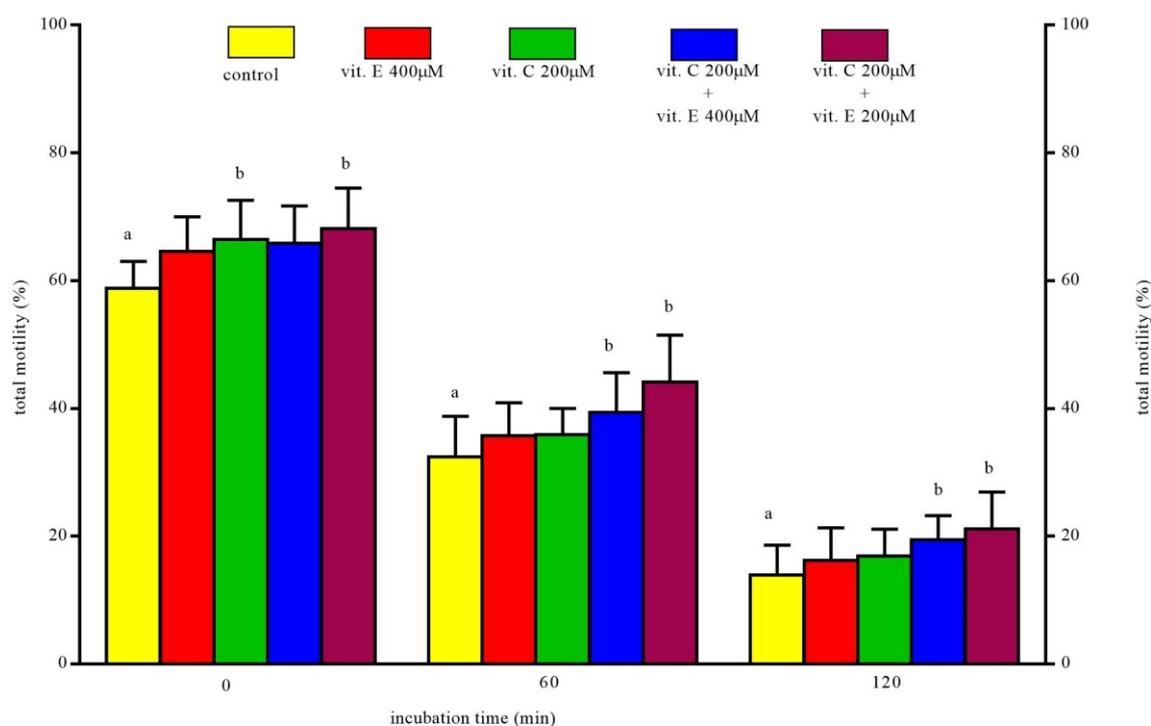


Fig. 1. The effect of semen extender supplementation with different concentrations and combinations of Trolox (water-soluble vitamin E) and L-ascorbic acid (vitamin C) on the total motility of frozen-thawed canine spermatozoa during 2 h of incubation. The presented values are means \pm SD for 20 ejaculates from four dogs. Values marked with different letters (a, b) differ significantly at $P \leq 0.05$

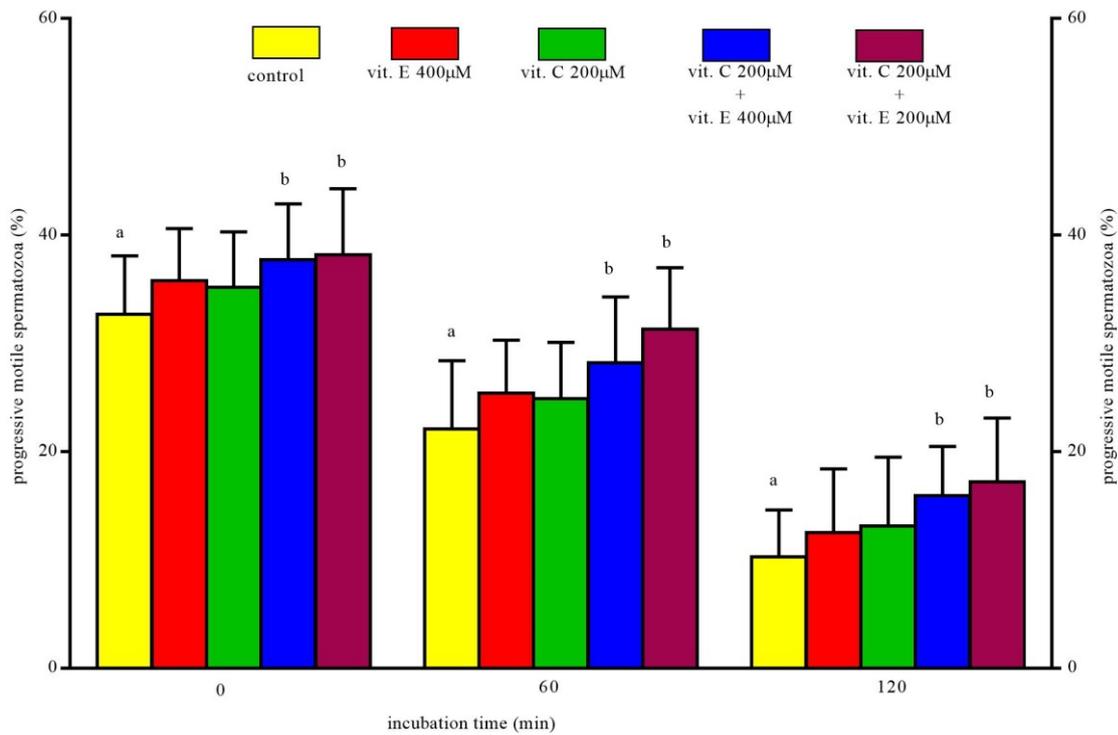


Fig. 2. The effect of semen extender supplementation with different concentrations of low-molecular-weight antioxidants on the progressive motility of frozen-thawed canine spermatozoa during 2 h of incubation. The presented values are means \pm SD for 16 ejaculates from four dogs. Values marked with different letters (a, b) differ significantly at $P \leq 0.05$

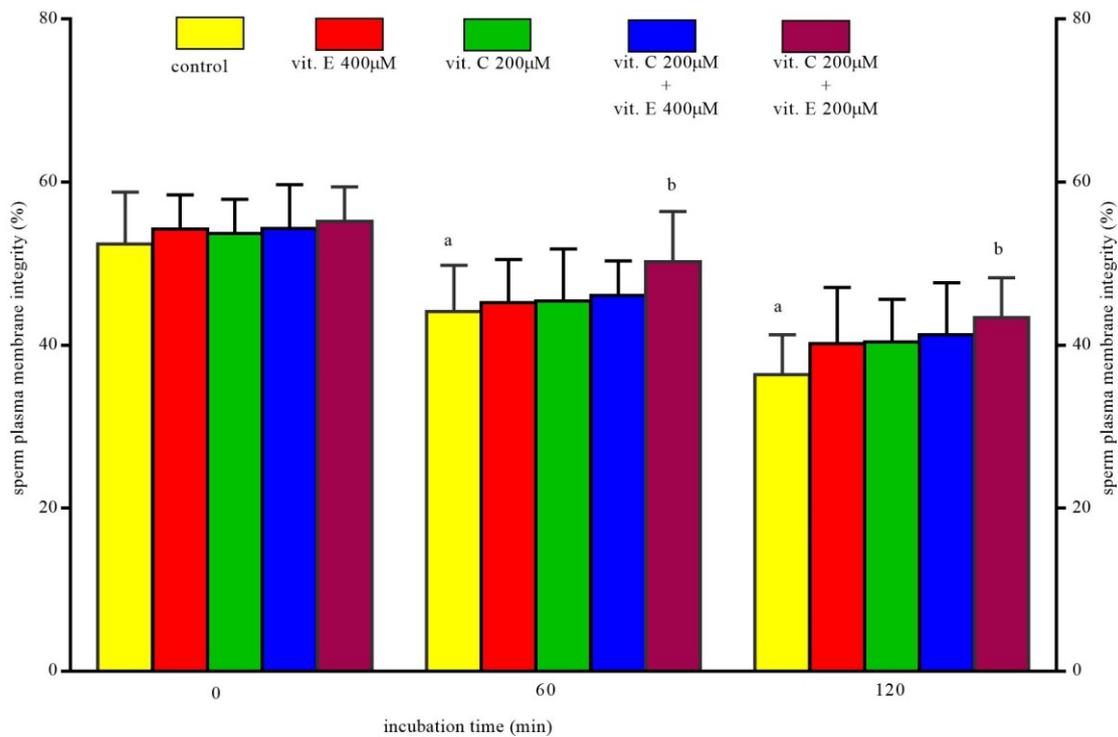


Fig. 3. The effect of semen extender supplementation with different concentrations of low-molecular-weight antioxidants on plasma membrane integrity in frozen-thawed canine spermatozoa during 2 h of incubation. The presented values are means \pm SD for 16 ejaculates from four dogs. Values marked with different letters (a, b) differ significantly at $P \leq 0.05$

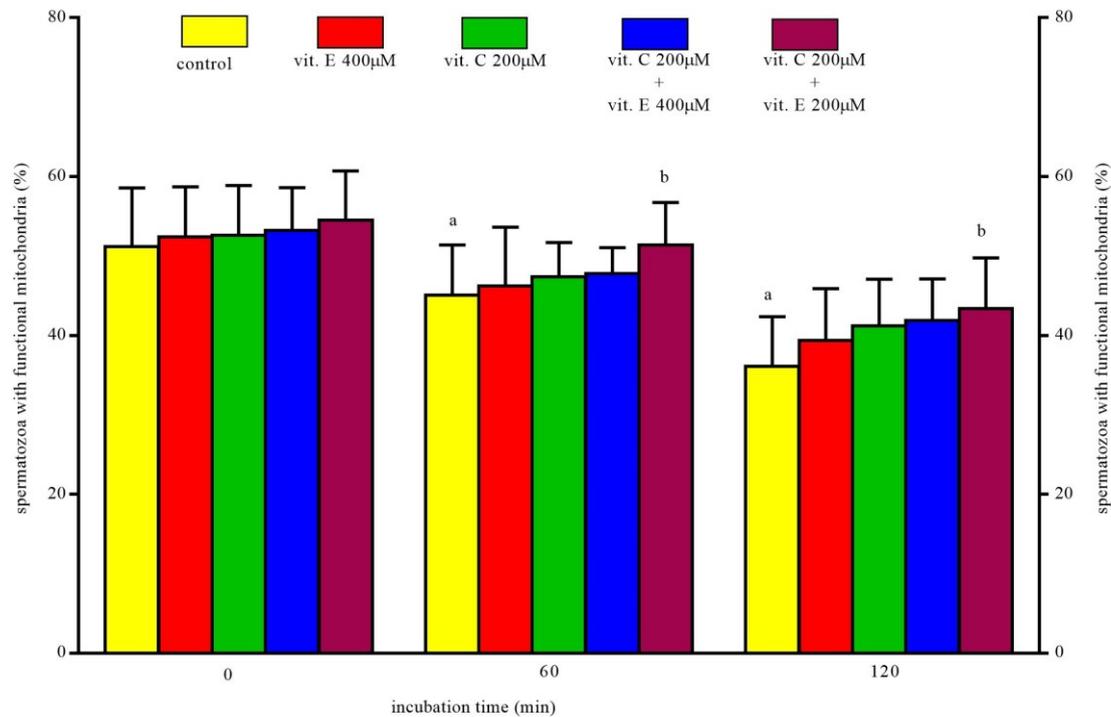


Fig. 4. The effect of semen extender supplementation with different concentrations of low-molecular-weight antioxidants on mitochondrial activity in frozen-thawed canine spermatozoa during 2 h of incubation. The presented values are means \pm SD for 16 ejaculates from four dogs. Values marked with different letters (a, b) differ significantly at $P \leq 0.05$

Discussion

The balance between ROS generation and the activity of the antioxidant defence system containing both enzymatic and non-enzymatic components determines homeostasis in living organisms. Excessive ROS generation during cryopreservation compromises the quality of thawed spermatozoa (35). High antioxidant potential of cryopreserved semen could have a positive effect on the motility, viability, and energy status of sperm cells and their ability to bind to the *zona pellucida* of oocytes.

Vitamins E and C are natural antioxidants. The effect of semen extender supplementation with different concentrations and combinations of Trolox (water-soluble vitamin E) and L-ascorbic acid (vitamin C) on selected quality parameters of frozen-thawed canine spermatozoa was determined in this study.

Vitamin E is the primary component of the antioxidant system of sperm cells and one of the key compounds protecting plasma membranes against peroxidative damage (41). Vitamin E is soluble in lipids, and it could serve as the first line of defence against the peroxidation of polyunsaturated fatty acids on phospholipids with a membranous structure (5). Furthermore, vitamin E neutralises free radicals, protects the cell membrane against ROS, and enhances the functions of other antioxidants (12). Lipid peroxidation reactions are inhibited in membranes by eliminating peroxy ($\text{ROO}\cdot$), alkoxy ($\text{RO}\cdot$), and other

free radicals which are generated during the conversion of lipid hydroperoxides in the peroxidative chain reaction (3, 25). Ascorbic acid is a water-soluble antioxidant that acts as a key cofactor in hydroxylation and amidation processes (13). Ascorbic acid and vitamin E are involved in the synthesis of collagen, proteoglycan, and components of the intercellular matrix (33, 12). The addition of vitamin C to extenders could improve sperm performance by minimising cell damage caused by radical scavenging (3).

In the present study, the addition of different concentrations of Trolox did not exert a significant influence on selected sperm parameters regardless of incubation time. However, Trolox-enhanced samples were characterised by a somewhat higher percentage of cryopreserved motile spermatozoa and sperm cells exhibiting linear motility without incubation time bearing upon this motility. A significant improvement of progressive motile sperm was shown in humans after the addition of vitamin E ($40 \mu\text{mol/L}$) to the freezing medium (30). These results were similar to those of other studies. For example, Mohammed *et al.* (19) noted a significant improvement of progressive motile sperm and sperm viability after the addition of $40 \mu\text{mol}$ of Trolox during the cryopreservation processes. On the other hand, Taylor *et al.* (37), found that vitamin E supplementation to semen cryopreservation medium at a concentration of $200 \mu\text{mol}$ significantly improved semen post-thaw motility, but not vitality. Furthermore, in cryopreserved boar semen, vitamin E

supplementation enhanced sperm motility, mitochondrial membrane potential, and membrane integrity in different ejaculate fractions (23, 24). Moreover, it was reported that addition of α -tocopherol to fresh and stored boar semen samples significantly increased all kinematic parameters (15). Zeitoun and Al-Damegh (42) demonstrated that optimal post-thaw motility is achieved when vitamin E concentration in ram semen extender does not exceed 5 IU/mL. In frozen ram semen, low concentrations of vitamin E also improved the quality of post-thaw sperm (2).

The concentrations of vitamin E applied in this study could be sub-optimal for preserving the viability of canine sperm cells, and the observed effects could differ with the concentration of the antioxidant supplement. In comparison with the control, the total percentage of motile spermatozoa cryopreserved with the addition of vitamin C ($200 \mu\text{M}/1 \times 10^9$ spermatozoa) was significantly higher only in rapidly thawed semen, which could be attributed to lower lipid peroxidation damage. Similar results were reported in other studies where extender supplementation with vitamin C increased the motility of spermatozoa in canine semen (40), bull semen (27, 33, 34, 35), and ram semen (4). Furthermore, Verma and Kanwar (39) showed a positive effect on human sperm motility of semen incubation with 800 mmol/L of ascorbic acid. However, paradoxically they found that higher doses of vitamin C ($>1,000 \text{ mmol/L}$) increased ROS levels, which decreased sperm motility.

Regardless of incubation time, the percentage of cryopreserved motile spermatozoa ($P \leq 0.05$) and the percentage of spermatozoa with active mitochondria and intact plasma membranes ($P \leq 0.05$) were significantly higher after 60 and 120 min incubation of post-thawed semen cryopreserved with the addition of vitamins C + E ($200 + 200 \mu\text{M}/1 \times 10^9$ spermatozoa). Similar results were noted in Mangalitsa boars where the combined addition of vitamins E and C had the most beneficial effect on the motility of frozen-thawed sperm (38). Mittal *et al.* (18) demonstrated that extender supplementation with vitamins C and E improved the motility, viability, and plasma membrane integrity of cryopreserved spermatozoa of Bhadawari bulls. In boars, extender supplementation with vitamins E and C together had more beneficial effect on the quality of frozen-thawed sperm than these non-enzymatic antioxidants when applied separately (14).

In conclusion, the results of this study indicate that total sperm motility, percentage of sperm cells exhibiting linear motion, and percentage of spermatozoa with intact plasma membranes and active mitochondria were higher when a supplemented extender for the cryopreservation of canine semen was improved with a combination of vitamins C + E at a concentration of $200 + 200 \mu\text{M}/1 \times 10^9$ spermatozoa than with vitamins E and C separately.

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Animal Rights Statement: The experiment was approved by the Local Ethics Committee at the University of Warmia and Mazury in Olsztyn.

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