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Original Paper

Effects of Encapsulated Rosemary Extract on Turkey Semen Quality Characteristics During Short-Term Low Temperature Storage

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The use of fresh semen stored at low temperatures for artificial insemination is common in avian and especially turkey commercial farms due to its more practicality and compromised sperm structural integrity and functional activity of cryopreserved semen. Various natural compounds have been used to preserve the sperm quality and fertility during short term storage. This study aimed to evaluate the *in vitro* effects of rosemary extract, in both un-encapsulated and encapsulated forms, on turkey spermatozoa during short-term low-temperature storage. Semen samples collected from mature male turkeys were treated with different concentrations (0, 5, 10, and 25 μg.mL^{-1}) of either form of rosemary extract. The effects on sperm motility, progressive motility, membrane integrity, mitochondrial activity and membrane potential, apoptosis, necrosis, reactive oxygen species (ROS), superoxide production, lipid and protein oxidation, DNA fragmentation, and bacterial load were evaluated at 0, 2, and 24 hours of storage. Supplementation with 5 and 10 μg.mL^{-1} rosemary extract improved sperm motility, mitochondrial activity, and membrane potential ($P \leq 0.0001$). However, 25 μg.mL^{-1} had a detrimental effect. Encapsulated rosemary extract yielded better sperm quality outcomes than the un-encapsulated form, especially at the 25 μg.mL^{-1} dose and after 24 hours of storage. These findings suggest that rosemary extract in semen extender can enhance sperm quality. Moreover, encapsulation may mitigate the toxicity of high extract doses and support prolonged preservation of sperm fertility.

Keywords: turkey semen, oxidative stress, rosemary extract, encapsulation

1 *Introduction*

Artificial insemination in commercial farms of turkey is an undeniable prerequisite for optimum fertility as due to extensive genetic selection for bright weight, natural mating possibility was reduced and eliminated for male turkey (Di Iorio et al., 2020). Oxidative damage to sperm resulting from reactive oxygen species generated by the cellular components of semen is one of the main causes for the decline in motility and fertility of spermatozoa during short and long term storage (Bansal & Bilaspuri, 2011). Sperm cells, due to their lower antioxidant production potential and higher polyunsaturated fatty acid content in their membrane phospholipids, are more vulnerable to ROS production during oxidative stress (Cerolini et al., 2006).

Short-term liquid storage of semen is used in avian species commercial farms due to more feasibility, however cryopreservation of avian semen is available to facilitate the management of genetic resources, including the preservation of rare and economically important

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breeds (Blesbois & Brillard, 2007). Sperm fertilizing capacity is dramatically lost by increasing storage time at 4 °C harming the insemination efficiency. Hence, strategies were acquired to mitigate the detrimental effects of cold shock and oxidative stress particularly on the sperm cell membrane (Blesbois & Brillard, 2007). Various antioxidants with different characteristics and specificity are used in semen extenders to control ROS production and their subsequent deleterious effects on the sperm functionality (Sapanidou et al., 2023). Antioxidants are commonly included in semen extenders to reduce ROS production and limit oxidative damage. Natural antioxidants are particularly favored due to their cost-effectiveness and reduced side effects (Kowalczyk, 2022).

Rosmarinus officinalis has been reported to have strong antioxidant, anti-inflammatory and anti-carcinogenic properties due to the presence of considerable amount of polyphenols and volatile oil including carnosic, rosmarinic acids, and camphor in it (Di Iorio et al., 2020). Supplementation of *R. officinalis* powder in rooster feed (Seyfi et al., 2023) and also the diet of rams (Ali et al., 2024) has significantly improved the sperm functional parameters. Moreover, addition of *R. officinalis* essential oil into semen extender showed positive effects on sperm quality characteristics in roosters (Touazi et al., 2018), bulls (Daghigh-Kia et al., 2014). In the study of Daghigh-Kia et al. (2014), 5 and 10 $g.L^{-1}$ of rosemary water solubilized extract supplementation into bulls semen extender caused positive effects on semen quality characteristics, whereas addition of 870 $\mu g.ml^{-1}$ of hydro-distilled rosemary extract in rooster semen extender caused negative effects on semen parameters (Touazi et al., 2018) demonstrating a high variability in response to rosemary extract possibly due to type of extract, extraction procedure, content of active metabolites, type of animal species and weather the semen is cryopreserved or kept at 4 °C. Moreover, high concentrations of metabolites in extracts with antioxidant activity might be toxic to sperm cells immediately after supplementation, whereas by prolonging the storage time and enhancement in ROS production, the necessity of the presence of metabolites with antioxidant activity would increase.

Essential oils are hydrophobic and volatile, which limits their stability and functionality in aqueous environments like semen extenders. Encapsulation offers a solution by enclosing these compounds within biocompatible materials, allowing controlled release and enhanced solubility (Sundar & Parikh, 2023). Therefore, this study aimed to evaluate the efficacy of both encapsulated and un-encapsulated *Rosmarinus officinalis* essential oil (ROEO) in preserving the functional and oxidative parameters of turkey spermatozoa during short-term storage at 4 °C.

2 Materials and Methods

2.1 Sample Collection

Semen samples were obtained from the turkey breeding company Branko Nitra, a.s. (Nitra, Slovakia). The samples were collected by cloacal massage from 40 adult Big 6 males and transported to the laboratory in a thermal container (37 °C; M&G Int, Renate, Italy) within 30 min. Only ejaculates meeting established quality standards were processed further in the experiments. These included a minimum volume of 0.2–0.3 mL, sperm concentration of at least 4–5 $\times$ 10^9 $sperm.mL^{-1}$, total motility ≥80%, and progressive motility ≥55%. Only clean ejaculates, free of fecal or urinary contamination, were used to ensure sample integrity and reproducibility of results. Animal handling procedures complied with the ethical guidelines of the Slovak Animal Protection Regulation RD 377/12, in accordance with European Directive 2010/63/EU (Lenický et al., 2021).

Each ejaculate was divided into 8 equal aliquots and each aliquot was diluted either with the control or experimental diluent using a dilution ratio of 1 : 50–1 : 70 depending on the initial sperm concentration. The 8 experimental treatments were included 0, 5, 10 and 25 $\mu g.ml^{-1}$ of either un-encapsulated or encapsulated forms of ROEO. The semen samples were stored at 4 °C and sperm parameters and bacteriological assessments were evaluated at time of 0 (immediately after dilution; control group), 2 and 24 h post-dilution. The semen samples from each treatment were pre-warmed to 37 °C prior to each assessment round for specific analysis and 100 µL of sample from each treatments was transferred into a sterile Eppendorf tube and kept at -20 °C for subsequent bacteriological examination.

The encapsulation of ROEO was done through formation of the *Rosmarinus officinalis* essential oils (ROEO)/β-CD inclusion system prepared by co-precipitation method. Briefly, 500 mg of β-cyclodextrin (β-CD, Sigma Aldrich, Germany) was dissolved in 5 mL of an ethanol/millipore-water mixture (1 : 2, v/v) under continuous stirring induced by a magnetic stirrer at 55 ±3 °C until complete dissolution. Then, 125 mg of ROEO (weight ratio of 20 : 80 of ROEO to β-CD) was added to the β-CD solution at the same temperature and then resulting dispersion was cooled to 4 °C in a refrigerator for 1 hour. The precipitated ROEO/β-CD was collected by vacuum filtration using a Hirsch funnel and washed twice with ethanol to remove the essential oil adsorbed on the surface of β-CD. After drying under vacuum for 3 h at 40 °C, 387 mg of ROEO/β-CD inclusion system was obtained, transferred to a well closed vial and stored at 4 °C until further biological evaluation. The chemical composition of ROEO is presented in Table 1.

Table 1 Chemical composition of *Rosmarinus officinalis* essential oils

Components	(%)	Components	(%)	Components	(%)
1,8-cineole	40.4	γ-terpinene	1.2	3-octanone	–
menthol	–	(Z)-β-farnesene	–	α-phellandrene	0.2
linalool acetate	–	(E)-β-ocimene	0.1	δ-3-carene	0.2
linalool	1.2	hexyl butanoate	–	viridiflorol	–
menthone	0.1	geranyl acetate	–	*n*-amyl isovalerate	–
camphor	11.9	α-humulene	0.7	*n*-hexanol	–
menthyl acetate	–	3-carvomenthenone	–	pinocarvone	0.1
α-pinene	8.7	α-terpinene	0.6	tricyclene	0.1
β-pinene	6.9	caryophyllene oxide	0.6	*p*-cimene	0.1
neo-menthol	–	sabinene	0.4	β-elemene	–
(E)-caryophyllene	5.3	β-bourbonene	–	carvone	–
methofuran	–	δ-cadinene	0.3	isopulegol	–
borneol	3.9	α-thujene	0.4	*cis*-3-hexenol	–
camphene	3.5	α-terpinolene	0.4	β-thujone	Tr
isomenthone	–	α-copaene	0.4	α-ylangene	Tr
α-terpineol	2.7	neryl acetate	–	aromadendrene	Tr
α-limonene	2.4	iso-menthyl acetate	–	3-octanol	–
ocimene	2.2	(E)-β-farnesene	–	ethyl hexanoate	–
lavandulyl acetate	–	α-amorphene	0.2	*cis*-linalool oxide	–
germacrene D	Tr	hexyl tiglate	–	capryl acetate	–
pulegone	–	α-bisabolol	–	nerol	–
cis-sabinene hydrate	0.2	3-octanol	–	caryophyllene oxide	–
β-myrcene	1.5	isomenthol	–	epi-α-cadinol	–
bornyl acetate	1.4	bicyclogermacrene	–	Total	99.5
4-terpineol	1.1	*trans*-linalool oxide	–		

2.2 Motility Evaluation

The computer-assisted sperm analysis (CASA) system (version 14.0 TOX IVOS II, Hamilton-Thorne Biosciences, Beverly, CA, USA) was used to evaluate the sperm motion characteristics as previously described (Lenický et al., 2021). Total and progressive motility were defined as the proportion of spermatozoa moving at a velocity greater than 5 $\mu m.s^{-1}$ and proportion of spermatozoa moving at speeds higher than 20 $\mu m.s^{-1}$ respectively.

2.3 Sperm Membrane Integrity

A triple fluorescent staining protocol was used to assess sperm membrane integrity. The cells were stained with CFDA (carboxylfluorescein diacetate; Sigma-Aldrich, St. Louis, MO, USA) for quantification of cellular esterase activity as an indicator of cell viability. The staining protocol was included PI (propidium iodide; Sigma-Aldrich, St. Louis, MO, USA; 5 $\mu g.mL^{-1}$ in PBS), as an indicator of dead cells, and the nucleic acid dye DAPI (4'6-diamidine-2-phenylindole; Sigma-Aldrich, St. Louis, MO, USA) for counting the number of spermatozoa. The membrane integrity quantification was done by Glomax Multi + spectro-fluoro-luminometer (Promega, Madison, WI, USA).

2.4 Mitochondrial Membrane Potential

The mitochondrial membrane potential was evaluated by JC-1 Assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 100 µL of the sample was stained with 5 µL of JC-1 working solution and incubated for 30 min at °C. Then, the samples were centrifuged for 5 min at 150 × g at 25 °C and washed twice with a washing buffer provided by the commercial kit. Finally, the samples were transferred to a dark 96-chamber plate and analyzed by a combined GloMax-Multi+ spectro-fluoro-luminometer (Promega, Madison, WI, USA) using appropriate filters (485 nm excitation and 535 nm for emission in case of the JC1 monomers, and 520–570 nm excitation and 570–610 nm for emission with respect to the JC1 polymers) (Duracka et al., 2019). The resulting

ΔΨm was expressed as the ratio of JC-1 complexes to JC-1 monomers (red/green ratio).

2.5 Mitochondrial Activity

Mitochondrial metabolic activity was assessed through Mitochondrial Toxicity Test (MTT). For this purpose, 20 µL of tetrazolium salt (Sigma-Aldrich, St. Louis, USA) was dissolved in PBS (Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride; Sigma-Aldrich, St. Louis, USA) with the concentration of 5 mg.mL^{-1} and added to each sample and incubated for 1 h at 37 °C. Subsequently, formazan crystals were dissolved using 80 µL of isopropanol (propan-2-ol; Centralchem, Bratislava, Slovakia). Optical density was measured by GloMax-Multi + (Promega Corporation, Madison, WI, USA) at wavelength of 570 nm against 620 nm as reference. The results were expressed as percentage of the control group set to 100% (Tvrdá et al., 2016a).

2.6 Superoxide Production

The nitroblue-tetrazolium (NBT) test was done for quantification of intracellular superoxide radical. The NBT salt was dissolved in PBS containing 1.5% DMSO (dimethyl sulfoxide, Sigma-Aldrich) to a final concentration of 1 mg.mL^{-1} and added to the cells (100 µL per well). After 1 h incubation (shaker, 37 °C, 95% air atmosphere, 5% CO_2), the cells were washed twice with PBS and centrifuged at 300 × g for 10 min. Then, the cells and formazan crystals were dissolved in 2 M KOH (potassium hydroxide; Centralchem) in DMSO. Optical density was determined at wavelength of 620 nm against 570 nm as reference by a micro-plate ELISA reader (Anthos MultiRead 400). Data was expressed in percentage of the SC Control (Control 1) set to 100% (Tvrdá et al., 2016b).

2.7 Sperm Chromatin Structure Assay

The susceptibility of sperm DNA to *in situ* denaturation following low pH treatment as an indicator of sperm DNA damage was assessed with the sperm chromatin structure assay (SCSA). Each sample was adjusted to 2 × 10^6 sperm.mL^{-1} with TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.4; Sigma-Aldrich, St. Louis, MO, USA), mixed with 0.4 mL of acid detergent (0.17% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.4; Sigma-Aldrich, St. Louis, MO, USA). Following 30 s, the cells were stained with acridine orange solution (0.1 m citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0; 6 µg.mL^{-1} acridine orange; Sigma-Aldrich, St. Louis, MO, USA). Stained samples were transferred to a 96 black well plate and analyzed using the GloMax-Multi+ combined spectro-fluoro-luminometer using appropriate filters to detect double-stranded DNA green fluorescence (530 ±30 nm) and single-stranded DNA red fluorescence (>630 nm).

2.8 Apoptotic Spermatozoa

The externalization of phosphatidylserine as an indicator of sperm apoptosis was quantified using the Annexin V-FLUOS kit (Roche, Basel, Switzerland) in combination with propidium iodide (PI) to differentiate apoptotic and necrotic cells. The samples were washed in binding buffer and adjusted to a concentration of 1 × 10^6 spermatozoa.mL^{-1}. Annexin V (5 µL) was added to 100 µL of the suspension and incubated at room temperature for 20 min, followed by addition of 5 µL PI and further incubation for at least 10 min. The stained samples were analyzed using the GloMax-Multi+spectro-fluoro-luminometer (Promega Corporation, Madison, WI, USA). using appropriate filters (~494 nm excitation and ~518 nm for emission in case of AV, and ~535 nm excitation and ~617 nm for emission with respect to PI). Spermatozoa were classified into three subpopulations:

1. viable (AV^-/PI^-),
2. apoptotic (AV^+/PI^-),
3. necrotic (AV^-/PI^+),

with results expressed as percentages. Cells exhibiting both AV and PI positivity were treated as necrotic (Najafi et al., 2024).

2.9 ROS Measurement

Reactive oxygen species (ROS) production in semen samples was measured by chemiluminescence procedure described by Lenický et al. (2021). The luminescent signal produced through interaction of spermatozoa and luminol (Sigma-Aldrich, St. Louis, MO, USA) was quantified by GloMax-Multi + spectro-fluoro-luminometer. The results were expressed as relative light units (RLU)/s/10^6 spermatozoa.

2.10 Protein Oxidation and Lipid Peroxidation

The samples were washed by PBS and centrifuged at 300 × g for 10 min. The resulting pellets were lysed overnight in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Then the lysates were centrifuged at 5,000 × g for 10 min and a semi-automated Monza photometric analyzer (Randox Laboratories, Crumlin, UK) was used for measuring total protein content using the total protein kit (DiaSys, Holzheim, Germany) (Lenický et al., 2021). Protein carbonyl (PC) as an indicator of protein oxidation were measured using dinitrophenylhydrazine (DNPH) assay (Weber et al., 2015) with some modification (Lenický et al., 2021). The amount of PC expressed as nmol PC.mg^{-1} protein considered as

protein oxidation index. Lipid peroxidation was evaluated by the thiobarbituric acid-reactive substances (TBARS) procedure for measuring malondialdehyde (MDA) as a predominant lipid peroxidation by-product. A standard multi-plate protocol was acquired and lipids oxidative damage was expressed as μmol.g^{-1} of MDA (Lenický et al., 2021).

2.11 Statistical Analysis

Data were analyzed by GLM procedure of SAS software in a 3 × 4 × 2 factorial arrangement. Data were tested for normal distribution through the univariate procedure. The model included the fixed effects of storage time (0, 2 and 24 h of storage times), ROEO supplementation level (0, 5, 10 and 25 μg.mL^{-1} of ROEO), type of ROEO (un-encapsulated and encapsulated forms of ROEO) and their interaction effects. Least-square means were computed and tested for differences by the Tukey's test. Difference between least-squared means was considered to be significant at P <0.05.

3 Results and Discussion

3.1 Sperm Motility

Prolonging the storage time significantly reduced sperm motility (Table 2). Increasing ROEO concentration up to 10 μg.mL^{-1} improved sperm motility, whereas further enhancement up to 25 μg.mL^{-1} reduced sperm motility comparing to control group (Table 3). Encapsulation of ROEO improved sperm motility (Table 4). At time 0, various concentrations of ROEO caused no difference in sperm motility comparing to control group, whereas after 2 h of storage, 5 and 10 μg.mL^{-1} of ROEO improved and 25 μg.mL^{-1} of ROEO reduced sperm motility comparing to control group (Table 5). After 24 h of storage, 10 μg.mL^{-1} of ROEO showed the highest improvement in sperm motility followed by 5 μg.mL^{-1} of ROEO, whereas 25 μg.mL^{-1} of ROEO caused sperm motility similar to control treatment. There was no difference in types of ROEO in supplementation levels of 0, 5 and 10, whereas in 25 μg.mL^{-1} of ROEO, encapsulated form was more beneficial on sperm motility comparing to un-encapsulated form (Table 6). Forms of ROEO caused no difference in sperm motility at 0 and 2 h of storage time, whereas after 24 h of storage, encapsulated ROEO showed significant improvement in sperm motility comparing to un-encapsulated form (Table 7).

Progressive motility decreased in response to prolonging the storage time from 0 to 24 h (Table 2). Moreover, 10 μg.mL^{-1} of ROEO caused highest progressive motility fallowed by 5 and 25 μg.mL^{-1} of ROEO comparing to control group (Table 3). Encapsulation of ROEO caused higher progressive motility (Table 4). Although 5 and 10 μg.mL^{-1} of ROEO caused higher progressive motility comparing to control group at time 0, these concentrations of ROEO showed more profound effect on progressive motility comparing to the control group at 2 and 24 h of storage (Table 4). There was no difference in progressive motility affected by types of ROEO in 0 and 2 h of storage time, whereas after 24 h of storage, encapsulated form showed higher progressive motility comparing to un-encapsulated form (Table 7).

3.2 Membrane Integrity

Membrane integrity was reduced in response to enhancement in storage time (Table 2). Levels of 5 and 10 μg.mL^{-1} of ROEO significantly increased

Table 2 Effects of storage time (h) on semen quality parameters

Storage time (h)	0	2	24	SEM[1]	*P*-value
Motility (%)	86.59^{a}	75.42^{b}	32.17^{c}	0.39	0.0001
Progressive motility (%)	36.88^{a}	32.74^{b}	14.58^{c}	0.35	0.0001
Membrane integrity (%)	83.84^{a}	76.78^{b}	57.76^{c}	0.37	0.0001
Necrotic spermatozoa (%)	6.31^{c}	10.52^{b}	13.34^{a}	0.23	0.0001
Mitochondrial membrane potential (JC-1 Units)	0.88^{a}	0.75^{b}	0.53^{c}	0.005	0.0001
Mitochondrial activity (%)	104.40^{c}	114.65^{b}	127.10^{a}	1.02	0.0001
Superoxide production (%)	96.90^{a}	92.42^{b}	82.85^{c}	0.63	0.0001
Apoptotic spermatozoa (%)	9.85^{c}	13.95^{b}	21.17^{a}	0.42	0.0001
Sperm DNA fragmentation (%)	8.91^{c}	13.21^{b}	21.66^{a}	0.26	0.0001
ROS (RLU.sec^{-1}.10^{6})	6.44^{c}	13.26^{b}	16.00^{a}	0.20	0.0001
Protein oxidation (nanomole protein carbonyls.mg^{-1} protein)	1.44^{c}	2.91^{b}	4.15^{a}	0.04	0.0001
Lipid oxidation (MDA; μmol.gr^{-1} protein)	1.19^{c}	1.82^{b}	2.58^{a}	0.03	0.0001

1 – standard error of the mean; a, b, c, d – values in columns with different letters differ significantly (P ≤0.0001)

Table 3 Effects of Rosemary extract levels (μg.mL^{-1}) on semen quality parameters

Rosemary extract levels (μg.mL^{-1})	0	5	10	25	SEM[1]	*P*-value
Motility (%)	60.06^{c}	68.89^{b}	71.33^{a}	58.64^{c}	0.45	0.0001
Progressive motility (%)	22.21^{d}	30.65^{b}	33.14^{a}	26.27^{c}	0.41	0.0001
Membrane integrity (%)	70.33^{b}	74.32^{a}	75.74^{a}	70.78^{b}	0.43	0.0001
Necrotic spermatozoa (%)	12.42^{a}	8.69^{c}	8.43^{c}	10.70^{b}	0.27	0.0001
Mitochondrial membrane potential (JC-1 Units)	0.67^{b}	0.75^{a}	0.76^{a}	0.69^{b}	0.006	0.0001
Mitochondrial activity (%)	100.00^{c}	129.20^{b}	134.63^{a}	97.70^{c}	1.18	0.0001
Superoxide production (%)	100.00^{a}	80.40^{b}	80.30^{b}	102.20^{a}	0.72	0.0001
Apoptotic spermatozoa (%)	17.68^{a}	13.16^{b}	11.90^{b}	17.22^{a}	0.48	0.0001
Sperm DNA fragmentation (%)	16.96^{a}	12.57^{b}	12.14^{b}	16.71^{a}	0.30	0.0001
ROS (RLU.sec^{-1}.10^{6})	12.72^{b}	10.32^{c}	9.47^{c}	15.09^{a}	0.23	0.0001
Protein oxidation (nanomole protein carbonyls.mg^{-1} protein)	3.19^{a}	2.62^{b}	2.29^{c}	3.23^{a}	0.05	0.0001
Lipid oxidation (MDA; μmol.gr^{-1} protein)	1.96^{b}	1.70^{c}	1.58^{c}	2.22^{a}	0.04	0.0001

1 – standard error of the mean; a, b, c, d – values in columns with different letters differ significantly ($P \leq 0.0001$)

Table 4 Effects of Type of rosemary extract on semen quality parameters

Type of rosemary extract	UE[1]	EN[2]	SEM[3]	*P*-value
Motility (%)	63. 26^{b}	66.20^{a}	0.32	0.0001
Progressive motility (%)	27.59^{b}	28.55^{a}	0.29	0.02
Membrane integrity (%)	72.17^{a}	73.42^{b}	0.30	0.005
Necrotic spermatozoa (%)	10.31	9.81	0.19	0.06
Mitochondrial membrane potential (JC-1 Units)	0.71	0.73	0.004	0.06
Mitochondrial activity (%)	114.30	116.46	0.83	0.07
Superoxide production (%)	92.03^{a}	89.41^{b}	0.51	0.0005
Apoptotic spermatozoa (%)	15.45	14.52	0.34	0.05
Sperm DNA fragmentation (%)	15.04 ^{a}	14.15^{b}	0.21	0.004
ROS (RLU.sec^{-1}.10^{6})	11.91	11.89	0.16	0.93
Protein oxidation (nanomole protein carbonyls.mg^{-1} protein)	2.93^{a}	2.74^{b}	0.03	0.001
Lipid oxidation (MDA; μmol.gr^{-1} protein)	1.87	1.86	0.02	0.95

1 – un-encapsulated; 2 – encapsulated; 3 – standard error of the meana; b, c, d – values in columns with different letters differ significantly ($P \leq 0.0001$)

membrane integrity, whereas 25 μg.mL^{-1} of ROEO caused no difference comparing to control group (Table 3). Moreover, encapsulation of ROEO improved membrane integrity regardless of storage time and ROEO supplementation level (Table 4). Supplementation of ROEO into semen extender caused no difference in membrane integrity at time 0 of storage, whereas at times 2 and 24 h of storage, 5 and 10 but not 25 μg.mL^{-1} of ROEO significantly improved membrane integrity comparing to control group (Table 5). Moreover, encapsulated form of ROEO caused higher membrane integrity comparing to un-encapsulated form in highest supplementation level (Table 7). Both un-encapsulated and encapsulated forms of ROEO showed similar membrane integrity at 0 and 2 h of storage, whereas after 24 h of storage, encapsulated ROEO showed higher membrane integrity comparing to un-encapsulated form (Table 6).

3.3 Mitochondrial Potential

The MMP was reduced in response to enhancement in time of storage (Table 2). Levels of 5 and 10 μg.mL^{-1} of ROEO improved MMP, however 25 μg.mL^{-1} of ROEO caused no effect on MMP comparing to control group (Table 3). Various levels of ROEO caused no effect on MMP at 0 h of storage, whereas at 2 and 24 h of storage times, increasing concentration of ROEO improved MMP comparing to control group, however 25 μg.mL^{-1} of ROEO showed no effect comparing to control group at 24 h of storage (Table 5).

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Table 5 Interactions of storage time (h) and Rosemary extract levels (µg.mL^{-1}) on semen quality parameters

Storage time (h)	0				2				24				SEM1	*P*-value
Rosemary extract levels (µg.mL^{-1})	0	5	10	25	0	5	10	25	0	5	10	25		
Motility (%)	83.48^{bc}	88.22^{a}	88.37^{a}	86.32^{ab}	74.30^{e}	79.62^{d}	82.07^{cd}	65.72^{f}	22.40^{i}	38.84^{h}	43.55^{g}	23.89^{i}	0.78	0.0001
Progressive motility (%)	33.60^{bc}	38.17^{a}	39.32^{a}	36.45^{ab}	27.74^{d}	36.15^{ab}	36.11^{ab}	30.97^{cd}	5.50^{h}	17.64^{f}	23.99^{e}	11.40^{h}	0.71	0.0001
Membrane integrity (%)	85.68^{a}	82.58^{ab}	84.28^{a}	82.82^{ab}	74.00^{e}	78.49^{cd}	79.34^{bc}	75.32^{de}	51.32^{g}	61.90^{f}	63.60^{f}	54.22^{g}	0.75	0.0001
Necrotic spermatozoa (%)	6.88^{ef}	6.22^{f}	6.17^{f}	6.00^{f}	14.00^{b}	8.68^{de}	8.84^{de}	10.59^{cd}	16.40^{a}	11.17^{c}	10.30^{cd}	15.52^{ab}	0.47	0.0001
Mitochondrial membrane potential (JC-1 Units)	0.87^{a}	0.90^{a}	0.89^{a}	0.88^{a}	0.68^{d}	0.78^{bc}	0.80^{b}	0.74^{c}	0.48^{f}	0.58^{e}	0.60^{e}	0.44^{f}	0.01	0.0001
Mitochondrial activity (%)	100.00^{d}	104.80^{d}	106.10^{d}	106.70^{d}	100.00^{d}	127.90^{c}	132.60^{c}	98.10^{d}	100.00^{d}	154.90^{b}	165.20^{a}	88.30^{e}	2.05	0.0001
Superoxide production (%)	100.00^{bc}	96.20^{c}	95.80^{c}	95.60^{c}	100.00^{bc}	82.30^{d}	84.40^{d}	103.00^{ab}	100.00^{bc}	62.70^{e}	60.70^{e}	108.00^{a}	1.26	0.0001
Apoptotic spermatozoa (%)	10.00^{d}	10.94^{d}	9.48^{d}	9.00^{d}	16.86^{b}	12.30^{cd}	10.75^{d}	15.90^{bc}	26.18^{a}	16.26^{bc}	15.47^{bc}	26.77^{a}	0.845	0.0001
Sperm DNA fragmentation (%)	8.70^{e}	9.22^{de}	8.82^{e}	8.93^{e}	16.42^{b}	11.97^{c}	11.52^{cd}	12.96^{c}	25.78^{a}	16.53^{b}	16.09^{b}	28.24^{a}	0.52	0.0001
ROS (RLU.sec^{-1}.10^{6})	6.66^{e}	6.26^{e}	6.31^{e}	6.54^{e}	13.58^{c}	11.32^{d}	10.34^{d}	17.80^{b}	17.94^{b}	13.38^{c}	11.78^{cd}	20.93^{a}	0.41	0.0001
Protein oxidation (nanomole protein carbonyls.mg^{-1} protein)	1.53^{e}	1.39^{e}	1.37^{e}	1.46^{e}	3.26^{bc}	2.82^{c}	2.10^{d}	3.45^{b}	4.79^{a}	3.65^{b}	3.39^{b}	4.78^{a}	0.09	0.0001
Lipid oxidation (MDA; µmol.gr^{-1} protein)	1.13^{f}	1.21^{f}	1.24^{f}	1.20^{f}	1.85^{e}	1.63^{e}	1.60^{e}	2.21^{cd}	2.90^{b}	2.27^{c}	1.91^{de}	3.26^{a}	0.06	0.0001

1– standard error of the mean; a, b, c, d – values in columns with different letters differ significantly ($P \leq 0.0001$)

Table 6 Interactions of storage time (h) and type of Rosemary extract on semen quality parameters

Storage time (h)	0		2		24		SEM1	*P*-value
Type of rosemary extract	UE[1]	EN[2]	UE[1]	EN[2]	UE[1]	EN[2]		
Motility (%)	86.16^{a}	87.03^{a}	74.44^{b}	76.41^{b}	29.18^{c}	35.15^{d}	0.55	0.0001
Progressive motility (%)	37.39^{a}	36.38^{a}	32.42^{b}	33.06^{b}	12.95^{d}	16.21^{c}	0.50	0.0002
Membrane integrity (%)	83.37^{a}	84.30^{a}	77.51^{b}	76.06^{b}	55.62^{d}	59.90^{c}	0.53	0.0001
Necrotic spermatozoa (%)	5.90^{d}	6.73^{d}	10.91^{c}	10.14^{c}	14.14^{a}	12.55^{b}	0.33	0.001
Mitochondrial membrane potential (JC-1 Units)	0.88	0.89	0.75	0.75	0.51	0.54	0.007	0.22
Mitochondrial activity (%)	104.60^{d}	104.20^{d}	115.45^{c}	113.85^{c}	122.85^{b}	131.35^{a}	1.14	0.001
Superoxide production (%)	97.05^{a}	96.75^{a}	94.15^{ab}	90.70^{b}	84.90^{c}	80.80^{d}	0.89	0.07
Apoptotic spermatozoa (%)	9.80	9.91	14.55	13.35	22.02	20.32	0.59	0.29
Sperm DNA fragmentation (%)	8.72^{d}	9.11^{d}	13.38^{c}	13.05^{c}	23.04^{a}	20.28^{b}	0.37	0.0001
ROS (RLU.sec^{-1}.10^{6})	6.44	6.44	12.95	13.57	16.34	15.67	0.29	0.09
Protein oxidation (nanomole protein carbonyls.mg^{-1} protein)	1.45^{d}	1.43^{d}	2.95^{c}	2.87^{c}	4.39^{a}	3.92^{b}	0.06	0.002
Lipid oxidation (MDA; µmol.gr^{-1} protein)	1.15^{c}	1.24^{c}	1.82^{b}	1.82^{b}	2.63^{a}	2.53^{a}	0.04	0.13

1 – un-encapsulated; 2 – encapsulated; 3 – standard error of the mean; a, b, c, d – values in columns with different letters differ significantly ($P \leq 0.0001$)

3.4 Mitochondrial Activity

Mitochondrial activity was increased in response to prolonging storage time (Table 2). Supplementation of 10 μg.mL^{-1} of ROEO caused the highest mitochondrial activity fallowed by 5 μg.mL^{-1} of ROEO, whereas 25 μg.mL^{-1} of ROEO showed no difference comparing to control group (Table 3). Levels of 5 and 10 μg.mL^{-1} of ROEO at 2 and 24 h of storage increased mitochondrial activity comparing to control group, whereas this effect was not evidenced in 0 h of storage (Table 5). In addition, 25 μg.mL^{-1} of ROEO lowered mitochondrial activity comparing to control group at 24 h of storage. There was no difference in mitochondrial activity affected by type of ROEO at 0 and 2 h of sperm storage, whereas at 24 h of storage, encapsulated form of ROEO increased mitochondrial activity comparing to un-encapsulated form (Table 6). Moreover, mitochondrial activity was not affected by type of ROEO in 0, 5 and 10 μg.mL^{-1} of supplementation levels, however encapsulated form of ROEO enhanced mitochondrial activity at 25 μg.mL^{-1} of supplementation level (Table 7).

3.5 DNA Fragmentation

Enhancement in turkey semen storage time increased sperm DNA fragmentation (Table 2). Levels of 5 and 10 μg.ml^{-1} of ROEO reduced DNA fragmentation, whereas 25 μg.ml^{-1} of ROEO caused no change comparing to control group (Table 3). The DNA fragmentation was lower in encapsulated comparing to un-encapsulated ROEO (Table 4). Supplementation of ROEO caused no difference in sperm DNA fragmentation at 0 h of storage, whereas at 2 and 24 h of storage times, ROEO supplementation reduced DNA fragmentation comparing to control group, however the highest level of ROEO caused no difference at 24 h of storage (Table 5). Encapsulation of ROEO caused no difference in DNA fragmentation at times 0 and 2 h of storage, whereas at 24 h of storage, ROEO encapsulation lowered DNA fragmentation (Table 6). Encapsulated ROEO lowered DNA fragmentation comparing to un-encapsulated form in 25 μg.ml^{-1} of ROEO, whereas there was no difference caused by type of ROEO at 0, 5 and 10 μg.ml^{-1} of supplementation level (Table 7).

3.6 Apoptotic Spermatozoa

Prolongation of storage time considerably increased the percentage of apoptotic spermatozoa (Table 2). Levels of 5 and 10 μg.ml^{-1} of ROEO reduced the proportion of apoptotic sperm (Table 3). At time of 0, ROEO supplementation caused no difference in apoptotic spermatozoa, whereas in times 2 and 24 h of storage, 5 and 10 but not 25 μg.ml^{-1} of ROEO significantly reduced apoptotic spermatozoa comparing to control group (Table 5). Moreover, at highest concentration of ROEO, encapsulated form of ROEO was more influential in reducing apoptotic spermatozoa proportion (Table 7).

3.7 Necrotic Spermatozoa

Necrotic spermatozoa proportion increased in response to enhancement in sperm storage time (Table 2). Supplementation of ROEO reduced the proportion of necrotic spermatozoa comparing to control group, however the efficacy of 25 μg.mL^{-1} was lower than 5 and 10 μg.mL^{-1} of ROEO. At time of 0, there was no difference in necrotic spermatozoa affected by ROEO supplementation, whereas at times 2 and 24 h of storage, 5 and 10 but not 25 μg.mL^{-1} of ROEO reduced the proportion of necrotic spermatozoa comparing to control group (Table 5). Moreover, at time 24 h of storage, encapsulated form of ROEO reduced the proportion of necrotic spermatozoa (Table 6). In 25 μg.mL^{-1} of ROEO supplementation level, encapsulated form of ROEO lowered necrotic spermatozoa (Table 7).

3.8 Protein Oxidation

Protein oxidation increased in response to enhancement in storage time (Table 2). Supplementation levels of 5 and 10 but not 25 μg.mL^{-1} of ROEO significantly reduced protein oxidation comparing to control group (Table 3). Moreover, encapsulated form of ROEO reduced protein oxidation (Table 4). Various supplementation levels of ROEO caused no difference in protein oxidation in 0 time of storage whereas level of 5 and levels of 5 and 10 μg.mL^{-1} of ROEO respectively in 2 and 24 h of storage times, reduced protein oxidation comparing to control group (Table 5). There was no difference in protein oxidation at 0 and 2 h of storage affected by type of ROEO, whereas encapsulated ROEO reduced protein oxidation at 24 h of storage (Table 6). There was no difference in protein oxidation caused by type of ROEO in supplementation levels of 0, 5 and 10 μg.mL^{-1}, whereas in 25 μg.mL^{-1} of ROEO supplementation level, encapsulated ROEO was more beneficial in reducing protein oxidation (Table 7).

3.9 Antioxidant Activity

The concentration of MDA was increased in response to enhancement in storage time (Table 2). Moreover, levels of 5 and 10 μg.mL^{-1} of ROEO reduced MDA concentration, whereas 25 μg.mL^{-1} of ROEO increased MDA comparing to control group (Table 3). Although there was no difference in MDA content affected by various ROEO supplementation levels at 0 h of storage, 5 and 10 μg.mL^{-1} of ROEO lowered MDA level comparing to control group at 2 and 24 h of storage, however this reduction was not statistically significant at 2 h of storage

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Table 7 Interactions of Rosemary extract levels (µg.mL^{-1}) and Type Rosemary extract on semen quality parameters

Rosemary extract levels (µg.mL^{-1})	0		5		10		25		SEM1	*P*-value
Type of rosemary extract	UE[1]	EN[2]	UE[1]	EN[2]	UE[1]	EN[2]	UE[1]	EN[2]		
Motility (%)	60.06^{d}	60.06^{d}	69.00^{b}	68.78^{b}	70.11^{ab}	72.54^{a}	53.88^{e}	63.40^{c}	0.62	0.0001
Progressive motility (%)	22.21	22.21	30.11	31.19	32.43	33.84	25.60	26.94	0.58	0.59
Membrane integrity (%)	70.33^{cd}	70.33^{cd}	74.12^{ab}	74.52^{ab}	75.00^{ab}	76.47^{a}	69.21^{d}	72.36^{bc}	0.61	0.05
Necrotic spermatozoa (%)	12.42^{a}	12.42^{a}	8.72^{b}	8.66^{b}	8.28^{b}	8.58^{b}	11.84^{a}	9.56^{b}	0.38	0.004
Mitochondrial membrane potential (JC-1 Units)	0.67	0.67	0.75	0.76	0.76	0.76	0.67	0.71	0.009	0.09
Mitochondrial activity (%)	100.00^{bc}	100.00^{bc}	128.60^{a}	129.80^{a}	134.86^{a}	134.40^{a}	93.73^{c}	101.66^{b}	1.67	0.04
Superoxide production (%)	100.00^{b}	100.00^{b}	81.20^{c}	79.60^{c}	81.80^{c}	78.80^{c}	105.13^{a}	99.26^{b}	1.02	0.03
Apoptotic spermatozoa (%)	17.68^{ab}	17.68^{ab}	13.22^{cd}	13.11^{cd}	11.91^{d}	11.88^{d}	19.02^{a}	15.42^{bc}	0.69	0.02
Sperm DNA fragmentation (%)	16.96^{a}	16.96^{a}	12.26^{c}	12.88^{c}	12.56^{c}	11. 72^{c}	18.38^{a}	15.03^{b}	0.42	0.0001
ROS (RLU.sec^{-1}.10^{6})	12.72	12.72	10.12	10.52	9.41	9.54	15.39	14.78	0.33	0.50
Protein oxidation (nanomole protein carbonyls.mg^{-1} protein)	3.19^{ab}	3.19^{ab}	2.72^{cd}	2.53^{de}	2.30^{e}	2.28^{e}	3.50^{a}	2.97^{bc}	0.07	0.003
Lipid oxidation (MDA; µmol.gr^{-1} protein)	1.96	1.96	1.70	1.71	1.57	1.60	2.25	2.19	0.05	0.90

1 – un-encapsulated; 2 – encapsulated; 3 – standard error of the mean; a, b, c, d – values in columns with different letters differ significantly ($P \leq 0.0001$)

(Table 5). Moreover, 25 µg.mL^{-1} of ROEO significantly increased MDA content comparing to control group at 2 and 24 h of storage times.

Superoxide production significantly reduced in response to prolongation of storage time (Table 2). Levels of 5 and 10 µg.mL^{-1} of ROEO reduced superoxide production, whereas 25 µg.mL^{-1} of ROEO caused no difference comparing to control group (Table 3). Moreover, encapsulation of ROEO reduced superoxide production comparing to un-encapsulated form (Table 4). Superoxide production was not affected by ROEO supplementation at 0 h of storage, however after 2 and 24 h of storage, 5 and 10 µg.mL^{-1} of ROEO considerably reduced superoxide production compared to control group, whereas in both of these storage time, 25 µg.mL^{-1} of ROEO increased superoxide production compared to control group (Table 5). Encapsulated and un-encapsulated forms of ROEO showed no difference in superoxide production in 0, 5 and 10 µg.mL^{-1} of ROEO levels, however in supplementation level of 25 µg.mL^{-1} of ROEO, encapsulated form reduced superoxide production (Table 7).

The produced ROS substantially increased in response to prolonging storage time (Table 2). Moreover, 5 and 10 µg.mL^{-1} of ROEO reduced ROS production, whereas 25 µg.mL^{-1} of ROEO increased ROS production comparing to control group (Table 3). Various supplementation levels of ROEO caused no difference in ROS concentration at 0 h of storage, whereas at 2 h after storage, levels of 5 and 10 µg.mL^{-1} of ROEO reduced ROS concentration and this proportional inhibition of ROS production comparing to control group was even more profound at 24 h of storage (Table 5). In addition, 25 µg.mL^{-1} of ROEO increased ROS production comparing to control group at both 2 and 24 h of storage times.

3.10 Discussion

Oxidative stress caused during semen storage either in short-term low temperature storage or cryopreservation induced by various environmental factors during semen handling, is the main detrimental effector of sperm quality characteristics (Wang et al., 2025). Synthetic antioxidants are used commonly in semen extenders of various species for controlling oxidative stress. Concerns related to toxicity, biocompatibility and costs changed interest toward utilization of natural antioxidants derived from plants. The essential oils can exert major antioxidant properties. The rosemary extract as a natural plants source product, has been shown to have strong antioxidant activities making it a suitable candidate for application in semen extender (Kloy et al., 2020).

Supplementation of ROEO has been reported to have beneficial effects on sperm motility characteristics and lipid peroxidation (Daghigh-Kia et al., 2014), sperm viability and abnormality, DNA fragmentation, apoptosis and fertility potential (Ali et al., 2024). Addition of 10 mg.ml^{-1} of rosemary extract in bulls semen extender caused significant improvement in sperm motility after thawing fallowing cryopreservation (Daghigh-Kia et al., 2014). In the study of Touazi et al. (2018) in which rosemary extract was supplemented in rooster semen extender, total and progressive motility was improved during short term low temperature storage. These beneficial effects on sperm motility characteristics was also exhibited when rosemary extract and rosemary leaf powder was supplemented in the diets of rams and rooster respectively (Seyfi et al., 2023). Rosemary aqueous extract supplementation into semen extender of buck semen improved sperm motility, membrane integrity and mitochondrial activity and reduced sperm abnormality, MDA production and apoptotic rate (Zanganeh et al., 2013). The detrimental effect of ROEO on sperm quality characteristics in treatments with 25 µg.ml^{-1} in current study is in accordance with the study of Touazi et al. (2018) possibly due to spermicidal effects of ROEO through disruption of cell membrane and acrosome integrity. Detrimental effect of high ROEO dosage has been reported to originate from depolarization of mitochondrial membrane induced by impaired membrane potential and affected ionic cycling (Bakkali et al., 2008). The concentration of ROEO with detrimental effect on motility was far higher compared to current research. The composition and proportional concentration of essential oils in ROEO can be affected by several factors including the concentration of essential oils, plant genotype, soil type, climate conditions, extraction method and harvesting procedure. In addition, there are some other factors such as type of species, animal basal diet, semen extender composition and storage procedure which can affect the final consequences of ROEO supplementation.

The lowered MDA production as a consequence of ROEO supplementation in current study is in agreement with Zanganeh et al. (2013) in which rosemary extract was administered in buck semen extender. However rosemary extract supplementation in semen extender of bulls showed no effects on SOD concentration after thawing fallowing cryopreservation (Daghigh-Kia et al., 2014). In addition to lipid peroxidation, excessive oxidative stress can lead to protein oxidation leading to impaired functions of key proteins responsible for sperm structure and motility, sperm metabolism and energy production (Wang et al., 2025). The lowered ROS and SOD production alongside with reduced protein and lipid oxidation in response to ROEO supplementation in current work can be related to presence of active antioxidant compounds in ROEO. The isoprenoid quinones present in ROEO act

as a chain terminator of free radicals and also as chelators of ROS. In addition, phenolic compounds of ROEO act as primary antioxidants when reacting with the lipid and hydroxyl radicals converting them into stable products and act as metal ion chelators leading to reduction in formation of reactive species derived from oxygen (Fang & Wada, 1993). Carnosic acid and carnosol as scavengers of peroxyl radicals has been reported to be more effective than BHA, BHT and propyl gallate on peroxidation of membrane lipids (Chen et al., 1992). Carnosic acid and carnosol has been reported to be responsible for more than 90% of antioxidant properties of ROEO, however rosmarinic acid and hesperidin also was reported as radical scavenger (Yang et al., 2013).

In spermatozoa, ROS is primarily produced through electron leakage from mitochondrial electron transport chain, cytosolic L-amino acid oxidases and NADPH oxidase system (Aitken, 2017). Over-production of ROS can be triggered by environmental stress such as ambient temperature, incubation and centrifugation during sperm preparation and due to sperm cell limited cytosol, cell membrane high polyunsaturated fatty acids level, lack of endogenous and cytoplasmic antioxidant enzymes and DNA repair mechanisms, this enhanced ROS production can lead to oxidative stress which can further exacerbate ROS production. Increased ROS production can trigger lipid peroxidation and consequently disturb membrane fluidity and integrity leading to impaired sperm motility, capacitation, acrosome reaction and fertilization (Aitken, 2017). Lipid peroxidation can cause apoptosis through activation of Nuclear Factor Kappa B, Mitogen-Activated Protein Kinase, and Protein Kinase C signaling pathways. Moreover, ROS induced lipid peroxidation in plasma membrane can lead to a type of regulated cell death termed ferroptosis which is characterized by an iron-dependent lipid peroxidation. Apoptotic stimuli can trigger the transcription of pro-apoptotic genes, including Bax and subsequently open mitochondrial permeable transition pore leading to mitochondrial swelling and cytochrome C release into cytoplasm. Upon entrance into the cytoplasm, cytochrome C activate caspases and subsequently specific endonucleases, causing sperm DNA fragmentation (Taylor et al., 2004). Moreover, cytochrome C can also cause DNA fragmentation through increased ROS production and oxidative DNA damage (Cui et al., 2000). In non-apoptotic spermatozoa, DNA fragmentation can occur independent of caspase activation (Hai et al., 2024). Increased exposure of the sperm cell to H2O2 and inactivation of caspase 8 was shown to cause a type of cell death with the morphological features typical of cell necrosis termed necroptosis (Hai et al., 2024).

Most sperm quality parameters evaluated in current work were positively affected by encapsulation of ROEO especially at 24 h of storage and also in supplementation level of 25 $\mu g.ml^{-1}$ of ROEO. Plants extract and essential oils are highly active, volatile and sensitive to environmental conditions such as oxidants, light, pH and temperature and also hydrophobic which make their incorporation in aqueous products challenging. Encapsulation technique was acquired to enhance water solubility, reduced their volatility and prolong their presence in medium and affectivity (Sundar & Parikh, 2023). Encapsulated rosemary extract has been reported to enhanced the oxidative and microbiological stability of beef meat during refrigerated storage (Rashidaie Abandansarie et al., 2019). To the best of our knowledge, this is the first experiment evaluating the encapsulated ROEO utilization in semen extender for short term storage. The overall positive effects of encapsulated ROEO on sperm quality characteristics can be related to its improved solubility in semen aqueous medium. Moreover, the higher beneficial effect of encapsulated ROEO at 25 $\mu g.ml^{-1}$ of supplementation compared to un-encapsulated form can be related to preventing the likely detrimental effects of active compounds on sperm cell immediately after administration and the slow, continuous and prolonged release of these active compounds from encapsulated form might be the reason for higher beneficial effects on sperm parameters at 24 h of storage.

4 Conclusion

In summary, rosemary essential oil (ROEO), particularly in its encapsulated form, demonstrated promising antioxidant properties that contributed to improved sperm quality in turkey semen during short-term storage. Lower concentrations (5 and 10 $\mu g.mL^{-1}$) proved most effective, while the encapsulation technique enhanced the safety and efficacy of ROEO, especially at higher doses and extended storage durations. These findings highlight the potential of encapsulated plant-based bioactives as practical supplements in poultry semen extenders. Future *in vivo* studies are needed to confirm the fertility-enhancing effects observed *in vitro* and to evaluate long-term implications for reproductive success and progeny health.

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Conflict of Interest

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Authors Contributions

Sara Ataei-Nazari: investigation, writing original draft and revision. Filip Benko: investigation and data curation. Michal Ďuračka: investigation. Tomáš Slanina: conceptualization, resources and investigation. Miroslava Kačániová: methodology, investigation, data curation and project administration. Nenad Vukovic: methodology, investigation and data curation. Eva Tvrda: conceptualization, investigation, data analysis and project administration.

AI and AI-Assisted Technologies Use Declaration

No AI or AI-assisted technologies were used in the writing, data analysis, or preparation of this manuscript.

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