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Research article

CRYOPROTECTIVE AND ANTIBACTERIAL EFFECTS OF RESVERATROL ON FROZEN-THAWED TURKEY SPERMATOZOA

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ABSTRACT

This study aimed to evaluate the effects of resveratrol (RES) supplementation in the cryopreservation medium on the conventional and non-conventional quality parameters, oxidative status, and microbial profile of cryopreserved turkey semen. Ejaculates (n = 40) were cryopreserved in a modified Beltsville extender either without RES (the cryopreserved control [$Ctrl_C$]) or with 5, 10, or 25 µM RES . Fresh semen served as a negative control ($Ctrl_N$). Post-thaw analyses included assessments of motility, viability, membrane functionality, mitochondrial activity, DNA fragmentation, apoptotic status, reactive oxygen species (ROS), protein carbonyl (PC), and malondialdehyde (MDA) levels. Bacteriological analysis was performed using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The results obtained indicate that the sperm quality, particularly the mitochondrial activity, was significantly improved following the administration of 5 µM RES compared to $Ctrl_C$ ($p < 0.05$). All RES doses were particularly effectvive in preventing the ROS overgeneration and associated lipid peroxidation relative to $Ctrl_C$ ($p<0.05$). The bacterial load decreased in a dose-dependent manner, whereas RES was found effective in enhancing the antibacterial efficacy of gentamicin in the frozen-thawed semen. In conclusion, this study demonstrates that supplementing the modified Beltsville extender with 5 or 10 µM RES improves post-thaw turkey semen quality.

Key words:
antioxidant, cryopreservation, semen, spermatozoa, turkey, resveratrol

Abbreviations:
AV – Annexin V; CASA – computer-assisted sperm analysis; DMSO – dimethyl sulfoxide; DNA – deoxyribonucleic acid; DNPH – dinitrophenylhydrazine; IF – index of fragmentation; MALDI-TOF – matrix-assisted laser desorption/ionization time-of-flight; MDA – malondialdehyde; mOsm/kg – milliosmoles per kilogram; MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; PC – protein carbonyls; PI – propidium iodide; RES – resveratrol; RLU – relative light units; SDS – sodium dodecyl sulfate; TBARS – thiobarbituric acid-reactive substances; TCA – trichloroacetic acid

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INTRODUCTION

The poultry industry has evolved significantly over the past decades, particularly in the efficiency of meat and egg production (Surai & Fisinin, 2016). However, consumer demand for turkey breast meat has led to excessive selective breeding aimed at increasing chest muscle mass, resulting in the loss of natural reproductive capability in males.
Unlike wild turkeys which have retained this ability, commercial turkey breeding depends on artificial insemination for an effective transmission of genes associated with a broad breast, a highly desirable trait in poultry production (Smyth and Leighton, 1953).
One of the most important advancements in artificial insemination is the application of frozen-thawed spermatozoa, which has broad applicability in both small-scale ("backyard farm") and large-scale industrial poultry production (Nikitkina et al., 2022; Zong et al., 2023). The overall fertility of the breeding flock is directly affected by the quality and quantity of spermatozoa, as well as the genetic and environmental factors responsible for their formation (Slanina et al., 2015; Restoux et al., 2022). Currently, sperm cryopreservation represents an effective means of preserving male genetic material in cryobanks (Iaffaldano et al., 2016). Conversely, further refinements in poultry sperm cryopreservation are necessary, including the optimization of equilibration, cooling, and freezing protocols, as well as improvements in semen extenders and cryoprotectants (Woelders et al., 2022). Cryopreserved poultry spermatozoa are known to exhibit limitations, including reduced post-thaw motility and fertilization potential, which may be attributed to cryotrauma, osmotic shock, or oxidative stress (Mphaphathi et al., 2012; Iaffaldano et al., 2016; Zong et al., 2023).
Recent research has led to the development of advanced semen cryopreservation media supplemented with cytoprotective and antioxidant molecules to mitigate cryoinjury and preserve sperm viability under low temperatures (Partyka and Niżański, 2021). In this study, the molecule of interest was resveratrol (3,5,40-trihydroxistilbene; RES), which is a polyphenol present in an ample array of fruits, nuts and wine, and which acts as a natural phytoalexin and phytoestrogen (Gambini et al., 2015). Over the past decade, RES has been a focus point of scientific attention for its potential as a novel supplement to semen extenders, primarily due to its antioxidant properties which may assist in the prevention and/or attenuation of sturctural or functional loss of biomolecules pivotal for the survival of male gametes under low temperatures. Studies on mammalian (Branco et al., 2010; Sun et al., 2020; Assunção et al., 2021; Lone et al., 2021) and avian (Rezaie et al., 2021) spermatozoa have reported improvements in motility, membrane integrity, and mitochondrial metabolism following RES supplementation during cryopreservation. Additionally, frozen-thawed semen enriched with RES has exhibited reduced DNA damage and lipid peroxidation (Branco et al., 2010; Tvrdá et al., 2015). Furthermore, RES possesses notable antimicrobial properties, making it a promising candidate for use alone or in combination with conventional antibiotics to prevent or control bacterial contamination in semen used for artificial insemination (Abedini et al., 2021; Lenický et al., 2021).
Despite substantial evidence supporting the beneficial effects of RES on sperm cryophysiology, accompanied by a lack of data on turkey spermatozoa, this study aimed to ellucidate the impact of RES on the conventional, non-conventional and oxidative characteristics of cryopreserved turkey spermatozoa. Moreover, the study examined the potential of RES to reduce bacterial contamination during the freezing and thawing process of turkey semen.

MATERIAL AND METHODS

Sample collection and cryopreservation

The Beltsville turkey semen extender (Continental, AG, Hanover, Germany) was used as the cryopreservation medium. Soybean lecithin (1% w/v; Sigma Aldrich, St. Louis, MO), gentamicin (0.25 g/L; Sigma Aldrich, St. Louis, MO), and glycerol (3% v/v; Sigma Aldrich, St. Louis, MO) were supplemented to the medium (340 mOsm/kg, pH 7.5) (Rezaie et al., 2021).
Turkey 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. 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 semen sample was divided into five aliquots. One aliquot served as negative control (fresh semen) and was diluted in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 40 million sperm/mL before immediate evaluation. The phosphate buffered saline used for the negative control dilution contained no antibiotics. The remaining aliquots were diluted in freshly

prepared extender to 40 million sperm/mL. For the positive (cryopreserved) control, 0.1% DMSO (dimethyl sulfoxide; Sigma-Aldrich, St. Louis, MO, USA) was added to the cryopreservation medium. In the experimental groups, the cryopreservation medium was supplemented with 5, 10, or 25 µM resveratrol (RES; Sigma-Aldrich, St. Louis, MO, USA), which had been pre-dissolved in DMSO. As in the cryopreserved control, all the experimental groups contained 0.25 g/L gentamicin. The diluted samples were cooled at 5°C for 2 h, then dispersed into 1.5 mL cryovials and stored in liquid nitrogen for one month. The frozen samples were thawed at 37°C for 90 s in a water bath, and immediately assessed (Lenický et al., 2021; Rezaie et al., 2021).

Sperm quality analysis

Sperm motion characteristics were analyzed using the computer-assisted sperm analysis (CASA) system (version 14.0 TOX IVOS II, Hamilton-Thorne Biosciences, Beverly, CA, USA) as previously described (Lenický et al., 2021). The total motility was defined as the percentage (%) of spermatozoa moving at a velocity greater than 5 µm/s, whereas the progressive motility encompassed spermatozoa moving at speeds exceeding 20 µm/s. In addition, secondary kinematic parameters were assessed, including the path velocity (VAP; µm/s), linear velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), lateral amplitude of head displacement (ALH; µm) and beat cross frequency (BCF; Hz).

Membrane integrity was assessed using differential eosin-nigrosine staining. A 2-µL aliquot of the sample was mixed with 4 µL of eosin (Eosin Y; Sigma-Aldrich, St. Louis, MO, USA) and 4 µL of nigrosine (Sigma-Aldrich, St. Louis, MO, USA), smeared onto a slide, and air-dried at room temperature. The slides were examined under a Leica DM IL LED microscope (Leica Microsystems, Wetzlar, Germany), with 300 cells counted per sample. Membrane integrity is expressed as the percentage (%) of viable spermatozoa (Slanina et al., 2018; Lenický et al., 2021).

Plasma membrane functionality was additionally evaluated using the hypo-osmotic swelling test (HOST) following the protocol of Rezaie et al. (2021). A 5-µL sperm suspension was added to 50 µL of hypo-osmotic solution and incubated at 37°C for 20 min. A total of 300 spermatozoa were evaluated under the Leica DM IL LED microscope to determine the percentage (%) of cells exhibiting tail swelling, indicative of intact membranes.

Acrosomal integrity was assessed using the fast green-rose bengal staining technique. A 20-µL aliquot of each sample was mixed with 20 µL of a staining solution containing fast green (Sigma-Aldrich, St. Louis, MO, USA) and rose bengal (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 70 s. A 10-µL aliquot of the stained sample was smeared onto a slide and air-dried. The slides were examined under a Leica DM IL LED microscope, with 300 cells counted per sample. The percentage (%) of spermatozoa with intact acrosomes was recorded (Lenický et al., 2021).

Mitochondrial activity was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, which measures the reduction of MTT tetrazolium salt to formazan by succinate-coenzyme Q reductase in functional mitochondria. MTT salt (Sigma-Aldrich, St. Louis, MO, USA) was added to each sample and incubated for 2 h. The reaction was terminated using isopropanol (Centralchem, Bratislava, Slovakia), and absorbance was measured at 570 nm against 620 nm using a Promega microplate photometer (Promega Corporation, Madison, WI, USA). Mitochondrial activity is expressed as a percentage (%) relative to the negative (fresh) control, which was set at 100% (Hazary & Wishart, 2001).

The sperm DNA fragmentation index was determined using the Halomax commercial kit (#HT-MG40, Halotech DNA, Madrid, Spain) following the manufacturer's instructions. A total of 300 spermatozoa per sample were analyzed using an epifluorescence microscope at ×40 magnification (Leica Microsystems, Wetzlar, Germany). The sperm DNA fragmentation is expressed as the DNA fragmentation index (%) (Blank et al., 2021).

The externalization of phosphatidylserine, indicative 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. Annexin V (5 µL) was added to 100 µL of the suspension and incubated at room temperature for 20 min, followed by the 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). Spermatozoa were classified into three subpopulations: (1) viable (AV^-/PI^-), (2) apoptotic (AV^+/PI^-), and (3) necrotic (AV^-/PI^+), with results expressed as percentages (%) (Najafi et al., 2024).

Reactive oxygen species (ROS) production by spermatozoa was quantified using a chemiluminescence assay as described previously (Lenický et al., 2021). The luminescent signal generated by spermatozoa interacting with luminol (Sigma-Aldrich, St. Louis, MO, USA) was measured using the Glomax Multi$^+$ spectro-fluoro-luminometer. The results are expressed as relative light units (RLU)/s/10^6 spermatozoa.

Preparation of cell lysates and assessment of oxidative damage

The samples were washed with PBS and centrifuged (Hettich Rotina 420, Tuttlingen, Germany) at 300× g for 10 min. The resulting pellets were lysed overnight in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysates were then centrifuged at 5,000× g for 10 min, and total protein content was quantified using the Total Protein kit (DiaSys, Holzheim, Germany) with a semi-automated Monza photometric analyzer (Randox Laboratories, Crumlin, UK) (Lenický et al., 2021; Baňas et al., 2024).

Protein oxidation, indicated by protein carbonyl (PC) levels, was assessed using a modified dinitrophenylhydrazine (DNPH) assay (Weber et al., 2015) as previously described (Lenický et al., 2021). Oxidative damage to proteins was expressed as nmol PC/mg protein.

Lipid peroxidation was quantified using the thiobarbituric acid-reactive substances (TBARS) assay to measure malondialdehyde (MDA), a predominant lipid peroxidation by-product. A standard multiplate protocol was followed (Lenický et al., 2021), and oxidative damage to lipids was expressed as µmol MDA/g protein.

Bacteriology

For the quantification and identification of bacterial colonies in the semen specimens, 100 µL of each sample was inoculated onto tryptone soya agar (TSA, Soyabean Casein Digest Agar; Merck, Darmstadt, Germany) or blood agar (BA, Blood Agar Base No. 2; Merck, Darmstadt, Germany) and incubated under conditions previously described (Kačániová et al., 2020). Bacterial species were identified using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Biotyper mass spectrometry (Bruker Daltonics, Bremen, Germany) as outlined by Lenický et al. (2021). Identification was performed with the Microflex LT instrument, flexControl software version 3.4, and the MALDI Biotyper Bruker Taxonomy database (Bruker Daltonics, Bremen, Germany) (Kačániová et al., 2020; Lenický et al., 2021).

Statistical analysis

Collected data were processed using GraphPad Prism (version 10.1.1 for Mac; GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed using a one-way ANOVA with Greenhouse-Geisser correction followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$. The following comparisons were conducted:

- The native control ($Ctrl_N$) was compared with the cryopreserved control ($Ctrl_C$).
- Groups supplemented with RES were compared with both $Ctrl_N$ and $Ctrl_C$.

RESULTS AND DISCUSSION

The supplementation of substances capable to protect spermatozoa subjected to thermal, osmotic or oxidative stress during the freeze-thaw process has become an attractive strategy in animal reproductive technologies. Several pivotal studies suggest that, in addition to their role as reactive oxygen species (ROS)-quenching molecules, vitamins C and E may protect the plasma membrane of frozen-thawed spermatozoa against cryo-induced premature activation. Consequently, numerous reports have emerged in recent years, proposing that ethnopharmacologically relevant biomolecules could enhance post-cryopreservation sperm quality more effectively than conventional antioxidants (Partyka and Niżański, 2021; Qamar et al., 2023). The present study was designed based on current evidence supporting the beneficial effects of resveratrol (RES) on male reproductive tissues and cells. The primary objective was to evaluate its potential in mitigating the cryo-induced loss of sperm viability in turkeys.

Sperm quality characteristics

Motility assessment revealed a significant ($p < 0.05$) reduction in the proportion of motile spermatozoa in the cryopreserved control ($Ctrl_C$) compared to fresh semen (the native control; $Ctrl_N$) (Table 1). Supplementation with 5 µM and 10 µM RES led to a significant improvement in the primary and secondary motion characteristics relative to $Ctrl_C$, except for the lateral amplitude and beat frequency. Significant differences in the motility were also observed between $Ctrl_N$ and all the RES-supplemented experimental groups ($p < 0.05$).

In addition to the improved post-thaw motility, a significantly higher proportion of spermatozoa with intact membranes and HOS-positivity was found in the cryopreserved groups treated with 5 µM and 10 µM RES in comparison with $Ctrl_C$ ($p < 0.05$) (Table 1). When compared to $Ctrl_N$, all the RES-supplemented groups exhibited significant changes in membrane stability ($p < 0.05$).

Detrimental effects of the freeze-thaw process were also observed with respect to the acrosome integrity. A significant decline in the integrity of the acrosome was found in both $Ctrl_C$ ($p < 0.05$) and all the RES-supplemented

groups (Table 1). However, all the RES-supplemented groups demonstrated significantly improved mitochondrial activity relative to $Ctrl_C$ ($p < 0.05$).

The detrimental impact of cryopreservation on sperm mitochondrial function was revealed by the MTT assay. Significant differences in the mitochondrial activity were observed between $Ctrl_N$ and $Ctrl_C$ ($p < 0.05$) and between $Ctrl_N$ and all the RES-supplemented groups ($p < 0.05$) (Table 1). However, all the RES-supplemented groups demonstrated significantly improved mitochondrial activity relative to $Ctrl_C$ ($p < 0.05$).

A high proportion of spermatozoa exhibiting cryoinjury was associated with increased sperm DNA damage, as indicated by significant differences in % IF between $Ctrl_N$ and $Ctrl_C$ ($p < 0.05$) (Table 1). The sperm chromatin dispersion test further revealed a significant increase in spermatozoa with DNA damage in all the RES-treated cryopreserved groups ($p < 0.05$). However, supplementation with 5 µM RES significantly reduced % IF compared to $Ctrl_C$ ($p < 0.05$).

The highest proportions of apoptotic and necrotic spermatozoa was found in $Ctrl_C$, with significantly higher values than $Ctrl_N$ ($p < 0.05$) (Table 1). Apoptotic and necrotic spermatozoa were also significantly more abundant in all the RES-supplemented groups relative to $Ctrl_N$ ($p < 0.05$). However, supplementation with 5 µM RES significantly mitigated sperm apoptosis and necrosis compared to $Ctrl_C$ ($p < 0.05$).

Table 1. Conventional and non-conventional quality parameters of turkey spermatozoa (n = 40) in the native state (negative control; $Ctrl_N$) and after cryopreservation in the absence (positive control; $Ctrl_C$) or presence of resveratrol (RES)

Parameter	Controls		Experimental groups		
	$Ctrl_N$	$Ctrl_C$	5 µM RES	10 µM RES	25 µM RES
Motility [%]	80.33±8.49	34.97±4.49^{*N}	52.33±6.60$^{*N; *C}$	47.30±4.64$^{*N; *C}$	40.00±4.56^{*N}
Progressive motility [%]	69.21±3.24	17.03±4.71^{*N}	30.56±5.26$^{*N; *C}$	27.51±3.44$^{*N; *C}$	24.11±2.78^{*N}
Path velocity [µm/s]	62.60±6.89	39.51±4.25^{*N}	50.50±4.96$^{*N; *C}$	47.33±4.78$^{*N; *C}$	42.19±5.23^{*N}
Linear velocity [µm/s]	44.32±3.96	30.24±2.59^{*N}	39.10±4.12$^{*N; *C}$	35.14±2.59$^{*N; *C}$	33.15±4.03^{*N}
Curvilinear velocity [µm/s]	108.22±9.36	90.05±8.85^{*N}	100.12±9.63$^{*N; *C}$	98.87±7.08$^{*N; *C}$	92.63±4.58^{*N}
Lateral amplitude [µm]	5.71±0.95	5.05±1.01	5.32±0.88	5.30±0.79	5.10±0.54
Beat cross frequency [Hz]	34.10±2.53	29.12±1.92	31.13±3.02	31.05±2.36	30.40±5.36
Membrane integrity [%]	73.76±4.13	50.33±4.98^{*N}	60.33±4.11$^{*N; *C}$	59.00±2.94$^{*N; *C}$	54.33±4.71^{*N}
HOS test positivity [%]	70.56±7.41	48.52±2.16^{*N}	57.00±8.32$^{*N; *C}$	59.35±4.19$^{*N; *C}$	53.81±4.08^{*N}
Acrosome integrity [%]	68.18±3.92	45.76±1.55^{*N}	57.39±3.73$^{*N; *C}$	55.45±2.76$^{*N; *C}$	49.39±4.28^{*N}
Mitochondrial activity [%]	100.00±1.63	37.70±2.05^{*N}	75.70±11.15$^{*N; *C}$	66.44±7.62$^{*N; *C}$	60.30±4.71$^{*N; *C}$
DNA fragmentation [%]	10.00±0.82	38.70±4.57^{*N}	26.70±4.03$^{*N; *C}$	32.00±1.63^{*N}	35.30±2.06^{*N}
Apoptotic spermatozoa [%]	18.73±4.87	51.95±1.62^{*N}	40.82±1.35$^{*N; *C}$	41.77±2.81$^{*N; *C}$	48.14±4.51^{*N}
Necrotic spermatozoa [%]	4.03±0.47	15.23±2.25^{*N}	8.75±3.38$^{*N; *C}$	9.05±3.90^{*N}	11.00±1.98^{*N}

Legend: significant if $p < 0.05$. ^{N} – vs. negative control; ^{C} – vs. positive control; RES – resveratrol; HOS – hypo-osmotic test

As shown in Table 2, exposure to low temperatures induced oxidative stress, as evidenced by the highest reactive oxygen species (ROS) levels recorded in $Ctrl_C$ ($p < 0.05$), which were significantly elevated compared to $Ctrl_N$.

Although none of the RES concentrations tested fully mitigated ROS overproduction, all the RES-treated groups exhibited significantly lower ROS levels compared to $Ctrl_C$ ($p < 0.05$). Correspondingly, the highest PC levels were detected in $Ctrl_C$, with a significant difference relative to $Ctrl_N$ ($p < 0.05$) (Table 2). While all the RES-treated groups exhibited reduced protein oxidation, a significant decrease in the protein carbonyl content was observed only in the group cryopreserved with 5 µM RES ($p < 0.05$) compared to $Ctrl_C$.

The degree of sperm lipid peroxidation mirrored a similar trend as recorded in the above-mentioned analyses of the oxidative profile (Table 2). Significantly higher MDA levels were recorded in $Ctrl_C$ against $Ctrl_N$ ($p < 0.05$). Inversely, a significantly decreased MDA concentration was noted in all the groups supplemented with RES ($p < 0.05$).

Table 2. Oxidative profile of turkey spermatozoa (n = 40) in the native state (negative control; $Ctrl_N$) and after cryopreservation in the absence (positive control; $Ctrl_C$) or presence of resveratrol (RES)

Parameter	Controls		Experimental groups		
	$Ctrl_N$	$Ctrl_C$	5 µM RES	10 µM RES	25 µM RES
ROS production [RLU/s/10^6 sperm]	4.33±0.49	17.97±2.10^{*N}	8.33±0.60$^{*N; *C}$	10.30±2.64$^{*N; *C}$	11.00±1.56$^{*N; *C}$
Protein oxidation [nmol PC/mg prot]	2.11±0.13	10.33±0.98^{*N}	7.30±0.91$^{*N; *C}$	8.00±0.94^{*N}	8.43±1.71^{*N}
Lipid peroxidation [µM MDA/g prot]	0.76±0.11	2.82±0.26^{*N}	0.94±0.22$^{*N; *C}$	1.05±0.19$^{*N; *C}$	1.31±0.28$^{*N; *C}$

Legend: significant if $p < 0.05$; ^{N} – vs. negative control; ^{C} – vs. positive control; RES – resveratrol; ROS – reactive oxygen species; RLU – relative light units; s – second; PC – protein carbonyls; MDA – malondialdehyde; prot – protein

As listed in Table 3, 23 bacterial species were detected and identified in the native turkey semen ($Ctrl_N$), including *Bacillus cereus* (*B. cereus*), *Bacillus subtilis* (*B. subtilis*), *Citrobacter braakii* (*C. braakii*), *Clostridium difficile* (*C. difficile*), *Empedobacter brevis* (*E. brevis*), *Enterococcus avium* (*E. avium*), *Enterococcus faecium* (*E. faecium*), *Escherichia coli* (*E. coli*), *Klebsiella oxytoca* (*K. oxytoca*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Morganella morganii* (*M. morganii*), *Proteus hauseri* (*P. hauseri*), *Proteus mirabilis* (*P. mirabilis*), *Proteus penneri* (*P. penneri*), *Proteus vulgaris* (*P. vulgaris*), *Pseudomonas putida* (*P. putida*), *Staphylococcus aureus* (*S. aureus*), *Staphylococcus chromogenes* (*S. chromogenes*), *Staphylococcus lentus* (*S. lentus*), *Staphylococcus saprophyticus* (*S. saprophyticus*), *Staphylococcus warneri* (*S. warneri*), *Streptococcus alactolyticus* (*S. alactolyticus*), and *Vibrio fluvialis* (*V. fluvialis*). The bacteriological analysis indicated that the gentamicin supplementation in the cryopreservation extender was insufficient to completely eradicate the bacterial contamination in the cryopreserved semen. However, a significant reduction in bacterial load was observed in $Ctrl_C$ compared to $Ctrl_N$ ($p < 0.05$). RES administration also contributed to the elimination of specific bacterial species, particularly *B. cereus*, *B. subtilis*, *E. faecium*, *S. chromogenes*, *S. lentus*, and *S. alactolyticus*. A significant reduction in the overall bacterial load was detected in all the RES-treated groups, with the most pronounced effect observed in the 25 µM RES group compared to both $Ctrl_C$ ($p < 0.05$) and $Ctrl_N$ ($p < 0.05$).

Table 3. Bacterial profile of turkey spermatozoa (n = 40) in the native state (negative control; $Ctrl_N$) and after cryopreservation in the absence (positive control; $Ctrl_C$) or presence of resveratrol (RES)

Parameter	**Controls**		**Experimental groups**		
	$Ctrl_N$	**$Ctrl_C$**	**5 µM RES**	**10 µM RES**	**25 µM RES**
Bacterial load [log CFU/mL]	14.00 ± 1.22	4.00±4.02^{*N}	2.10±0.28^{*N}	2.10±0.16^{*N}	1.35±0.10$^{*N; *C}$
Identified bacteria	*B. cereus* *B. subtilis* *C. braakii* *C. difficile* *E. brevis* *E. avium* *E. faecium* *E. coli* *K. oxytoca* *K. pneumoniae* *M. morganii* *P. hauseri* *P. mirabilis* *P. penneri* *P. vulgaris* *P. putida* *S. aureus* *S. chromogenes* *S. lentus* *S. saprophyticus* *S. warneri* *S. alactolyticus* *V. fluvialis*	*B. cereus* *B. subtilis* *C. difficile* *E. avium* *E. faecium* *S. chromogenes* *S. lentus* *S. alactolyticus*	*B. cereus* *B. subtilis* *C. difficile* *E. avium* *E. faecium* *S. chromogenes* *S. lentus* *S. alactolyticus*	*B. subtilis* *C. difficile* *E. avium* *S. chromogenes* *S. lentus* *S. alactolyticus*	*C. difficile* *E. avium*

Legend: significant if $p < 0.05$. ^{N} – vs. negative control; ^{C} – vs. positive control; RES – resveratrol; CFU – colony-forming units.

The evidence from previous studies highlights the diverse benefits of RES, attributed to its antioxidant, antibacterial, cytomodulatory, and anti-inflammatory properties (de la Lastra and Villegas, 2007). However, several *in vitro* studies suggest that RES exerts a dose-dependent effect on mammalian cells, acting as a dichotomous agent: low concentrations may promote cell viability, whereas high concentrations can induce cellular deterioration (Salehi et al., 2018).

The RES supplementation in this study enhanced the motility and viability of frozen-thawed turkey spermatozoa, consistent with findings by Rezaie et al. (2021), Kaeoket and Chanapiwat (2023), and Chen et al. (2024), who

reported similar beneficial effects of RES on cryopreserved rooster, boar, and ram spermatozoa, respectively. Nevertheless, our findings contrast with those of Collodel et al. (2011), who observed that supplementation with 100 μM RES in swim-up spermatozoa resulted in a complete loss of motility, whereas maximal progressive motility was recorded at 6 and 15 μM RES. Given these conflicting results, it is important to emphasize that RES may exert dichotomous effects across a broader concentration range. The RES doses selected in our study (5–25 μM) align with those identified as most effective in cryopreservation studies (Silva et al., 2016; Rezaie et al., 2021; Kaeoket and Chanapiwat, 2023; Galarza et al., 2024; Chen et al., 2024). Our CASA and MTT data are consistent with the findings of Mojica-Villegas et al. (2014), who reported an 8.0-fold increase in sperm motility and a 2.0-fold rise in viability following a pretreatment with 15 mg/mL RES before exposure to ferrous ascorbate (FeAA). Similarly, our previous studies on *in vitro* stored bull (Tvrdá et al., 2015) and mouse spermatozoa (Tvrdá et al., 2019) demonstrated that RES supplementation significantly preserved motility and membrane stability. In contrast, Silva et al. (2012) found that RES supplementation prior to freezing had no effect on the motility, membrane integrity, or acrosome functionality in ram spermatozoa.

Sperm motility is ATP-dependent and relies on mitochondrial function. Mojica-Villegas et al. (2014) reported that the pre-incubation with RES protected sperm mitochondria from FeAA-induced toxicity. Moreover, Silva et al. (2012) found that RES regulated the mitochondrial membrane potential in a dose-dependent manner without affecting the motility, membrane integrity, or acrosome functionality in cryopreserved ram spermatozoa. Another proposed mechanism by which RES stabilizes ATP levels in sperm mitochondria involves modulating calcium influx (Martín-Hidalgo et al., 2013), thus preventing cryocapacitation and enhancing sperm survival by preserving energy reserves (Li et al., 2018). Mitochondria are the primary endogenous source of ROS due to the involvement of the electron transport chain in autoxidation (Moretti et al., 2012). RES has been demonstrated to act as a potent *in vitro* antioxidant by directly preventing mitochondrial ROS overgeneration, protecting sperm lipids from oxidative damage, and modulating both ROS-producing and antioxidant enzymes (Gambini et al., 2015; Pasquariello et al., 2020). Another notable characteristic of RES is its ability to integrate into plasma membranes, stabilizing their fluidity and enhancing interactions with ROS during oxidative stress (Tadolini et al., 2000). Furthermore, Lagouge et al. (2006) reported that low RES concentrations induced the expression of genes regulating oxidative phosphorylation and mitochondrial biogenesis, thereby enhancing mitochondrial function. This suggests that low RES doses may strengthen the sperm's energy-producing machinery, preserving functional activity under cryogenic conditions. The protective effects of RES on the membrane and acrosome stability in frozen-thawed turkey spermatozoa observed in this study align with the findings by Longobardi et al. (2017) and Najafi et al. (2024), who reported that RES supplementation in cryopreserved buffalo and rooster spermatozoa preserved the membrane integrity and reduced the incidence of apoptotic and necrotic cells. Similarly, Li et al. (2018) found that RES supplementation improved the acrosome integrity in bovine sex-sorted spermatozoa.

Oxidative stress arising during cryopreservation is well known to negatively impact sperm membranous structures (Rui et al., 2017). Moreover, polyunsaturated fatty acids (PUFAs), which are integral to sperm membranes, are highly susceptible to peroxidative damage, leading to alterations in membrane fluidity, premature acrosome exocytosis, and impaired sperm-oocyte interactions (Partyka et al., 2012). The beneficial effects of RES on the post-thaw quality of turkey spermatozoa may be attributed primarily to its ability to suppress peroxidative modifications in membranous structures (Tadolini et al., 2000), a mechanism extensively documented in studies on animal spermatozoa (Tvrdá et al., 2015, 2019; Longobardi et al., 2017; Partyka & Niżański, 2021; Rezaie et al., 2021; Najafi et al., 2024). Polyphenols, including RES, readily permeate the lipid bilayer of sperm membranes (Tadolini et al., 2000), thus preventing oxidative chain reactions and the accumulation of lipid peroxidation byproducts. This protective effect stabilizes membrane integrity and preserves the intracellular environment of spermatozoa (Tadolini et al., 2000). Collectively, these findings support the observed higher proportion of turkey spermatozoa with intact cytoplasmic membranes and acrosomes following RES supplementation.

The presence of bacteria in poultry ejaculates, as previously reported (Lenický et al., 2021; Mohan et al., 2023; Tvrdá et al., 2023a, 2023b), necessitates the inclusion of effective antibacterial agents in extenders to protect spermatozoa from bacteriospermia. Consistent with earlier studies (Mohan et al., 2023; Tvrdá et al., 2023b; Rakha et al., 2024), the findings of this study indicate that bacterial contamination in extended ejaculates is a common occurrence and that antibiotic supplementation provides only limited control over bacterial persistence during cryopreservation and long-term semen storage. Bacteriological analysis suggests that gentamicin effectively eliminated Gram-negative bacteria, while its impact on Gram-positive bacteria was comparatively limited. These differences in bacterial susceptibility likely stem from the fundamental mode of action of gentamicin, which exhibits greater potency against Gram-negative bacteria but reduced efficacy against Gram-positive species (Chen et al., 2014).

RES has been identified as a highly effective antibacterial agent, primarily through the inhibition of ATP synthase activity in various bacteria, including *Escherichia coli* (Dadi et al., 2009), *Mycobacterium smegmatis* (Hotra et al., 2016), and *Arcobacter* spp. (Ferreira et al., 2014). Moreover, RES has been reported to induce bacterial DNA fragmentation, inhibit cell division (Hwang and Lim, 2015), and disrupt the plasma membrane, leading to increased potassium leakage and necrosis (Subramanian et al., 2014). A notable aspect of RES antibacterial activity lies in its ability to enhance the efficacy of aminoglycosides, such as gentamicin, by approximately 16-fold against *Staphylococcus aureus* and, to a lesser extent, against other Gram-positive bacteria, including *Staphylococcus epidermidis*, *Enterococcus faecium*, and *Enterococcus faecalis* (Nøhr-Meldgaard et al., 2018). Collectively, these findings suggest that RES may aid in preventing bacterial contamination in cryopreserved semen while offering a potentially lower spermatotoxicity compared to conventional antibiotics.

Finally, it is important to acknowledge that RES exhibits both antioxidant and prooxidant properties. Its effects are dose- and cell model-dependent, with high concentrations reported to induce ROS overproduction, leading to oxidative damage of critical biomacromolecules such as DNA, lipids, and proteins, particularly in the presence of transition metal ions like iron or copper (de la Lastra & Villegas, 2007). Accordingly, discrepancies in the reported effects of RES on spermatozoa may be attributed to multiple factors, including the animal species studied, the composition of the semen extender, the duration of exposure, and the administered dose, all of which influence its biological activity.

CONCLUSION

Our findings further support the *in vitro* membrane-stabilizing and motility-enhancing effects of RES at concentrations ranging from 5 to 10 μM in cryopreserved turkey spermatozoa. The development of novel semen extenders that selectively promote the survival of frozen-thawed spermatozoa while optimizing their energetic demands remains a key objective in poultry breeding. At low concentrations, RES may serve as a valuable supplement for reproductive technologies in poultry, particularly sperm cryopreservation and artificial insemination.

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Data protection (optional): The animals and sample collection were carefully handled in accordance with ethical guidelines as stated in the Slovak Animal Protection Regulation RD 377/12, which conforms to European Union Regulation 2010/63. Since semen collection is routinely performed at the company Branko Nitra, a.s., causing no harm or discomfort, a special Ethical Approval was not needed for this type of experiment. The samples were obtained directly from the company.

Data availability statement (optional): The data presented in this study are available upon reasonable request from the corresponding author.

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