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Article

Potential of Epicatechin as a Cryoadditive of Turkey Semen Storage Medium

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Abstract: This study examined the effectiveness of epicatechin (EPI) supplementation in enhancing the post-thaw quality of turkey semen. Semen samples collected from 40 adult Big 6 male turkeys were divided into five experimental groups: fresh semen, cryopreserved semen, and three cryopreserved groups with 10, 25, and 50 μM of EPI. Samples were cryopreserved in a Beltsville turkey semen extender containing soybean lecithin, kanamycin, and glycerol, then stored in liquid nitrogen for one month. Sperm motility, membrane integrity and functionality, acrosome integrity, mitochondrial activity, DNA fragmentation, apoptosis/necrosis, and ROS production were evaluated. Moreover, microbial analysis was performed on bacterial isolates using the MALDI Biotyper. Results demonstrated that EPI supplementation significantly improved all evaluated sperm quality parameters in a dose-dependent manner compared to the cryopreserved control, with 50 μM EPI yielding optimal results. At this concentration, sperm motility increased from 33.31% to 62.02%, membrane integrity from 52.67% to 67.33%, and mitochondrial activity from 43.56% to 65.79%. Additionally, 50 μM EPI reduced DNA fragmentation, apoptosis, and necrosis rates while demonstrating enhanced antimicrobial properties. These findings suggest that epicatechin enhances turkey sperm cryosurvival through multiple protective mechanisms, including antioxidant activity, membrane stabilization, and antimicrobial effects. EPI supplementation represents a promising strategy for improving the efficiency of turkey semen cryopreservation.

Keywords: sperm, reactive oxygen species, cryopreservation, antibacterial activity, sperm post-thaw quality

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1. Introduction

In bio-economic terms, cryopreservation allows long-term storage and transport of valuable genetic material, enabling strategic breeding decisions and the establishment of semen cryobanks for preserving diversity. Turkey semen cryopreservation enhances commercial breeding and genetic resource management while contributing to biosecurity. Freezing semen reduces bacterial concentrations approximately tenfold (1 log CFU/mL) and eliminates the necessity for transporting live birds. However, research indicates that certain Salmonella serovars demonstrate resistance to the freezing process and remain viable (Iaffaldano et al., 2010).

Although optimisation of the existing cryopreservation protocols has been extensively studied, turkey semen cryopreservation remains challenging, with fertility rates ranging from 15.8% to 84.3% after artificial insemination using frozen-thawed semen (Iaffaldano et al., 2016a). According to Zong et al. (2023), the cryosensitivity of turkey semen is caused by the combination of several factors, such as specific sperm head morphology, reduced cytoplasmic volume, high levels of polyunsaturated fatty acids, and low tolerance to changes in osmotic pressure. At the same time, thermal and osmotic stress induce the reactive oxygen species generation leading to peroxidative damage to the fatty acids present in the plasma membrane (Ansari et al., 2019). Therefore, the inclusion of bioactive substances of natural origin has become more popular in developing modern diluents.

Epicatechin (EPI) has shown promising results in bovine semen cryopreservation, enhancing sperm motility, membrane integrity, and DNA protection through its antioxidant properties (Banas et al., 2022; Baňas et al., 2023a). EPI also stabilizes transmembrane ion channels and prevents cryocapacitation, contributing to improved post-thaw sperm functionality (Baňas et al., 2023a). The positive effects on post-thaw quality have been demonstrated in buck semen when treated with quercetin (Almadaly et al., 2024) and in bull semen when treated with epigallocatechin-3-gallate (EGCG) (Parvizi Alan et al., 2022). These findings suggest that flavonoids, such as EPI, could potentially improve the post-thaw quality of turkey semen through similar antioxidant and cryoprotective mechanisms. However, only a limited number of studies have been published concerning the effect of bioactive substances on the cryopreservation of turkey semen (Benko et al., 2025; Torabi et al., 2025). Therefore, this study aimed to research the impact of epicatechin supplementation on sperm quality, oxidative characteristics, and bacterial load in freeze-stored turkey semen.

2. Materials and Methods

For cryopreservation, Beltsville turkey semen extender was used (Continental plastic, Delavan, WI, USA) containing 1% w/v soybean lecithin, 0.25 g/L gentamycin, and 3% v/v glycerol (all from Sigma Aldrich, St. Louis, MO). The final osmolality was 340 mOsm/kg and pH 7.5 (Rezaie et al., 2021).

Semen samples from 40 adult Big 6 male turkeys were collected at Branko Nitra, a.s. (Nitra, Slovakia) by cloacal massage. Semen samples for the purposes of this study were collected during the spring period, starting in late March and ending in mid-May to coincide with the peak gonadal activity, optimal spermatogenesis and in theory, the best semen quality in the BIG-6 line of domestic turkeys.

Fresh semen was handled at (35-37 °C) immediately after collection to prevent cold shock. The samples were transported to the laboratory in a thermal container. All manipulations, including dilution and initial evaluation were performed at 30-33 °C using pre-warmed equipment and media. Cooling only began once the extender was added and the sample was prepared for cryopreservation.

The animals were handled in accordance with Slovak Animal Protection Regulation RD 377/12 and European Directive 2010/63/EU ethical requirements (Lenický et al., 2021).

Only ejaculates meeting basic quality standards were processed. Semen samples were required to show ≥75% motility, membrane integrity exceeding 75%, and sperm concentration above 3–4 × 10^9 cells/mL. Ejaculates not meeting these criteria were excluded from further processing.

Each sample was divided into five aliquots. The fresh semen (negative control) was diluted with Dulbecco's phosphate-buffered saline, without calcium and magnesium (Sigma-Aldrich, St. Louis, MO, USA), to a concentration of 40×10^6 sperm/mL and directly examined. We diluted the rest of the aliquots in the same ratio with the extender prepared earlier. For positive control (cryopreserved semen), 0.1% DMSO (dimethyl sulfoxide) was added to the culture medium. The experimental groups were treated with 10, 25 or 50 μM epicatechin (EPI) pre-dissolved in DMSO. After dilution with the cryoprotective medium, the semen samples were gradually cooled to 5 °C by placing the tubes in a refrigerated water bath and lowering the temperature stepwise over approximately 2 hours to minimize cold shock. Once equilibrated, 1.5 mL aliquots of each sample were transferred into pre-labeled sterile cryovials using adjustable micropipettes. The cryovials were then positioned on a floating rack placed above liquid nitrogen to allow manual vapor-phase cooling (approximately −80 °C) for 10–15 minutes and subsequently immersed directly into liquid nitrogen (−196 °C) for long-term storage lasting one month prior to thawing and post-thaw evaluation.

The frozen samples were thawed in a 37°C water bath for 90 seconds and analyzed immediately after thawing (Lenický et al., 2021; Rezaie et al., 2021).

The sperm motility was evaluated using a CASA system (version 14.0 TOX IVOS II, Hamilton-Thorne Biosciences), evaluating total sperm motility with velocity >5 μm/s.

Membrane integrity and functionality were evaluated using eosin-nigrosin staining and hypo-osmotic swelling test (HOS test). Two μL of the sample was mixed with four μL of eosin and four μL of nigrosine on a microscopic slide, smeared, and allowed to air dry at room temperature. Under a Leica DM IL LED microscope, 300 cells/sample were counted to determine the percentage of living spermatozoa.

For the HOS test, 5 μL of sperm suspension was diluted in 50 μL hypo-osmotic solution and incubated at 37°C for 20 minutes. Three hundred cells were evaluated for the percentage of spermatozoa with swollen tails, indicating intact membranes.

For assessing acrosome integrity, we used Fast Green-Rose Bengal staining. Twenty μL of the staining solution was added to twenty μL of the sample, incubated for 70 seconds, and smeared. Three hundred cells were assessed and the percentage of sperm with intact acrosome was calculated.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used for the mitochondrial activity assessment. To each sample, we added MTT salt and incubated for 2 hours and then quenched the reaction using isopropanol. With a Promega microplate photometer, absorbance at 570 nm compared to 620 nm was determined, with results reported as a percentage of negative control.

DNA fragmentation was quantified using the Halomax commercial kit (Halotech DNA, Madrid, Spain). We counted 300 spermatozoa per sample under an epifluorescence microscope at 40× magnification and expressed results as the percentage DNA fragmentation index.

To measure apoptosis and necrosis, we used the Annexin V-FLUOS kit in combination with propidium iodide (PI). After preparing and staining the samples, we measured them using a Glomax Multi+ spectro-fluoro-luminometer. We categorized cells as: viable (Annexin V-negative (AV-) and propidium iodide-negative (PI-)), apoptotic (Annexin V-positive (AV+) and PI-), or necrotic (AV- and propidium iodide-positive (PI+)), expressing results as percentages.

Lastly, ROS production was quantified using a chemiluminescence technique with luminol. Readings were quantified with the Glomax Multi+ spectro-fluoro-luminometer and reported as relative light units (RLU)/s/10^6 spermatozoa.

Bacterial species were analysed using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Biotyper mass spectrometry (Bruker Daltonics, Bremen, Germany). Semen (100 μL per sample) was inoculated and incubated on tryptic soy agar (TSA, Soyabean Casein Digest Agar; Merck, Darmstadt, Germany). Colonies were counted and purified by the four-way streak plate method. The purified colonies were processed, and spectra were analysed using the Microflex LT, flexControl software version 3.4, and MALDI Biotyper Bruker Taxonomy database (Bruker Daltonics, Bremen, Germany) (Ďuračka et al., 2020). The threshold score was set to ≥ 2.0.

Statistical analyses were performed in the GraphPad Prism statistical software (version 10.1.1 for Mac; GraphPad Software, La Jolla, CA, USA) using a one-way ANOVA with Greenhouse-Geisser correction followed by Tukey's post hoc test. The native control (CtrlN) was compared to the cryopreserved control (CtrlC), while all EPI-supplemented groups were compared with both controls, CtrlN and CtrlC.

3. Results

Results obtained from the CASA analysis (Table 1) showed the highest motility in native semen samples (83.88±4.31, $Ctrl_N$), while the cryopreserved group (CtrlC) showed a significant decrease (33.31±4.98, $P<0.05$). EPI treatment improved motility in a dose-dependent manner, with the highest motility observed at 50 μM EPI (62.02±4.63), significantly higher than CtrlC ($P<0.05$).

Membrane integrity analysis showed highest preserved membranes in the CtrlN (77.78±2.56), while cryopreserved one (CtrlC) showed significantly ($P<0.05$) lower membrane quality (52.67±1.99). The MI improved with increasing EPI concentration, reaching 67.33±4.27 at 50 μM EPI.

Functionality of membrane was measured by HOS test, while CtrlN had the best membrane integrity (73.02±3.56). The lowest membrane functionality was observed in the CtrlC. Similar to other parameters, the best results within cryopreserved groups were observed at 50 μM (62.44±2.67).

The results indicate the CtrlN group showing the highest mitochondrial activity within the groups. In contrast, the CtrlC group demonstrated significantly reduced mitochondrial activity at 43.56±5.54. The EPI treatment appears to have a positive effect on restoring mitochondrial function, with the 50 μM EPI treatment reaching 65.79±1.87.

The DNA integrity results reveal that the CtrlN exhibited minimal DNA damage at 10.50±2.11%, while the CtrlC group showed significantly higher ($P<0.05$) DNA damage at 37.20±1.58%. EPI treatment demonstrated a protective effect against DNA damage in sperm cells, with the 50 μM EPI concentration being most effective among the treatment groups by reducing DNA damage to 21.22±2.67%, which is substantially improved over the CtrlC ($P<0.05$).

The Annexin V results demonstrated that the CtrlN exhibited minimal early apoptotic activity at 14.24±4.28%, whereas the cryopreserved control group manifested significantly elevated apoptotic markers at 44.24±4.28%. EPI intervention attenuated apoptotic processes in spermatozoa, with the 50 μM EPI concentration eliciting the most pronounced anti-apoptotic effect among treatment cohorts, reducing early apoptosis to 27.30±2.28%.

The Propidium Iodide analysis indicated that the CtrlN group displayed negligible necrotic cell death at 6.67±6.24%, but the CtrlC exhibited significantly increased necrosis at 19.00±8.62%. The administration of EPI led to a dose-dependent decrease in necrotic sperm, with the most effective cytoprotective effects noted at a concentration of 50 μM, resulting in necrosis values of 12.70±5.24%.

Table 1. Effects of cryopreservation and EPI addition to cryopreservation medium on turkey sperm quality.

	CtrlN (40)	**CtrlC (40)**	**10 μM EPI (40)**	**25 μM EPI (40)**	**50 μM EPI (40)**
MOT [%]	83.88±4.31	33.31±4.98[a]	46.33±5.43[a; b]	53.64±5.11[a; b]	62.02±4.63[a; b]
MI [%]	77.78±2.56	52.67±1.99[a]	64.67±2.32[a; b]	65.33±6.94[a; b]	67.33±2.47[a; b]
HOS [%]	73.02±3.56	51.67±4.41[a]	58.67±6.26[a]	63.21±2.65[a; b]	66.44±4.78[a; b]
AI [%]	71.82±1.96	51.49±3.72[a]	64.29±1.93[a; b]	64.69±1.83[a; b]	65.77±2.78[a; b]
MA [%]	100.00±5.12	43.56±6.54[a]	58.33±2.76[a]	64.00±1.93[a; b]	65.69±1.83[a; b]
DNA [%]	10.50±2.11	37.20±1.58[a]	22.73±7.34[a; b]	21.28±2.67[a; b]	21.21±1.14[a; b]
Apoptotic sperm [%]	14.24±4.28	44.24±4.28[a]	31.52±3.73[a; b]	31.21±2.14[a; b]	27.30±2.28[a; b]
Necrotic sperm [%]	6.67±6.24	19.00±8.62[a]	15.00±6.48[a]	13.70±2.05[a; b]	12.70±5.24[a; b]

MOT – sperm motility, MI – membrane integrity, HOS - Hypoosmotic Swelling Test, AI – acrosome integrity, MTT – mitochondrial activity, DNA – sperm DNA fragmentation, CtrlN – control (native sperm quality), CtrlC – cryopreserved control, EPI – cryopreserved groups with addition of epicatechin. [a] – compared to CtrlN, [b] – compared to CtrlC.

Reactive oxygen species (ROS) demonstrated a statistically significant elevation ($P<0.05$) in post-thaw spermatozoa when compared to CtrlN. The experimental groups receiving 25 and 50 μM EPI supplementation exhibited a significant reduction ($P<0.05$) in ROS generation relative to the CtrlC group (Table 2). Similar patterns were observed in the malondialdehyde (MDA) quantification analysis. While the CtrlC group manifested significantly higher MDA content in comparison to CtrlN, EPI concentrations equal to or exceeding 25 μM substantially attenuated MDA levels.

The analysis of protein carbonyl (PC) formation revealed a dose-dependent relationship, wherein increasing concentrations of EPI corresponded with decreasing concentrations of PC. Statistically significant reductions in PC were observed when 50 μM EPI was administered prior to the freezing procedure.

Table 2. Effects of cryopreservation and EPI addition to cryopreservation medium on oxidative stress parameters.

	CtrlN (40)	CtrlC (40)	10 μM EPI (40)	25 μM EPI (40)	50 μM EPI (40)
ROS [RLU/sec/10^6 sperm]	5.30±0.60	21.00±2.46[a]	16.32±3.86[a]	14.00±1.41[a; b]	13.30±1.25[a; b]
PC [nmol PC/mg prot]	1.92±0.16	8.30±0.49[a]	7.64±4.28[a]	6.70±2.89[a]	5.60±3.39[a; b]
MDA [μmol MDA/g prot]	1.34±0.05	3.85±0.11[a]	3.82±0.46[a]	2.53±0.26[a; b]	2.35±0.10[a; b]

ROS – reactive oxygen species, PC – protein carbonyls, MDA – malondialdehyde concentration. CtrlN – control (native sperm quality), CtrlC – cryopreserved control, EPI – cryopreserved groups with addition of epicatechin. [a] – compared to CtrlN, [b] – compared to CtrlC

All cryopreserved groups demonstrated a significant reduction in bacterial occurrence ($P<0.05$) compared to native semen, while 14 bacterial species were found in CtrlN, 5 species in CtrC and 10 μM EPI, 4 species in 25 μM EPI and 3 species in 50 μM EPI. The addition of EPI to cryopreservation medium showed a dose-dependent trend with increasing concentration. EPI administration contributed to eradication of *C. braakii* and *S. epidermidis* when treated by 50 μM. As showed in Table 3, 14 bacterial species were identified in native Turkey semen samples including *A. baumannii*, *C. jejuni*, *C. braakii*, *E. faecalis*, *E. faecium*, *E. coli*, *K. pneumoniae*, *M. luteus*, *P. mirabilis*, *P. vulgaris*, *S. chromogenes*, *S. epidermidis*, *S. lentus* and *S. gallactolyticus*.

Table 3. Effects of cryopreservation and EPI addition to cryopreservation medium on bacterial concentration and species composition.

	CtrlN (40)	**CtrlC (40)**	**10 μM EPI (40)**	**25 μM EPI (40)**	**50 μM EPI (40)**
Bacterial load [log CFU/mL]	10.55±1.58	1.99±1.02[a]	1.51±0.33[a]	1.10±0.70[a]	0.90±0.25[a]
Species	*Acinetobacter baumannii (A. baumannii)* *Campylobacter jejuni (C. jejuni)* *Citrobacter braakii (C. braakii)* *Enterococcus faecalis (E. faecalis)* *Enterococcus faecium (E. faecium)* *Escherichia coli (E. coli)* *Klebsiella pneumoniae (K. pneumoniae)* *Micrococcus luteus (M. luteus)* *Proteus mirabilis (P. mirabilis)* *Proteus vulgaris (P. vulgaris)* *Staphylococcus chromogenes (S. chromogenes)* *Staphylococcus epidermidis (S. epidermidis)* *Staphylococcus lentus (S. lentus)* *Streptococcus galactolyticus (S. galactolyticus)*	*E. coli* *E. faecalis* *E. faecium* *C. braakii* *S. epidermidis*	*E. coli* *E. faecalis* *E. faecium* *C. braakii* *S. epidermidis*	*E. coli* *E. faecalis* *E. faecium* *C. braakii*	*E. coli* *E. faecalis* *E. faecium*

[a] – compared to CtrlN.

4. Discussion

Turkey semen cryopreservation has been studied to improve post-thaw sperm quality and fertility rates. Various cryoprotectants, extenders, and freezing methods have been evaluated (Iaffaldano et al., 2016b; Gloria et al., 2019; Iorio et al., 2020). The potential use of frozen turkey semen for artificial insemination could benefit turkey production. Factors affecting cryopreservation success include breeding line, freezing method, extender composition, and equilibration time. Despite progress, fertility rates from frozen-thawed turkey semen remain suboptimal, highlighting the need for further research to improve cryopreservation techniques for effective germplasm preservation.

Oxidative mechanisms occurring during cryopreservation have been studied to negatively impact sperm membrane structure (Rui et al., 2017). Moreover, the polyunsaturated fatty acid components comprising phospholipid bilayers exhibit significant susceptibility to peroxidative damage, leading to modifications in membrane fluidity, disturbances in the acrosomal exocytotic

process, and impaired gamete interaction dynamics (Partyka et al., 2012).

The integration of bioactive compounds that offer protective effects against oxidative stressors during cryopreservation procedures is emerging as a highly promising strategy in the field of animal reproductive biotechnologies. Pivotal studies have shown that ascorbic acid and α-tocopherol serve not only as effective scavengers of ROS but also protect the sperm plasma membrane from premature activation during cryogenic processes. However, a significant volume of research has recently indicated that ethnopharmacologically relevant phytochemicals may improve post-thaw sperm parameters more effectively than traditional antioxidant treatments (Partyka, Niżański, 2021; Qamar et al., 2023). This study is based on recent findings regarding the positive impact of epicatechin on male reproductive tissues and cellular components, aiming to assess its potential to counteract the reduction in sperm viability parameters caused by cryopreservation (Baňas et al., 2023; Yu et al., 2010). EPI, similar to other flavonoids, has ability to donate hydrogen atoms or electrons to neutralize ROS and peroxyl radicals, thereby protect lipids, proteins, and DNA from oxidative damage (Zahra et al., 2024).

EPI has shown promising results in bovine spermatozoa, effectively reducing oxidative damage to proteins, lipids, and DNA while preserving motility and viability. EPI supplementation during cryopreservation also prevented the loss of crucial proteins involved in sperm capacitation (Baňas et al., 2023). Similarly, quercetin supplementation in rooster semen extender improved post-thaw sperm quality, including motility (by 38.94%), membrane functionality (by 37.27%), and mitochondrial activity (by 26.92%) when compared to control. Moreover, MDA concentration was reduced by 52.04%, accompanied by a 57.47% reduction in dead/necrotic sperm (Siari et al., 2022). Our study is in agreement with previous studies cited above when all sperm quality parameters were significantly improved compared to cryopreserved control. Specifically, motility increased by 86.19%, membrane functionality by 28.58%, membrane integrity by 27.83% and mitochondrial activity by 50.80%, while MDA concentration and the proportion of necrotic sperm cells decreased by 38.96% and 33.15%, respectively, suggesting positive overall effect on the long-term preservation of turkey semen. The recent study by Rezaie et al. (2021) recorded significant improvements in post-thaw sperm quality regarding sperm motility (by 18.02%), mitochondrial activity (by 22.83%), and membrane integrity (by 23.37%), while MDA content and necrotic cell rate were reduced by 51.15% and 12.64%, respectively. Quantitatively, EPI demonstrated the greatest improvements in sperm motility and mitochondrial activity compared with the findings of Siari et al. (2022) and Rezaei et al. (2021), while other parameters improved similarly.

The microbiological contamination of avian seminal fluid, as stated by previous studies (Lenický et al., 2021; Mohan et al., 2023; Tvrdá et al., 2023a, 2023b), has required the integration of effective antimicrobial agents into semen extenders to ensure sufficient protection against bacteriospermia. The present study, consistent with previous research (Mohan et al., 2023; Tvrdá et al., 2023b; Rakha et al., 2024), reveals that bacterial contamination in extended ejaculates is common, and antimicrobial supplementation exhibits limited effectiveness in mitigating bacterial occurrence after cryopreservation, respectively extended post-thawed semen.

As shown by Shiota et al. (1999), EPI significantly decreased the minimum inhibitory concentration of several antibiotics against methicilin-resistant *Staphyloccocus aureus* making them bactericidal. Our data suggests that EPI enhanced the antibacterial efficacy of kanamycin. Specifically, in the cohort administered 50 mM EPI, S. epidermidis and C. braakii were eliminated in comparison to the cryopreserved control. According to Mita et al. (2025), catechins bind to bacterial lipids and proteins, altering membrane fluidity, damaging its integrity. This effect relates to interaction with their phenolic hydroxyl groups, which are also responsible for scavenging free radicals. On the other hand, catechins can induce oxidative stress via H_2O_2, leading to DNA damage in bacteria. Both direct antimicrobial and synergistic effects of catechins with antibiotics were previously noted (Mita et al., 2025). Based on our results, we may hypothesize that epicatechin could effectively reduce antibiotic usage in semen extenders, while reducing antibiotic overuse should be a global goal. The synergistic properties of epicatechin with conventional antibiotics may enhance the antimicrobial efficacy and, at the same time, reduce the antibiotic dosage without threat to microbial

safety, which was supported in previous studies (Miklasińska et al., 2016; Wu, Brown, 2021; Alkufeidy et al., 2024; Tvrdá et al., 2025).

The present study is in agreement with the research above, as all evaluated spermatozoa quality parameters exhibited statistically significant enhancement related to cryopreserved control specimens, thus indicating a positive effect on the long-term preservation of turkey semen. Our evaluation of functional and structural spermatozoa characteristics revealed enhancement in cellular viability, plasma membrane integrity, mitochondrial activity, and acrosomal status following the implementation of EPI in cryopreservation medium.

5. Conclusions

The experimental results detailed in this study offer evidence for the membrane-stabilizing and motility-enhancing effects of epicatechin at a concentration of 50 μM on cryopreserved turkey spermatozoa. The creation of innovative semen extender formulations that provide specific benefits for post-thaw sperm viability while also meeting their metabolic needs is a crucial focus in the field of avian reproductive biotechnology. These findings enhance the existing information on successful cryoprotective measures in avian reproductive science and may guide future advancements in spermatozoa cryopreservation techniques for domestic and endangered avian species.

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

6. References

1. Alkufeidy RM, Ameer Altuwijri L, Aldosari NS, Alsakabi N, Dawoud TM (2024): Antimicrobial and synergistic properties of green tea catechins against microbial pathogens. Journal of King Saud University - Science 36, 103277. https://doi.org/10.1016/j.jksus.2024.103277.
2. Almadaly EA, Elbendary MA, Sahwan FM, Shehabeldin AM, El-Domany WB, Ismail I. El-Kon II, Ramoun AA (2024): Quercetin Supplementation in Tris Egg Yolk Extender Improves the Post-Thaw Quality of Zaraibi Buck Semen. Egyptian Journal of Veterinary Sciences 1–9. https://doi.org/10.21608/ejvs.2024.332755.2467.
3. Ansari MS, Rakha BA, Akhter S, Blesbois E, Santiago-Moreno J (2019): Effect of Cryopreservation on Lipid Peroxidation, Antioxidant Potential, Chromatin Integrity, and Mitochondrial Activity of Indian Red Jungle Fowl (Gallus gallus murghi) Semen. Biopreservation and Biobanking 17, 288–295. https://doi.org/10.1089/bio.2018.0111.
4. Baňas Š, Benko F, Ďuračka M, Lukáč N, Tvrdá E (2023a): Epicatechin Prevents Cryocapacitation of Bovine Spermatozoa through Antioxidant Activity and Stabilization of Transmembrane Ion Channels. International Journal of Molecular Sciences 24, 2510. https://doi.org/10.3390/ijms24032510.
5. Baňas Š, Benko F, Ďuračka M, Lukáč N, Tvrdá E (2023b): Epicatechin Prevents Cryocapacitation of Bovine Spermatozoa through Antioxidant Activity and Stabilization of Transmembrane Ion Channels. International Journal of Molecular Sciences 24, 2510. https://doi.org/10.3390/ijms24032510.

6. Banas S, Duracka M, Benko F, Ziarovska J, Lukac N, Tvrda E (2022): Epicatechin Improves Frozen Sperm Vitality by Its Antioxidant and Cryoprotective Actions. Journal of microbiology, biotechnology and food sciences 12, e9490–e9490. https://doi.org/10.55251/jmbfs.9490.
7. Benko F, Slanina T, Ďuračka M, Kačániová M, Tvrdá E (2025): Cryopreservative and antimicrobial properties of kaempferol on the post-thaw quality of turkey spermatozoa. Czech Journal of Animal Science 70, 404–413. https://doi.org/10.17221/79/2025-CJAS.
8. Ďuračka M, Kováčik A, Kačániová M, Lukáč N, Tvrdá E (2020): Bacteria May Deteriorate Progressive Motility of Bovine Spermatozoa and Biochemical Parameters of Seminal Plasma. Journal of microbiology, biotechnology and food sciences 9, 844–847. https://doi.org/10.15414/jmbfs.2020.9.4.844-847.
9. Gloria A, Toscani T, Robbe D, Parrillo S, De Amicis I, Contri A (2019): Cryopreservation of turkey spermatozoa without permeant cryoprotectants. Animal Reproduction Science 211, 106218. https://doi.org/10.1016/j.anireprosci.2019.106218.
10. Iaffaldano N, Di Iorio M, Cerolini S, Manchisi A (2016a): Overview of Turkey Semen Storage: Focus on Cryopreservation – A Review. Annals of Animal Science 16, 961–974. https://doi.org/10.1515/aoas-2016-0026.
11. Iaffaldano N, Di Iorio ,M., Miranda ,M., Zaniboni ,L., Manchisi ,A., and Cerolini S (2016b): Cryopreserving turkey semen in straws and nitrogen vapour using DMSO or DMA: effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality. British Poultry Science 57, 264–270. https://doi.org/10.1080/00071668.2016.1148261.
12. Iaffaldano N, Reale A, Sorrentino E, Coppola R, Di Iorio M, Rosato MP (2010): Risk of *Salmonella* transmission via cryopreserved semen in turkey flocks. Poultry Science 89, 1975–1980. https://doi.org/10.3382/ps.2009-00573.
13. Iorio MD, Rusco G, Iampietro R, Colonna MA, Zaniboni L, Cerolini S, Iaffaldano N (2020): Finding an Effective Freezing Protocol for Turkey Semen: Benefits of Ficoll as Non-Permeant Cryoprotectant and 1:4 as Dilution Rate. Animals : an Open Access Journal from MDPI 10, 421. https://doi.org/10.3390/ani10030421.
14. Lenický M, Slanina T, Kačániová M, Galovičová L, Petrovičová M, Ďuračka M, Benko F, Kováč J, Tvrdá E (2021): Identification of Bacterial Profiles and Their Interactions with Selected Quality, Oxidative, and Immunological Parameters of Turkey Semen. Animals 11, 1771. https://doi.org/10.3390/ani11061771.
15. Miklasińska M, Kępa M, Wojtyczka RD, Idzik D, Dziedzic A, Wąsik TJ (2016): Catechin Hydrate Augments the Antibacterial Action of Selected Antibiotics against Staphylococcus aureus Clinical Strains. Molecules 21, 244. https://doi.org/10.3390/molecules21020244.
16. Mita SR, Muhtar NI, Kusuma SAF, Sriwidodo S, Hendrawan RP (2025): Catechins as Antimicrobial Agents and Their Contribution to Cosmetics. Cosmetics 12, 11. https://doi.org/10.3390/cosmetics12010011.
17. Mohan J, Kolluri ,G., Srivastava ,V., Tyagi ,J. S., and Tiwari AK (2023): Bacterial contamination of poultry semen, its dilution and storage. World's Poultry Science Journal 79, 593–617. https://doi.org/10.1080/00439339.2023.2225793.
18. Partyka A, Łukaszewicz E, Niżański W (2012): Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. Theriogenology 77, 1497–1504. https://doi.org/10.1016/j.theriogenology.2011.11.006.
19. Partyka A, Niżański W (2021): Supplementation of Avian Semen Extenders with Antioxidants to Improve Semen Quality-Is It an Effective Strategy? Antioxidants (Basel, Switzerland) 10, 1927. https://doi.org/10.3390/antiox10121927.
20. Parvizi Alan A, Ayen E, Khaki A, Soleimanzadeh A (2022): The Effects of Epigallocatechin-3-Gallate (Egcg) on the Quality of Fresh and Frozen-Thawed Semen by Two Different Cryopreservation Methods in Simmental Bulls. https://doi.org/10.2139/ssrn.4269474.
21. Qamar AY, Naveed MI, Raza S, Fang X, Roy PK, Bang S, Tanga BM, Saadeldin IM, Lee S, Cho J (2023): Role of antioxidants in fertility preservation of sperm — A narrative review. Animal Bioscience 36, 385–403. https://doi.org/10.5713/ab.22.0325.
22. Rakha BA, Zafar Z, Ansari MS, Akhter S, Qadeer S, Akhter A, Waseem K, Santiago-Moreno J (2024): Influence of Bacterial Contamination and Antibiotic Sensitivity on Cryopreserved Sperm Quality of Indian Red Jungle Fowl. Biopreservation and Biobanking 22, 21–28. https://doi.org/10.1089/bio.2022.0029.
23. Rezaie FS, Hezavehei M, Sharafi M, Shahverdi A (2021): Improving the post-thaw quality of rooster semen using the extender supplemented with resveratrol. Poultry Science 100, 101290. https://doi.org/10.1016/j.psj.2021.101290.

24. Rui BR, Shibuya FY, Kawaoku AJT, Losano JDA, Angrimani DSR, Dalmazzo A, Nichi M, Pereira RJG (2017): Impact of induced levels of specific free radicals and malondialdehyde on chicken semen quality and fertility. Theriogenology 90, 11–19. https://doi.org/10.1016/j.theriogenology.2016.11.001.
25. Shiota S, Shimizu M, Mizushima T, Ito H, Hatano T, Yoshida T, Tsuchiya T (1999): Marked reduction in the minimum inhibitory concentration (MIC) of beta-lactams in methicillin-resistant Staphylococcus aureus produced by epicatechin gallate, an ingredient of green tea (*Camellia sinensis*). Biological & Pharmaceutical Bulletin 22, 1388–1390. https://doi.org/10.1248/bpb.22.1388.
26. Siari S, Mehri M, Sharafi M (2022): Supplementation of Beltsville extender with quercetin improves the quality of frozen-thawed rooster semen. British Poultry Science 63, 252–260. https://doi.org/10.1080/00071668.2021.1955331.
27. Torabi SSS, Mohammadi T, Rezaei M (2025): The impact of procyanidin supplementation on Turkey semen quality, oxidative stress parameters, and fertilization during preservation at −196 °C. Cryobiology 120, 105286. https://doi.org/10.1016/j.cryobiol.2025.105286.
28. Tvrdá E, Baňas Š, Ďuračka M, Benko F, Čmiková N, Kačániová M (2025): Epicatechin as a potential supplement for frozen bovine semen: sperm-protecting and antibacterial properties. Animal Reproduction Science 272, 107698. https://doi.org/10.1016/j.anireprosci.2024.107698.
29. Tvrdá E, Petrovičová M, Benko F, Ďuračka M, Kováč J, Slanina T, Galovičová L, Žiarovská J, Kačániová M (2023a): Seminal Bacterioflora of Two Rooster Lines: Characterization, Antibiotic Resistance Patterns and Possible Impact on Semen Quality. Antibiotics 12, 336. https://doi.org/10.3390/antibiotics12020336.
30. Tvrdá E, Petrovičová M, Ďuračka M, Benko F, Slanina T, Galovičová L, Kačániová M (2023b): Short-Term Storage of Rooster Ejaculates: Sperm Quality and Bacterial Profile Differences in Selected Commercial Extenders. Antibiotics 12, 1284. https://doi.org/10.3390/antibiotics12081284.
31. Wu M, Brown AC (2021): Applications of Catechins in the Treatment of Bacterial Infections. Pathogens 10, 546. https://doi.org/10.3390/pathogens10050546.
32. Yu P-L, Pu H-F, Chen S-Y, Wang S-W, Wang PS (2010): Effects of catechin, epicatechin and epigallocatechin gallate on testosterone production in rat leydig cells. Journal of Cellular Biochemistry 110, 333–342. https://doi.org/10.1002/jcb.22541.
33. Zahra M, Abrahamse H, George BP (2024): Flavonoids: Antioxidant Powerhouses and Their Role in Nanomedicine. Antioxidants 13, 922. https://doi.org/10.3390/antiox13080922.