Ovozoa 11 No. 3, December 2022 p-ISSN 2302-6464 e-ISSN 2722-967X
Available at https://e-journal.unair.ac.id/OVZ/index

Original article

Green tea (*Camellia sinensis*) leaf extract maintained spermatozoa plasma membrane integrity, viability, and motility of mice (*Mus musculus*) exposed to cigarette smoke

Maryana Siska Silviani[1], Moh. Sukmanadi[2], Rochmah Kurnijasanti[2], Sri Pantja Madyawati[3], Epy Muhammad Luqman[4]

[1] Veterinary Profession Interest Group, Surabaya. [2] Division of Basic Veterinary, [3] Division of Veterinary Reproduction, [4] Division of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga
[*] Corresponding author, e-mail: silvianimaryanasiska@gmail.com

Open access under CC BY – SA license, DOI: 10.20473/ovz.v11i3.2022.130-136
Received July 26 2022, Revised November 11 2022, Accepted November 21 2022
Published online December 2022

How to cite this article: Silviani MS, Sukmanadi M, Susilowati S, Madyawati SP, Luqman EM, Kurnijasanti R. 2022. Green tea (*Camellia sinensis*) leaf extract maintained spermatozoa plasma membrane integrity, viability, and motility of mice (*Mus musculus*) exposed to cigarette smoke. Ovozoa: Journal of Animal Reproduction 11: 130-136.

ABSTRACT

Cigarette smoke chemicals caused oxidative stress by increasing Reactive Oxygen Species (ROS). ROS exposure could be neutralized by antioxidants, such as green tea (*Camellia sinensis*) leaf extract. This study aimed to determine the effect of green tea extract (GTE) on spermatozoa plasma membrane integrity, spermatozoa viability, and spermatozoa motility of mice (*Mus musculus*) exposed to cigarette smoke. Twenty-five mice were randomly divided into five groups. Group C- mice were given a placebo (1% Sodium carboxymethyl cellulose, Na-CMC). Group C+, T1, T2, and T3 mice were exposed to cigarette smoke and given 0, 20, 40, and 60 mg/kg BW GTE respectively. Cigarette smoke exposure used a clove cigarette per day. GTE in 1% Na-CMC solution was administered at 0.5 mL orally using a gastric probe. The treatment was conducted daily for 36 days, and on day 37, all mice were euthanized for spermatozoa evaluation. The results showed that all parameters evaluated in the C+ group were lower ($p < 0.05$) than in C- group. Administration of GTE in the T2 group increased ($p < 0.05$) all parameters compared to mice in the C+ group. However, administration of GTE to mice in the T3 group caused a decrease ($p < 0.05$) in all parameters than those of the T2 group and was not significantly different ($p > 0.05$) compared to those of the C+ group. It could be concluded that the administration of GTE at 40 mg/kg BW has maintained the spermatozoa plasma membrane integrity, spermatozoa viability, and spermatozoa motility of mice exposed to cigarette smoke.

Keywords: cigarette smoke, green tea extract, motility, plasma membranes integrity, viability

INTRODUCTION

Cigarette smoke contained compounds that can harm health. Burned cigarette produced about 4000 chemicals, including nicotine, tar, carbon monoxide, nitrosamines, nitrogen oxides, and polynuclear aromatic hydrocarbon compounds (Cheng *et al*., 2022). Cigarette smoke chemicals would enter quickly into the lungs and then spread throughout the body through the circulatory system, including to the testicular tissue, causing oxidative stress by increasing the formation of Reactive Oxygen Species (ROS) (Caliri *et al*., 2021). ROS first

oxidized polyunsaturated fatty acids (PUFA) in the plasma membrane, produced lipid peroxidase, unstable spermatozoa plasma membrane, and produced malondialdehyde, a toxic product for cells (Ayala *et al*., 2014). The plasma membrane of spermatozoa, which is composed of fatty acids, is susceptible to oxidative damage due to free radicals (Durairajanayagam *et al*., 2021). Damage to the plasma membrane of spermatozoa would be followed by protein inactivation and DNA damage which would lead to the death of spermatozoa (Panner Selvam *et al*., 2021).

ROS exposure could be neutralized by antioxidants. Antioxidants were components that could inhibit the oxidation of fats, nucleic acids, or other molecules by preventing oxidation through chain reactions. The antioxidative mechanism was the donation some of their electrons to oxidant molecules to protect tissues from the harmful effects of ROS (Lü *et al*., 2010). Changes in antioxidant molecules after releasing electrons did not turn them into reactive molecules (Kurutas, 2016). Green tea contained polyphenolic compounds, namely catechins, which were helpful as antioxidants that could neutralize free radicals. Tea catechins varied widely, consisting of epicatechin, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3-gallate. Green tea also contained vitamins C and E (Prasanth *et al*., 2019). Vitamin C is a compound that dissolves easily in water and could act as a free radical scavenger. In addition, vitamin E could protect cell membranes against lipid peroxidase by capturing free radicals (Vladika *et al*., 2019). There has been no report on the use of green tea extract (GTE) as an antioxidant to maintain semen quality in mice exposed to cigarette smoke. Therefore, this study aims to determine the effect of GTE on spermatozoa plasma membrane integrity, spermatozoa viability, and spermatozoa motility of mice (*Mus musculus*) exposed to cigarette smoke.

MATERIALS AND METHODS

This research procedure was approved by the Animal Care and Use Committee, Universitas Airlangga, Surabaya, Indonesia, No. 324/HRECC.FORM/IV/2020.

Experimental animals

This study used 25 male mice (*Mus musculus*) aged 12 weeks weighing 20-25 grams, in good health, and had never been used for research. Mice were kept in cages which were given sawdust for the base of the cage. Mice were fed pellets and drinking water *ad libitum*.

Green tea leaf extraction

Green tea leaves (*Camellia sinensis*) were obtained from Wonosari tea garden, Malang Regency, East Java, Indonesia. The extraction was carried out by maceration method using ethanol solvent. Green tea leaf powder weighing 1000 g of was soaked in eight liters of 96% ethanol for three days. The maceration results were then evaporated using a rotary evaporator at 50˚C, 45 rpm for 5 hours to obtain a thick extract, then freeze-dried (Khoirunnisa *et al*., 2019). Extract powder was redissolved in 1% Na-CMC and adjusted according to the dosages.

Treatment of animals

Mice were acclimatized in cages for one week, then treated for 36 days. Twenty five mice (*Mus musculus*) were randomly and evenly divided into five groups. In the C- (negative control) group, mice were not exposed to cigarette smoke and were not given GTE (only 0.5 mL of 1% Na-CMC). In groups C+, T1, T2, and T3, mice were exposed to cigarette smoke and given respectively 0, 20, 40, and 60 mg/kg BW of GTE. GTE was administered orally using a gastric probe at 0.5 mL/mouse/day. Cigarette smoke exposure to mice was carried out in an exposure chamber (31 x 19 x 22 cm) which had two holes, each for smoke input and output. Cigarette smoke was derived from a clove cigarette per day (Morales-Mantilla *et al*., 2020). On day 37, all mice were euthanized by cervical dislocation and dissected to collect the testes and cauda epididymis. The cauda epididymis was separated by cutting the proximal part of the corpus epididymis and the distal part of the vas deferens. The cauda epididymis was placed in a petri dish containing 0.2 mL of 0.9% NaCl solution. Spermatozoa was stripped out of the cauda gently, then mixed with the solution to be a suspension of spermatozoa for examination.

Plasma membrane integrity evaluation

To examine the integrity of the spermatozoa plasma membrane 0.1 mL of spermatozoa suspension was pipetted into a microtube containing 0.9 mL hypoosmotic solution. After being gently mixed, the mixture was incubated at 37˚C for 30 minutes and then spermatozoa were evaluated using a light microscope with a magnification of 400x. Spermatozoa tails with intact membranes appeared swollen or coiled, whereas spermatozoa with damaged membranes showed straight tail. Plasma membrane integrity of spermatozoa was counted as a percentage of spermatozoa with coiled tails (Puspita *et al*., 2020).

Spermatozoa viability evaluation

Examination of spermatozoa viability was carried out by dripping a drop of spermatozoa suspension and a drop of eosin-nigrosine solution onto a clean object glass. Spermatozoa and eosin nigrosine were then mixed homogeneously, smeared and briefly dried over a flame. The slides were observed under a light microscope (Olympus® CX-41) with 400x magnification. Live spermatozoa cells appeared transparent, while dead spermatozoa appeared purplish red in its head (Octaviani *et al*., 2021). Viability of spermatozoa was counted as a percentage of spermatozoa with transparent head out of 100 spermatozoa examined (Widiantoro *et al*., 2021).

Spermatozoa motility evaluation

Examination of spermatozoa motility was carried out by dripping a drop of spermatozoa suspension and a drop of 0.9% NaCl solution onto a clean object glass, then mixed homogeneously, covered, and examined under a light microscope (Olympus® CX-41) with 400x magnification (Octaviani *et al*., 2021). Spermatozoa motility was calculated based on the percentage of individual movement of a progressive out of 100 spermatozoa examined (Widiantoro *et al*., 2021).

Data analysis

The data obtained were analyzed using ANOVA followed by Duncan's multiple range test. Statistical analysis was performed using the Statistical Program and Service Solution (SPSS) version 23 software for Windows.

RESULTS

Plasma membrane integrity, spermatozoa viability, and motility in group C+ mice were lower ($p < 0.05$) than in C- group mice. Administration of GTE to mice in the T2 group increased ($p < 0.05$) plasma membrane integrity, spermatozoa viability, and motility compared to mice in the C+ group. However, administration of GTE to mice in the T3 group caused a decrease ($p < 0.05$) in plasma membrane, spermatozoa viability, and motility compared to the T2 group and was not significantly different ($p > 0.05$) compared to the C+ group (Table 1). Microscopic examination of intact or normal spermatozoa plasma membranes and damaged spermatozoa plasma membranes could be seen in Figure 1. Microscopic examination of live and dead spermatozoa with eosin-nigrosine staining could be seen in Figure 2.

Table 1 The integrity of spermatozoa plasma membrane, spermatozoa viability, and spermatozoa motility of mice (*Mus musculus*) after exposure to cigarette smoke and green tea extract administration

	C-	C+	T1	T2	T3
IPM	52.1 ± 8.73 ^{b}	20.95 ± 7.64 ^{a}	31.05 ± 6.86 ^{a}	44.82 ± 5.30 ^{b}	31.92 ± 10.84^{a}
viability	62.73 ± 16.56 ^{b}	9.91 ± 5.57 ^{a}	44.32 ± 10.84 ^{ab}	55.72 ± 21.68 ^{b}	50.88 ±16.45 ^{ab}
motility	38.00 ± 13.04 ^{c}	14.00 ± 4.18 ^{a}	19.00 ± 8.22 ^{ab}	27.00 ± 5.70 ^{bc}	23.00 ± 7.58 ^{ab}

Different superscripts in one line indicate a significant difference ($p < 0.05$); C-: mice were not exposed to cigarette smoke and were not GTE administration (0.5 mL of 0.1 % Na-CMC); C+, T1, T2, and T3: mice were exposed to cigarette smoke followed by the administration of 0, 20, 40, and 60 mg/kg BW in 1% Na-CMC 0.5 mL.

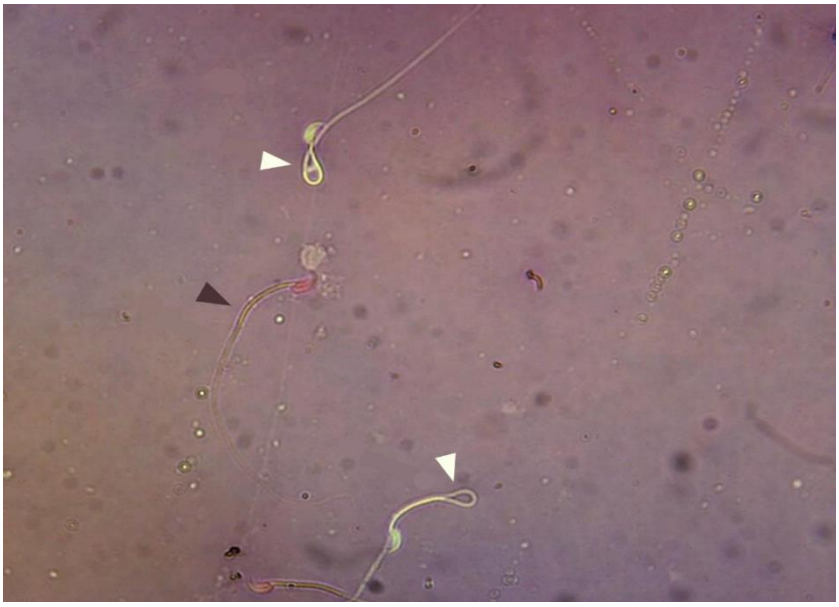

Figure 1 Examination of spermatozoa plasma membrane integrity of mice (*Mus musculus*) after exposure to cigarette smoke and green tea extract administration showing spermatozoa with intact plasma membrane (coiled tail, white arrowheads) and spermatozoa with damaged plasma membrane (straight tail, purple arrowhead); examined under a light microscope (Olympus® CX-41) with 400x magnification.

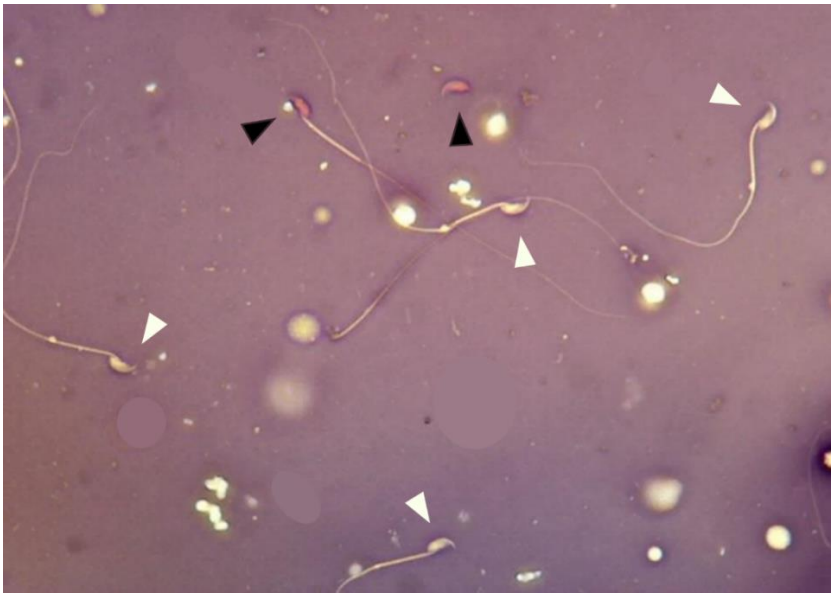

Figure 2 Eosin nigrosine staining of spermatozoa of mice (*Mus musculus*) after exposure to cigarette smoke and green tea extract administration showing live spermatozoa with transparent head (white arrowheads) and dead spermatozoa with purplish red head (black arrowheads); examined under a light microscope (Olympus® CX-41) with 400x magnification.

DISCUSSION

Plasma membrane integrity, spermatozoa viability, and motility in mice exposed to cigarette smoke were lower than in normal mice. Cigarette smoke increased free radicals in the form of ROS such as superoxide, hydroxyl radicals, and peroxyl radicals (Valavanidis *et al*., 2009). ROS could cause lipid peroxidation and disrupted the integrity of the spermatozoa plasma membrane, nuclear membrane, and mitochondrial membrane (Alahmar, 2019). Nuclear membrane damage could be followed by DNA fragmentation and cell death (Chen *et al*.,

2020). Meanwhile, disruption of the integrity of the mitochondrial membrane caused metabolic disorders that produce ATP which interfered with spermatozoa motility (Durairajanayagam *et al*., 2021). Cigarette smoke induced high ROS (Caliri *et al*., 2021) which caused damage to the spermatozoa plasma membrane (Sabeti *et al*., 2016). ROS were oxidants with high reactivity to polyunsaturated fatty acid (PUFA) in cell membranes. The toxic effects of ROS were lipid peroxidation, protein ionization and inactivation, and DNA damage (Juan *et al*., 2021). After the addition of the hypo-osmotic solution, the damaged spermatozoa plasma membrane is seen straight tail shape (Prochowska *et al*., 2022).

Mice exposed to cigarette smoke and administered 40 mg/kg BW showed the best results in maintaining spermatozoa plasma membrane integrity. GTE contained vitamins C, and E, flavonoids that act as antioxidants (Prasanth *et al*., 2019). Vitamin C is a compound that dissolved easily in water and could act as a free radical inhibitor (Padayatty and Levine, 2016). Vitamin E in GTE could protect cell membranes against lipid peroxidation by capturing free radicals (Howard *et al*., 2011). Meanwhile, flavonoids inhibited the formation of free radicals because they acted as antioxidants, inhibited many oxidation reactions, and protected membrane lipids from reactions that damaged them (Vladika *et al*., 2019).

This decrease in spermatozoa viability occured due to the chemical content in cigarette smoke, such as nicotine, tar, and carbon monoxide that had the potential to increase the production of free radicals (Harlev *et al*., 2015). This increase in free radicals would damage spermatogenic cells membranes, disrupted ion transport essential for spermatogenic cells proliferation and growth, damaged spermatozoa DNA and increased spermatozoa apoptosis (Takeshima *et al*., 2020). In addition, the chemical content in cigarette smoke can also inhibit the process of spermatogenesis resulting in lower spermatozoa concentrations (Ahmadnia *et al*., 2007). Administration of 40 mg/kg BW showed the best results in maintaining spermatozoa viability.

Spermatozoa motility in this study decreased due to exposure to cigarette smoke. Free radical compounds in cigarette smoke increased lipid peroxidase and caused disruption to the plasma membrane of spermatozoa, thereby reducing motility. Free radicals also cause low mitochondrial ATP production, thereby reducing the frequency of spermatozoa tail movement (Alahmar, 2019). ATP was needed by spermatozoa for their motility; when ATP production was inadequate, the movement of spermatozoa fibrils would stop (Davila *et al*., 2016).

Green tea contained catechins and flavanols could act as free radical scavengers by donating electrons to ROS to reduce their reactivity (Bernatoniene and Kopustinskiene, 2018). Antioxidants protected the plasma membrane from lipid peroxidation by ROS. The integrity of the spermatozoa membrane maintained mitochondrial metabolism for ATP production. Thereby, administration of antioxidants increased semen parameters, including the percentage of motile spermatozoa (Ahmadi *et al*., 2016). Antioxidants in green tea could prevent oxidative damage due to free radicals on several spermatozoa parameters, such as motility, abnormalities, and concentration (Mahmoudi *et al*., 2018). However, administration higher dose (60 mg/kg BW) of GTE showed a decrease in the percentage of spermatozoa plasma membrane integrity, viability, and motility. Higher administration of antioxidants caused an increase in osmotic pressure and causes damage to the plasma membrane (Henkel *et al*., 2019). The high content of quercetin in GTE decreased the expression of mitochondrial copy number (Chen *et al*., 2014). In addition, administration of higher doses of antioxidants would shift antioxidants to pro-oxidant that significantly reduced male fertility (Majzoub and Agarwal, 2018).

CONCLUSION

Administration of green tea extract at a dose of 40 mg/kg BW was effective for maintaining spermatozoa plasma membrane integrity, spermatozoa viability and spermatozoa motility of mice exposed to cigarette smoke.

REFERENCES

Ahmadi S, Bashiri R, Ghadiri-Anari A, Nadjarzadeh A. 2016. Antioxidant supplements and semen parameters: An evidence based review. Int J Reprod Biomed. 14: 729-36.

Ahmadnia H, Ghanbari M, Moradi MR, Khaje-Dalouee M. 2007. Effect of cigarette smoke on spermatogenesis in rats. Urol J. 4: 159-63.

Alahmar AT. 2019. Role of Oxidative Stress in Male Infertility: An Updated Review. J Hum Reprod Sci. 12: 4-18.

Ayala A, Muñoz MF, Argüelles S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014: 360438.

Bernatoniene J, Kopustinskiene DM. 2018. The Role of Catechins in Cellular Responses to Oxidative Stress. Molecules 23: 965.

Caliri AW, Tommasi S, Besaratinia A. 2021. Relationships among smoking, oxidative stress, inflammation, macromolecular damage, and cancer. Mutat Res Rev Mutat Res. 787: 108365.

Chen NY, Kim PH, Fong LG, Young SG. 2020. Nuclear membrane ruptures, cell death, and tissue damage in the setting of nuclear lamin deficiencies. Nucleus 11: 237-49.

Chen R, Lin J, Hong J, Han D, Zhang AD, Lan R, Fu L, Wu Z, Lin J, Zhang W, Wang Z, Chen W, Chen C, Zhang H. 2014. Potential toxicity of quercetin: The repression of mitochondrial copy number *via* decreased *POLG* expression and excessive *TFAM* expression in irradiated murine bone marrow. Toxicol Rep. 30: 450-58.

Cheng T, Reilly SM, Feng C, Walters MJ, Holman MR. 2022. Harmful and Potentially Harmful Constituents in the Filler and Smoke of Tobacco-Containing Tobacco Products. ACS Omega 7: 25537-54.

Davila MP, Muñoz PM, Bolaños JM, Stout TA, Gadella BM, Tapia JA, da Silva CB, Ferrusola CO, Peña FJ. 2016. Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. Reproduction 152: 683-94.

Durairajanayagam D, Singh D, Agarwal A, Henkel R. 2021. Causes and consequences of sperm mitochondrial dysfunction. Andrologia. 53: e13666.

Harlev A, Agarwal A, Gunes SO, Shetty A, du Plessis SS. 2015. Smoking and Male Infertility: An Evidence-Based Review. World J Mens Health 33: 143-60.

Henkel R, Sandhu IS, Agarwal A. 2019. The excessive use of antioxidant therapy: A possible cause of male infertility? Andrologia 51: e13162.

Howard AC, McNeil AK, McNeil PL. 2011. Promotion of plasma membrane repair by vitamin E. Nat Commun. 2: 597.

Juan CA, Pérez de la Lastra JM, Plou FJ, Pérez-Lebeña E. 2021. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. Int J Mol Sci. 22: 4642.

Khoirunnisa I, Susilowati S, Maslachah L, Suprayogi TW, Kurnijasanti R, Ratnani H. 2019. The effect of the addition of green tea extract (*Camellia sinensis*) in egg yolk citrate diluent for quality Sapudi Sheep spermatozoa storage on cold temperature. Ovozoa: J Anim Reprod. 8: 127-31.

Kurutas EB. 2016. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutr J. 15: 71.

Lü JM, Lin PH, Yao Q, Chen C. 2009. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. J Cell Mol Med. 14: 840-60.

Mahmoudi R, Azizi A, Abedini S, Hemayatkhah Jahromi V, Abidi H, Jafari Barmak M. 2018. Green tea improves rat sperm quality and reduced cadmium chloride damage effect in spermatogenesis cycle. J Med Life 11: 371-80.

Majzoub A, Agarwal A. 2018. Systematic review of antioxidant types and doses in male infertility: Benefits on semen parameters, advanced sperm function, assisted reproduction and live-birth rate. Arab J Urol. 16: 113-24.

Morales-Mantilla DE, Huang X, Erice P, Porter P, Zhang Y, Figueroa M, Chandra J, King KY, Kheradmand F, Rodríguez A. 2020. Cigarette Smoke Exposure in Mice using a Whole-Body Inhalation System. J Vis Exp. 164: 10.3791/61793.

Octaviani NAA, Utomo B, Sunarso A, Hamid IS, Madyawati SP, Suprayogi TW. 2021. The effect of Moringa (Moringa oleiferaLam.) leaf extract on the motility and viability of spermatozoa of heat exposed rats (*Rattus norvegicus*). Ovozoa: J Anim Reprod. 10: 65-71.

Padayatty SJ, Levine M. 2016. Vitamin C: the known and the unknown and Goldilocks. Oral Dis. 22: 463-93.

Prasanth MI, Sivamaruthi BS, Chaiyasut C, Tencomnao T. 2019. A Review of the Role of Green Tea (*Camellia sinensis*) in Antiphotoaging, Stress Resistance, Neuroprotection, and Autophagy. Nutrients 11: 474.

Prochowska S, Niżański W, Fontbonne A. 2022. Hypo-Osmotic Swelling Test (HOST) for Feline Spermatozoa: The Simplified Procedure and the Aspect of Sperm Morphology. Animals (Basel) 12: 903.

Puspita SAC, Susilowati S, Soeharsono S, Sardjito T, Samik A, Triana IN. 2020. Addition of alpha-tocopherol in skim milk-egg yolk extender on the quality of Sapudi ram spermatozoa stored at 5°C. Ovozoa: J Anim Reprod 20: 69-76.

Sabeti P, Pourmasumi S, Rahiminia T, Akyash F, Talebi AR. 2016. Etiologies of sperm oxidative stress. Int J Reprod Biomed. 14: 231-40.

Selvam MKP, Ambar RF, Agarwal A, Henkel R. 2020. Etiologies of sperm DNA damage and its impact on male infertility. Andrologia 53: e13706.

Takeshima T, Usui K, Mori K, Asai T, Yasuda K, Kuroda S, Yumura Y. 2020. Oxidative stress and male infertility. Reprod Med Biol. 20: 41-52.

Valavanidis A, Vlachogianni T, Fiotakis K. 2009. Tobacco smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles. Int J Environ Res Public Health 6: 445-62.

Vladika MA, Warsito SH, Susilowati S, Hernawati T, Kurnijasanti R, Restiadi TI. 2019. The influence of addition of green tea (*Camellia sinensis*) extract in diluent skim milk for sperm quality sheep Sapudi stored at a cool temperature. Ovozoa: J Anim Reprod 8: 116-21.

Widiantoro K, Madyawati SP, Warsito SH, Sardjito T, Hernawati T, Triana IN. 2021. Post-thawing quality of Merino ram semen in skim milk-egg yolk based diluent supplemented with crude protein Tyrosine Kinase isolate. Ovozoa: J Anim Reprod. 10: 39-45.

