AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

71 (2023) 3–4, 219–222

DOI:
10.1556/004.2023.00954

SHORT COMMUNICATION

Effects of ergothioneine supplementation on the quality of liquid-preserved and frozen-thawed boar semen

SUPITCHA KAEWMA[1,2,3], ZHAO NAMULA[1,4], SUONG THI NGUYEN[5], QINGYI LIN[1,6], NANAKA TORIGOE[1,6], BIN LIU[1,6], MEGUMI NAGAHARA[1,6], MASAHIRO NII[7], MASAYASU TANIGUCHI[5*] and TAKESHIGE OTOI[1,6]

[1] Bio-Innovation Research Center, Tokushima University, Tokushima, Japan

[2] Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

[3] Faculty of Veterinary Science, Prince of Songkla University, Songkhla, Thailand

[4] College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang China

[5] Joint Graduate School of Veterinary Sciences, Yamaguchi University, Yamaguchi, Japan

[6] Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima, Japan

[7] Tokushima Prefectural Livestock Research Institute, Tokushima, Japan

Received: 29 June 2023 • Accepted: 3 December 2023

ABSTRACT

This study examined the effects of ergothioneine (EGT) supplementation as an antioxidant on the quality of boar spermatozoa when using liquid and frozen preservation methods. In the first experiment, boar semen was preserved in an extender supplemented with 0, 50, 100 and 200 μM EGT, at 15 °C, part of the samples for one and another part for three weeks. In comparison with the control (without EGT), EGT supplementation at 100 μM significantly increased the percentage of total motility of spermatozoa that were preserved as a liquid both for one and three weeks ($P < 0.05$). EGT supplementation did not affect the quality of preserved spermatozoa, irrespective of the EGT concentration. In the second experiment, semen was frozen and thawed in the freezing extender supplemented with 0, 50, 100 and 200 μM EGT. In comparison with the control, the 100 μM EGT supplementation significantly increased the percentages of total and progressive motility of frozen-thawed spermatozoa ($P < 0.05$). EGT (100 μM) supplementation did not affect the viability, the plasma membrane integrity, or the acrosomal integrity of frozen-thawed spermatozoa. These findings indicate that supplementing extenders with 100 μM EGT may improve the motility of boar sperm in both liquid and freezing preservation methods.

KEYWORDS

antioxidant, boar, preservation, quality, semen

The preservation method decreases the temperature of semen to 15–18 °C or lower. Boar semen is extremely sensitive to cold damage. In addition, reactive oxygen species (ROS) play a key role in sperm impairment within the cryo-environment. ROS affects sperm function, acrosome integrity, and lipid peroxidation (Awda et al., 2009) in the cryo-environment, thereby reducing sperm motility, plasma membrane integrity, and mitochondrial function in post-thawed sperm (Fraser et al., 2007). In addition, ROS can cause DNA damage (Bennetts and Aitken, 2005) and inhibit sperm-oocyte fusion (Aitken et al., 1989), resulting in fertility reduction. It has been demonstrated that the addition of antioxidants improved sperm quality after thawing, by improving the success of sperm-oocyte binding and fertilisation (Contri et al., 2011; Nagahara et al., 2023).

*Corresponding author.
Tel./fax: +81 83 933 5911.
E-mail: masa0810@yamaguchi-u.ac.jp

Ergothioneine (EGT; 2-mercaptohistidine trimethylbetaine) is a natural antioxidant derived from microorganisms, non-yeast fungi, mycobacteria and cyanobacteria and has potent antioxidant activity that reduces oxidative stress in many tissues and cells (Cheah and Halliwell, 2012; Fu and Shen, 2022). Seminal plasma also contains EGT, a non-enzymatic antioxidant that mitigates oxidative damage (Storey, 1997; Strzezek et al., 1999). Therefore, supplementation with EGT as an antioxidant may improve the quality of porcine sperm after liquid storage or freezing. This study aimed to investigate the effects of EGT supplementation in semen extenders during liquid- and freeze-preservation on the quality of boar semen.

Each fresh semen sample collected from three mature, fertile large white boars, was diluted threefold with a modified Modena extender as described previously (Namula et al., 2014). The diluted samples were centrifuged at 600×*g* for 10 min. After removing the supernatant, pellets from the semen samples of the three boars were pooled into one tube to eliminate individual differences.

In the liquid-preservation experiment, the mixed samples were preserved at 15 °C for one or three weeks, according to the method described by Nagahara et al. (2023) with minor modifications. Briefly, the mixed samples were diluted in a modified Modena extender to a final concentration of 2×10^8 cells/mL at 25 °C. The extended semen samples (3 mL) were transferred to 15 mL conical polystyrene tubes and preserved at 15 °C in a Cool-Incubator A1201 (Ikuta Sangyo, Nagano, Japan) within an uncontrolled atmosphere.

In the freeze-preservation experiment, the mixed samples were cryopreserved according to the method described by Namula et al. (2019) with minor modifications. Briefly, semen samples were diluted to a concentration of 4×10^8 cells/mL with the first extender. Semen samples were then cooled to 5 °C from room temperature for approximately 2 h and immediately diluted to a final concentration of 2×10^8 cells/mL with the second extender. The spermatozoa were frozen in liquid nitrogen and thawed at 38 °C for 10 s on the examination day.

Liquid-preserved and frozen-thawed semen samples were tested for total sperm motility, progressive motility, sperm viability, plasma membrane integrity and acrosome integrity according to the methods described by Taniguchi et al. (2014). We performed triplicate measurements for each sperm parameter. Motility analyses were performed using a computer-assisted sperm analysis system with a Sperm Class Analyser ® (SCA® v.4.2) device (MICROPTIC, Barcelona, Spain). The analysis of total (TM, curvilinear velocity >10 μm s^{-1}) and progressive motility (RAP, curvilinear velocity >100 μm s^{-1}) was based on examining 25 consecutive digitised images obtained from 3 to 5 fields using a × 10 phase contrast objective. At least 500 spermatozoa per sample were analysed at an image capture speed of 40 ms. Analyses of cell viability, plasma membrane integrity and acrosome integrity were conducted using a live/dead stain combination (SYBR-14/propidium iodide (PI); LIVE/DEAD Sperm Viability Kit; Molecular Probes, Inc., Eugene, OR, USA), a hypo-osmotic swelling test (Ahmad et al., 2003), and fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Vector Laboratories, Inc., Burlingame, CA, USA), respectively.

To examine the effects of EGT supplementation on the quality of spermatozoa during liquid preservation, the semen samples were preserved in the modified Modena extender supplemented with 0, 50, 100 and 200 μM EGT (Wako Pure Chemical Industries, Osaka, Japan) at 15 °C for one or three weeks.

To examine the effects of EGT supplementation on the quality of post-thawed spermatozoa during the freezing and thawing processes, the semen samples were frozen and thawed in the freezing extender supplemented with 0, 50, 100 and 200 μM EGT. Semen samples frozen for at least 3 days were assessed within 5 min of thawing.

Mean sperm quality values were analysed using the analysis of variance (ANOVA) method, followed by the Fisher's protected least significant difference (PLSD) test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). The data were subjected to an arcsine transformation prior to statistical analysis. Differences with a probability value of (P) ≤ 0.05 were considered statistically significant.

When compared to that of the control (without EGT), 100 μM EGT supplementation in semen extender during storage at 15 °C for both one and three weeks significantly increased the percentage of total motility of preserved spermatozoa ($P < 0.05$; Table 1). However, EGT supplementation did not affect progressive motility, viability, plasma membrane integrity, or acrosomal integrity of preserved spermatozoa, irrespective of EGT concentration. Similarly, 100 μM EGT supplementation in the freezing extender significantly increased the percentage of total and progressive motility of frozen-thawed spermatozoa ($P < 0.05$) compared to that of the control group; however, viability, plasma membrane integrity, or acrosomal integrity of frozen-thawed spermatozoa were not affected (Table 2).

The production of ROS may damage sperm cells and induce oxidative stress, leading to poor sperm quality in boars and other species by reducing sperm motility, sperm-oocyte fusion ability and fertilisation (Michael et al., 2008). Importantly, antioxidants play a key role in protecting sperm against the effects of ROS. In the present study, we observed that the supplementation of the extender with 100 μM EGT significantly improved the motility of liquid-preserved and cryopreserved spermatozoa. It has been described that oxidative damage by ROS may affect mitochondrial DNA and membrane integrity, causing movement interference in sperm (O'Flaherty and Scarlata, 2022). EGT has been found to protect sperm cells from oxidative damage and peroxidative chemicals, and to improve motility and DNA integrity by scavenging singlet oxygen and hydroxyl and peroxyl radicals in cryopreserved ram sperm (Najafi et al., 2014). A study focusing on the quality of post-thawed canine sperm has demonstrated that supplementation with 100 μM EGT increased sperm motility, movement kinetics and the number (?) of rapid sperms by reducing the levels of ROS (Usuga et al., 2021). These studies support our findings

Table 1. Effects of ergothioneine concentration on the quality of boar spermatozoa incubated at 15 °C for one and three weeks*

Storage duration	Groups (μM)**	Motility (%)		Viability (%)	Plasma membrane integrity (%)	Acrosome integrity (%)
		Total	Progressive			
One week						
	Control	86.8 ± 1.8[a]	71.5 ± 1.1	60.6 ± 1.0	51.2 ± 2.3	94.5 ± 1.2
	50	89.0 ± 1.2[a,b]	72.9 ± 1.6	60.5 ± 2.1	52.9 ± 3.0	94.3 ± 0.7
	100	91.4 ± 0.2[b]	74.0 ± 1.6	62.8 ± 2.0	53.3 ± 3.7	95.1 ± 0.8
	200	87.6 ± 1.8[a,b]	69.7 ± 1.9	59.2 ± 1.2	51.4 ± 2.2	93.3 ± 1.5
Three weeks						
	Control	41.4 ± 2.8[a]	20.0 ± 2.0	21.6 ± 2.3	32.3 ± 1.5	91.8 ± 1.0
	50	39.5 ± 1.3[a]	18.8 ± 1.9	19.7 ± 1.5	34.3 ± 2.3	92.8 ± 0.9
	100	53.8 ± 2.6[b]	28.7 ± 4.1	29.1 ± 1.5	35.9 ± 2.0	92.6 ± 0.8
	200	47.3 ± 6.6[a,b]	23.4 ± 5.5	24.7 ± 5.3	34.1 ± 1.3	92.1 ± 0.3

*Four replicates were performed. Percentages are expressed as the mean ± SEM.
**Semen samples were incubated with 0 (control), 50, 100, and 200 μM of ergothioneine.
[a–b]Values with different superscript letters in the same column within the same storage duration differ statistically significantly ($P < 0.05$).

Table 2. Effects of ergothioneine concentration on the quality of frozen-thawed boar spermatozoa*

Groups (μM)**	Motility (%)		Viability (%)	Plasma membrane integrity (%)	Acrosome integrity (%)
	Total	Progressive			
Control	59.0 ± 3.2[a,c]	34.0 ± 2.1[a]	31.3 ± 3.4[a,b]	32.8 ± 1.8	91.4 ± 0.7
50	56.5 ± 3.3[a]	34.2 ± 0.8[a]	30.0 ± 3.2[b]	34.2 ± 1.8	91.5 ± 0.7
100	70.3 ± 1.9[b]	44.6 ± 2.8[b]	40.5 ± 3.5[a]	37.6 ± 1.6	92.5 ± 0.3
200	66.4 ± 3.4[b,c]	41.1 ± 1.3[b]	37.3 ± 3.5[a,b]	36.5 ± 1.2	92.4 ± 0.8

*Four replicates were performed. Percentages are expressed as the mean ± SEM.
**Semen samples were frozen with 0 (control), 50, 100 and 200 μM of ergothioneine.
[a–c]Values with different superscript letters in the same column differ statistically significantly ($P < 0.05$).

that supplementation with 100 μM EGT enhances sperm motility in both liquid-preserved and cryopreserved spermatozoa. However, our study did not examine DNA or mitochondrial damage, which can also be caused by intracellular ROS. Further studies are necessary to evaluate the role of EGT in the prevention of DNA and mitochondrial damage and to better describe the mechanism of action of EGT on boar sperm.

Sperm viability and membrane integrity are important parameters for the assessment of sperm quality. ROS plays a crucial role in sperm damage, leading to sperm death during cryopreservation. Importantly, boar sperm cells are highly sensitive to peroxidative damage because their structures contain polyunsaturated fatty acids (Cerolini et al., 2000). In the present study, we observed that adding EGT had no beneficial effects on cell viability, plasma membrane integrity or acrosomal integrity in either semen preservation methods. Cheah and Halliwell (2012) have suggested that the *in vivo* oxidative activity of EGT may powerfully scavenge hydroxyl radicals, hydrogen peroxide, superoxide radicals, peroxynitrite and redox reactions of cation compounds. Many cells are known to contain various protective mechanisms against peroxidation through producing enzymatic antioxidative agents e.g. superoxide dismutase, glutathione peroxidase and glucose-6-phosphate dehydrogenase that play important roles in the lipid peroxidative protection of sperm cells (Nair et al., 2006; Walters et al., 2020). Since the effect of EGT, used in the present study, was due to non-enzymatic antioxidants (Cheah and Halliwell, 2012), this may be the reason why our results showed no substantial enhancement of the sperm quality parameters after EGT supplementation.

In conclusion, EGT supplementation in semen extender at a concentration of 100 μM is likely to improve sperm motility but no other quality parameters when using either liquid (15 °C) or freezing (−196 °C) preservation methods. Therefore in the future, it may be necessary to examine other parameters by which EGT supplementation is associated with sperm quality or fertilization ability.

ACKNOWLEDGMENTS

This study was supported in part by JSPS KAKENHI, grant numbers JP22H02499 and JP22K19896. We acknowledge the financial support from the Uzushio Program of Tokushima University.

REFERENCES

Ahmad, Z., Anzar, M., Shahab, M., Ahmad, N. and Andrabi, S. M. (2003): Sephadex and Sephadex ion-exchange filtration

improves the quality and freezability of low-grade buffalo semen ejaculates. Theriogenology **59**, 1189–1202.

Aitken, R. J., Clarkson, J. S. and Fishel, S. (1989): Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol. Reprod. **41**, 183–197.

Awda, B. J., Mackenzie-Bell, M. and Buhr, M. M. (2009): Reactive oxygen species and boar sperm function. Biol. Reprod. **81**, 553–561.

Bennetts, L. E. and Aitken, R. J. (2005): A comparative study of oxidative DNA damage in mammalian spermatozoa. Mol. Reprod. Dev. **71**, 77–87.

Cerolini, S., Maldjian, A., Surai, P. and Noble, R. (2000): Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim. Reprod. Sci. **58**, 99–111.

Cheah, I. K. and Halliwell, B. (2012): Ergothioneine; antioxidant potential, physiological function and role in disease. Biochim. Biophys. Acta **1822**, 784–793.

Contri, A., De Amicis, I., Molinari, A., Faustini, M., Gramenzi, A., Robbe, D. and Carluccio, A. (2011): Effect of dietary antioxidant supplementation on fresh semen quality in stallion. Theriogenology **75**, 1319–1326.

Fraser, L., Dziekońska, A., Strzezek, R. and Strzezek, J. (2007): Dialysis of boar semen prior to freezing-thawing: its effects on post-thaw sperm characteristics. Theriogenology **67**, 994–1003.

Fu, T. T. and Shen, L. (2022): Ergothioneine as a natural antioxidant against oxidative stress-related diseases. Front Pharmacol **13**, 850813.

Michael, A. J., Alexopoulos, C., Pontiki, E. A., Hadjipavlou-Litina, D. J., Saratsis, P., Ververidis, H. N. and Boscos, C. M. (2008): Quality and reactive oxygen species of extended canine semen after vitamin C supplementation. Theriogenology **70**, 827–835.

Nagahara, M., Namula, Z., Lin, Q., Takebayashi, K., Torigoe, N., Liu, B., Aihara, M., Nii, M. and Otoi, T. (2023): Effects of curcumin supplementation on quality of porcine spermatozoa irradiated with ultraviolet-C at 228 nm during liquid preservation. Reprod. Domest. Anim. **58**, 882–887.

Nair, S. J., Brar, A. S., Ahuja, C. S., Sangha, S. P. and Chaudhary, K. C. (2006): A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. Anim. Reprod. Sci. **96**, 21–29.

Najafi, A., Kia, H. D., Mohammadi, H., Najafi, M. H., Zanganeh, Z., Sharafi, M., Martinez-Pastor, F. and Adeldust, H. (2014): Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender. Cryobiology **69**, 68–73.

Namula, Z., Kodama, R., Tanihara, F., Morita, Y., Sato, Y., Wittayarat, M., Taniguchi, M. and Otoi, T. (2014): Effects of skim-milk supplementation on the quality and penetrating ability of boar semen after long-term preservation at 15 degrees C. Acta Vet. Hung. **62**, 106–116.

Namula, Z., Tanihara, F., Wittayarat, M., Hirata, M., Nguyen, N. T., Hirano, T., Le, Q. A., Nii, M. and Otoi, T. (2019): Effects of Tris (hydroxymethyl) aminomethane on the quality of frozen-thawed boar spermatozoa. Acta Vet. Hung. **67**, 106–114.

O'Flaherty, C. and Scarlata, E. (2022): Oxidative stress and reproductive function: the protection of mammalian spermatozoa against oxidative stress. Reproduction **164**, F67–F78.

Storey, B. T. (1997): Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol. Hum. Reprod. **3**, 203–213.

Strzezek, J., Lapkiewicz, S. and Lecewicz, M. (1999): A note on antioxidant capacity of boar seminal plasma. Anim. Sci. Pap. Rep. **17**, 181–188.

Taniguchi, M., Wittayarat, M., Morinaga, K., Sato, Y., Kin Do, L. T., Chatdarong, K., Techakumphu, M., Nii, M. and Otoi, T. (2014): Chelation of trace elements in preservation medium influences the quality of boar spermatozoa during liquid preservation at 5 degrees C for 4 weeks. Cryoletters **35**, 336–344.

Usuga, A., Tejera, I., Gómez, J., Restrepo, O., Rojano, B. and Restrepo, G. (2021): Cryoprotective Effects of Ergothioneine and Isoespintanol on Canine Semen. Animals (Basel) **11**.

Walters, J. L. H., Gadella, B. M., Sutherland, J. M., Nixon, B. and Bromfield, E. G. (2020): Male infertility: Shining a light on lipids and lipid-modulating enzymes in the male germline. J. Clin. Med. **9**.