Exp. Anim. 63(2), 149–154, 2014

—Original—

Effects of Cholesterol-Loaded Cyclodextrins on the Rate and the Quality of Motility in Frozen and Thawed Rabbit Sperm

Kazutoshi NISHIJIMA[1)], Shinji YAMAGUCHI[1)], Mai TANAKA[1)], Yusuke SAKAI[2)], Chihiro KOSHIMOTO[2)], Masatoshi MORIMOTO[1)], Teruo WATANABE[1)], Jianglin FAN[3)], and Shuji KITAJIMA[1)]

[1)]*Division of Biological Resources and Development, Analytical Research Center for Experimental Sciences, Saga University, 5–1–1 Nabeshima, Saga 849-8501, Japan*
[2)]*Department of Bio-resources, Division of Biotechnology, Frontier Science Research Center, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*
[3)]*Department of Molecular Pathology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan*

Abstract: The motility of sperm after freezing and thawing is critical for effective cryopreservation. It is known that supplementation with cholesterol-loaded cyclodextrin (CLC) improves cryosurvival of sperm in various animals. To clarify the effects of supplementation with CLC on rabbit sperm motility after freezing and thawing, rabbit sperm motility was analyzed using a computer-assisted sperm analysis system. Sperm motility with CLC supplementation was 29.4 ± 9.6% (mean ± SD), which was significantly higher than that of controls (20.8 ± 7.1%, $P<0.05$). The curvilinear velocity of sperm with CLC exceeded that of controls, whereas the values for linearity and wobble were significantly lower in sperm with CLC compared with controls. After artificial insemination, 44.3% of recovered ova were fertilized in the CLC-supplemented group, which was higher than the percentage in the control group (36.4%). The results indicate that supplementation with CLC improves the rate and quality of motility in rabbit sperm after freezing and thawing, and would be advantageous for successful cryopreservation.
Key words: computer-assisted sperm analysis, cryopreservation, motility, rabbit, sperm

Introduction

Rabbits have lipid metabolism characteristics that resemble those in humans, and are recognized as a useful animal model in studies of lipid metabolism and cardiovascular disorders [35]. A number of transgenic (Tg) rabbit strains have been established as human disease models and therapeutic protein bioreactors [5, 6], making the rabbit a valuable bioresource [20].

Freezing embryos or sperm is the preferred way of preserving animal strains due to the low maintenance costs and increased reliability as compared with the maintenance of live colonies. In the case of the rabbit, sperm cryopreservation can be the preferred choice, as ejaculated semen is readily collectable and artificial insemination is easily applied. Several studies have been performed to improve the cryosurvivability of rabbit sperm in terms of the extenders and protocols [13, 14, 22]. However, a universal protocol has not yet been established, and several methods are used in the cryopreservation of rabbit sperm. We have employed a protocol using an egg-yolk HEPES extender containing

(Received 17 July 2013 / Accepted 27 September 2013)
Address Corresponding: K. Nishijima, Division of Biological Resources and Development, Analytical Research Center for Experimental Sciences, Saga University, 5–1–1 Nabeshima, Saga 849-8501, Japan

acetamide [13] to preserve Tg rabbit sperm and have confirmed the viability of sperm cryopreserved in this way for up to 5.6 years [25].

One of the measures of the efficacy of sperm preservation is the motility of sperm after freezing and thawing. To improve the motility of sperm that has been frozen and then thawed, several supplements can be added to the freezing solution, including chemicals and proteins [9, 27, 31, 34]. It is known that freezing of sperm induces a lipid phase transition in the plasma membrane resulting in cholesterol depletion, which causes membrane destabilization [4]. Therefore, supplementation with cholesterol would reduce the damage to the sperm membrane during freezing. Cholesterol can be transferred easily into membranes down a concentration gradient using cyclodextrins, which encapsulate hydrophobic compounds [16]. Supplementation with cholesterol-loaded cyclodextrin (CLC) has been revealed to increase sperm motility after freezing and thawing in some animal species, as reviewed by Mocé *et al.* [21].

The present study was conducted to clarify the effects of supplementation with CLC on rabbit sperm motility after freezing and thawing, using a computer-assisted sperm analysis (CASA) system, with the aim of improving cryopreservation of rabbit sperm.

Materials and Methods

All animal experimental procedures were approved by the Animal Care Committee of Saga University and conformed with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health.

Semen collection

Ejaculated semen from six reproductively mature, male Japanese white (JW) rabbits (6–26 months old, Japan SLC, Inc., Hamamatsu, Japan) was collected using an artificial vagina. Sperm samples collected from 2–3 male rabbits were pooled and analyzed as one experiment, which was repeated 10 times (n=10).

The number of immobile spermatozoa was counted before counting the total number of spermatozoa post fixation with glutaraldehyde using an improved Neubauer hemocytometer (Sunlead Glass Corp., Koshigaya, Japan). Sperm motility was calculated by subtracting the number of immotile sperm from the total number of sperm. The concentration of motile sperm was adjusted to 600×10^6 sperm/ml, by condensation with centrifugation ($200 \times g$, 15 min) or dilution with tris-citrate acid-glucose (TCG) buffer (313.8 mM tris, 103.1 mM citric acid, and 33.3 mM glucose).

CLC solution preparation

The CLC was prepared as previously described by Purdy and Graham [28]. In brief, 200 mg of cholesterol was dissolved in 1 ml of chloroform (cholesterol solution). In a separate tube, 1 g of methyl-β-cyclodextrin was dissolved in 2 ml methanol, and 0.45 ml of the cholesterol solution was added to the cyclodextrin solution and vortexed. The solvents were evaporated using nitrogen gas to obtain white CLC powder. Then, 50 mg of CLC powder was dissolved in 1 ml of tris-buffer (276.5 mM tris base, 90.9 mM citric acid monohydrate, and 76.8 mM fructose) containing 3 mg/ml BSA. The CLC solution was incubated in a water bath at 39°C for 5 min.

Sperm freezing and thawing

The collected semen was divided into two groups. One group was incubated in CLC solution (3 mg/120×10^6 sperm) (the CLC group), and the other group was incubated in the same volume of TCG buffer, without CLC (the EYA group). Both groups of semen were incubated at 22°C for 15 min.

A five-fold volume of egg-yolk HEPES, containing acetamide (EYA; pH: 7.2, 125 mM glucose, 105 mM lactose, 91 mM raffinose, 10 mM HEPES, 6% (w/v) acetamide, 20% (v/v) egg-yolk, 1,000 U/ml streptomycin, 1,000 U/ml penicillin G) extender was added to both semen groups. The solution was cooled in a programmable incubator (Taitech, Saitama, Japan) at the rate of −0.2°C/min, from 25 to 5°C. The solution was then packed into 0.5 ml straws (Fujihira Industry, Tokyo, Japan) and left in a refrigerator set to 4°C for 30 min. The straws were frozen in liquid nitrogen vapor for 15 min and then stored in liquid nitrogen.

The frozen straws were thawed by immersion in a 37°C water bath for 30 sec just before the motility analysis.

Sperm motility analysis

For each sample, more than 200 sperm from the JW rabbits (n=10) were analyzed by computer-assisted sperm analysis (CASA; Sperm Class Analyzer, Microptic S.L., Spain) system to clarify the effects of CLC supplement on sperm movement pattern characteristics.

Table 1. Sperm motility (%) after freezing and thawing with/without CLC supplementation

	Total motility	Velocity			Static
		Rapid	Medium	Slow	
EYA	20.8 ± 7.5	11.8 ± 5.6	4.1 ± 2.2	4.9 ± 2.8	79.2 ± 7.5
CLC	29.4 ± 10.2*	15.5 ± 9.1	6.3 ± 3.3	7.6 ± 1.9*	70.6 ± 10.2

Numbers are mean ± SD (%), *: Significantly higher than EYA (P<0.05).

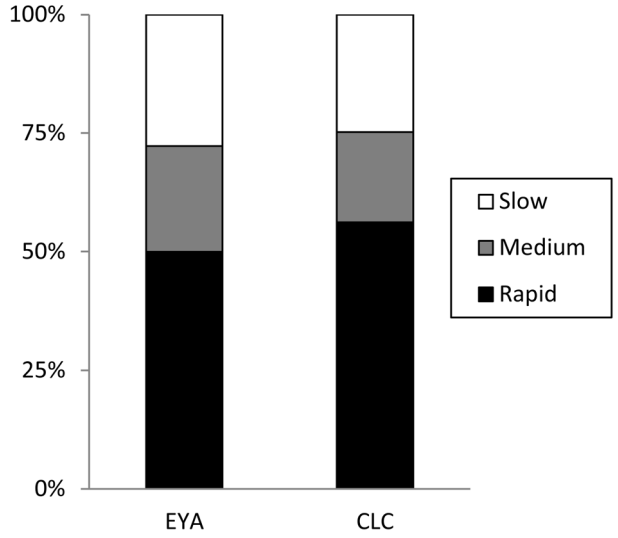

Fig. 1. Effect of CLC on the ratio of rapid, medium, or slow sperm after freezing and thawing.

The motile sperm were divided by their moving speed into rapid (>40 μm/s), medium (between 40 and 20 μm/s), and slow (between 20 and 10 μm/s) motility.

The values for curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), average velocity (VAP, μm/s), straightness index (STR, %), linearity index (LIN, %), and oscillation index of the sperm (WOB, %), together with the amplitude of lateral movement of the sperm heads (ALH, μm), were recorded.

Analysis of sperm fertilization capacity

Fifteen female JW rabbits (4–5 months of age, Japan SLC) for each group (EYA or CLC) were intramuscularly injected with 150 units (U) of pregnant mare's serum gonadotropin (Serotropin, ASKA Pharmaceutical, Tokyo, Japan) to induce superovulation 3 days before artificial inseminations (AI). The female rabbits were artificially inseminated with 40×10^6 motile sperm after freezing and thawing with/without CLC supplementation using a glass inseminating pipette [12]. Concurrently, the female rabbits were intravenously injected with 100 U of human chorionic gonadotrophin (Gonatropin, ASKA Pharmaceutical) to induce ovulation. At 17–19 h after artificial insemination, the rabbits were sacrificed using an anaesthetic overdose (thiamylal sodium; Kyorin Pharmaceutical, Tokyo, Japan). The ova collected by flushing the oviducts with an M2 medium [11], were transferred to an M199 medium (Sigma-Aldrich, St Louis, MO, USA) containing 20% fetal bovine serum (FBS; Sigma-Aldrich) and 0.1 g/l L-glutamine (Sigma-Aldrich). The numbers of collected ova and fertilized ova with male and female pronuclei were counted. The fertilized ova were utilized for a gene engineering experiment to produce transgenic rabbits as previously described [15].

Statistic analysis

Paired t-tests were employed to reveal any significant differences (P<0.05) between the EYA and CLC groups in terms of sperm motility, and for each motion parameter. For analysis of sperm fertilization capacity, significant differences (P<0.05) in percentages of fertile ova between the EYA and CLC groups were examined with Pearson's chi-squared test.

Results

The motility of the fresh sperm before freezing was 94.0 ± 1.9% (mean ± SD). The sperm motility of the CLC group was 29.4 ± 9.6%, which was significantly higher than that of the EYA group (20.8 ± 7.1%, P<0.05, Table 1). Although the proportion of slowly motile sperm in the CLC group (7.6 ± 1.8) was significantly greater than that of the EYA group (4.9 ± 2.7, P<0.05, Table 1), the ratio of rapid to medium to slow sperm for all motile sperm was not significantly different between the EYA and CLC groups (Fig. 1).

The VCL of the CLC group, especially in rapidly motile sperm, exceeded that of the EYA group, whereas the LIN and WOB were significantly lower in the CLC group than in the EYA group (Table 2). There were no signifi-

Table 2. Sperm values (mean ± SD) in frozen and thawed rabbit semen

		Total	Rapid	Medium	Slow
VCL (μm/s)	EYA	55.9 ± 10.2	72.1 ± 12.3	15.8 ± 6.9	7.4 ± 3.5
	CLC	63.1 ± 15.3	86.9 ± 12.7*	20.2 ± 4.7	9.4 ± 3.3
VSL (μm/s)	EYA	33.7 ± 8.0	45.0 ± 10.5	6.7 ± 4.4	2.1 ± 1.9
	CLC	33.5 ± 13.5	47.4 ± 17	8.6 ± 2.7	3.2 ± 1.7
VAP (μm/s)	EYA	39.1 ± 8.0	51.2 ± 9.7	9.8 ± 4.8	3.6 ± 2.4
	CLC	40.0 ± 13.5	55.5 ± 15.1	11.8 ± 3.1	7.0 ± 6.2
LIN (%)	EYA	59.5 ± 15.7	63.7 ± 15.7	36.8 ± 18.4	21.8 ± 17.8
	CLC	52.8 ± 17.1*	54.6 ± 18.4**	42.2 ± 9.6	33.1 ± 8.9*
STR (%)	EYA	85.7 ± 5.7	87.4 ± 6.9	63.7 ± 17.8	48.9 ± 22.5
	CLC	81.9 ± 8.4	83.6 ± 9.0	71.8 ± 8.1	60.6 ± 11.8
WOB (%)	EYA	70.6 ± 11.5	72.1 ± 13.1	61.7 ± 11.0	42.9 ± 20.6
	CLC	63.3 ± 14.9**	64.0 ± 15.9**	58.3 ± 8.4	54.3 ± 6.0
ALH (μm)	EYA	2.2 ± 0.6	2.2 ± 0.6	0.5 ± 0.3	–
	CLC	2.5 ± 0.5	2.7 ± 0.6	0.8 ± 0.3	–

*: $P<0.05$, **: $P<0.01$ Significantly different from EYA.

cant differences in the values of VSL, VAP, STR, and ALH between the CLC and EYA groups.

In the EYA group, 429 ova were recovered from 15 female rabbits, and 156 (36.4%) of them contained male and female pronuclei. In the CLC group, male and female pronuclei were found in 221 (44.3%) out of recovered 499 ova, which was significantly more than was found in the EYA group ($P<0.05$).

Discussion

The present study revealed that supplementation with CLC led to changes not only in the rate, but also the quality, of motility in frozen and thawed rabbit sperm.

The motility of frozen and thawed sperm greatly affects the efficiency of cryopreservation, since the number of motile sperm is directly related to reproductive outcome in animal strains. Thus, raising the motility of the sperm after freezing and thawing is an important concern. The characteristics of sperm differ among strains or species of animals, and there are different optimum freezing protocols for each animal [20, 22–24, 30].

CLC supplementation is known to increase sperm motility after freezing and thawing in several animal species, including the bull, stallion, donkey, goat, ram, boar and mouse, as reviewed by Mocé *et al.* [21]. This would be the result of high integrity of the plasma membrane, which was raised by introduction of cholesterol within the CLC [19]. In the present study, we found this to also be the case in the rabbit. CLC supplementation also increased sperm motility after freezing and thawing in human apolipoprotein AII [17] and human C-reactive protein [18] transgenic rabbits (data not shown). Therefore, CLC supplementation seemed to improve the efficacy of sperm cryopreservation in the rabbit, including transgenic strains.

With rabbit artificial insemination, the number of rapid motile sperm would affect the reproductive outcome, since the sperm need to reach the ova via a long reproductive tract. CASA revealed that the increased motility of rabbit sperm after freezing and thawing was not restricted of slow motility sperm only but also included rapid and medium motility sperm. This might indicate that CLC not only rescued the immotile sperm, enhancing them to slow motility sperm, but also raised the grade of each category of sperm.

As employed in the practical diagnosis of male sterility in humans, CASA provides an assessment of the motion properties of sperm, which is an indication of sperm quality and condition [7, 33]. Cancel *et al.* [3] reported significantly increased VCL and significantly decreased LIN in hyperactivated rat sperm. Sperm hyperactivation is thought to be critical for fertilization, as it is required for penetration of the zona pellucida [10]. In the present study, CLC supplementation led to increasing values for VCL, and decreasing values for LIN and WOB in the rabbit sperm. Though it is not certain if such changes in movement patterns indicate sperm hyperactivation, the changes might be advantageous for fertilization. It is reported that CLC enhances osmotic tolerance and inhibits the acrosome reaction in fresh sperm of some animals including the rabbit [1, 8, 26, 29, 32] and that freezing and thawing progresses the capacitation process [2]. Though reproductive efficiency was not examined

in the present study, we obtained fertilized ova by artificial insemination with CLC-supplemented frozen and thawed sperm at a higher rate than in the EYA group. This would indicate that capacitation of sperm proceeded during freezing and thawing or traveling through the female genital tract even when supplemented with CLC.

In conclusion, the present study indicates that supplementation with CLC improves the rate and quality of motility in rabbit sperm after freezing and thawing, and would be beneficial for successful cryopreservation of rabbits, especially for valuable strains including transgenic strains.

Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research C (Grant Number 22500386).

References

1. Aksoy, M., Akman, O., Lehimcioğlu, N.C., and Erdem, H. 2010. Cholesterol-loaded cyclodextrin enhances osmotic tolerance and inhibits the acrosome reaction in rabbitspermatozoa. *Anim. Reprod. Sci*. 120: 166–172. [CrossRef] [Medline]
2. Barbas, J.P. and Mascarenhas, R.D. 2009. Cryopreservation of domestic animal sperm cells. *Cell Tissue Bank*. 10: 49–62. [CrossRef] [Medline]
3. Cancel, A.M., Lobdell, D., Mendola, P., and Perreault, S.D. 2000. Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. *Hum. Reprod*. 15: 1322–1328. [CrossRef] [Medline]
4. Crockett, E.L. 1998. Cholesterol function in plasma membranes from ectotherms: membrane-specific roles in adaptation to temperature. *Amer. Zool*. 38: 291–304.
5. Duranthon, V., Beaujean, N., Brunner, M., Odening, K.E., Santos, A.N., Kacskovics, I., Hiripi, L., Weinstein, E.J., and Bosze, Z. 2012. On the emerging role of rabbit as human disease model and the instrumental role of novel transgenic tools. *Transgenic Res*. 21: 699–713. [CrossRef] [Medline]
6. Fan, J., Challah, M., and Watanabe, T. 1999. Transgenic rabbit models for biomedical research: current status, basic methods and future perspectives. *Pathol. Int*. 49: 583–594. [CrossRef] [Medline]
7. Garrett, C., Liu, D.Y., Clarke, G.N., Rushford, D.D., and Baker, H.W. 2003. Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. *Hum. Reprod*. 18: 1643–1649. [CrossRef] [Medline]
8. Galantino-Homer, H.L., Zeng, W.X., Megee, S.O., Dallmeyer, M., Voelkl, D., and Dobrinski, I. 2006. Effects of 2-hydroxypropyl-beta-cyclodextrin and cholesterol on porcine sperm viability and capacitation status following cold shock or incubation. *Mol. Reprod. Dev*. 73: 638–650. [CrossRef] [Medline]
9. Gibb, Z., Butler, T.J., Morris, L.H., Maxwell, W.M., and Grupen, C.G. 2013. Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and nonsorted stallion sperm. *Theriogenology* 79: 1001–1009. [CrossRef] [Medline]
10. Ho, H.C. and Suarez, S.S. 2001. Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction* 122: 519–526. [CrossRef] [Medline]
11. Hughes, M.A. and Anderson, G.B. 1982. Short-term *storage of rab*bit embryos at 4°C. *Theriogenology* 18: 275–282. [CrossRef] [Medline]
12. Kaneda, M., Fujii, S., Aoyama, H., and Teramoto, S. 1993. Usefulness of a simplified artificial insemination technique in the rabbit for teratology studies. *Jikken Dobutsu* 42: 217–220. [Medline]
13. Kashiwazaki, N., Okuda, Y., Seita, Y., Hisamatsu, S., Sonoki, S., Shino, M., Masaoka, T., and Inomata, T. 2006. Comparison of glycerol, lactamide, acetamide and dimethylsulfoxide as cryoprotectants of Japanese white rabbit spermatozoa. *J. Reprod. Dev*. 52: 511–516. [CrossRef] [Medline]
14. Kitajima, S. 2009. Improvement of rabbit production. pp. 3–12. *In*: Rabbit Biotechnology: Rabbit Genomics, Transgenesis, Cloning and Models (Houdebine, L.M.,and Fan, J., eds.), Springer, New York.
15. Kitajima, S., Morimoto, M., Watanabe, T., and Fan, J. 2003. Transgenic rabbits: production and application for human disease models. pp. 179–194. *In*: Recent Research Developments in Biophysics & Biochemistry (Pandalai, S.G. ed.), Research Signpost, Kerala.
16. Klein, U., Gimpi, G., and Fahrenholz, F. 1995. Alteration of the myometrial plasma membrane cholesterol content with cyclodextrin modulates the binding aYnity of the oxytocin receptor. *Biochemistry* 34: 13784–13793. [CrossRef] [Medline]
17. Koike, T., Kitajima, S., Yu, Y., Li, Y., Nishijima, K., Liu, E., Sun, H., Waqar, A.B., Shibata, N., Inoue, T., Wang, Y., Zhang, B., Kobayashi, J., Morimoto, M., Saku, K., Watanabe, T., and Fan, J. 2009. Expression of human apoAII in transgenic rabbits leads to dyslipidemia: a new model for combined hyperlipidemia. *Arterioscler. Thromb. Vasc. Biol*. 29: 2047–2053. [CrossRef] [Medline]
18. Koike, T., Kitajima, S., Yu, Y., Nishijima, K., Zhang, J., Ozaki, Y., Morimoto, M., Watanabe, T., Bhakdi, S., Asada, Y., Chen, Y.E., and Fan, J. 2009. Human C-reactive protein does not promote atherosclerosis in transgenic rabbits. *Circulation* 120: 2088–2094. [CrossRef] [Medline]
19. de Meyer, F. and Smit, B. 2009. Effect of cholesterol on the structure of a phospholipid bilayer. *Proc. Natl. Acad. Sci. USA* 106: 3654–3658. [CrossRef] [Medline]
20. Mazur, P., Leibo, S.P., and Seidel, G.E. Jr. 2008. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. *Biol. Reprod*. 78: 2–12. [CrossRef] [Medline]
21. Mocé, E., Blanch, E., Tomás, C., and Graham, J.K. 2010. Use of cholesterol in sperm cryopreservation: present moment and perspectives to future. *Reprod Domest Anim*. 45 (Suppl. 2): 57–66. [CrossRef] [Medline]
22. Mocé, E. and Vicente, J.S. 2009. Rabbit sperm cryopreser-

vation: a review. *Anim. Reprod. Sci*. 110: 1–24. [CrossRef] [Medline]
23. Nakagata, N. 2000. Cryopreservation of mouse spermatozoa. *Mamm. Genome* 11: 572–576. [CrossRef] [Medline]
24. Nakatsukasa, E., Kashiwazaki, N., Takizawa, A., Shino, M., Kitada, K., Serikawa, T., Hakamata, Y., Kobayashi, E., Takahashi, R., Ueda, M., Nakashima, T., and Nakagata, N. 2003. Cryopreservation of spermatozoa from closed colonies, and inbred, spontaneous mutant, and transgenic strains of rats. *Comp. Med*. 53: 639–641. [Medline]
25. Nishijima, K., Yamaguchi, S., Morimoto, M., Watanebe, T., and Kitajima, S. 2010. Examination of utility of sperm cryopreservation for preservation of transgenic rabbit lines: Fertility performance of transgenic rabbit's sperm cryopreserved for 5.6 years. *Kyushu J. Exp. Anim*. 26: 35–39 (in Japanese).
26. Oliveira, C.H., Vasconcelos, A.B., Souza, F.A., Martins-Filho, O.A., Silva, M.X., Varago, F.C., and Lagares, M.A. 2010. Cholesterol addition protects membrane intactness during cryopreservation of stallion sperm. *Anim. Reprod. Sci*. 118: 194–200. [CrossRef] [Medline]
27. Prathalingam, N.S., Holt, W.V., Revell, S.G., Mirczuk, S., Fleck, R.A., and Watson, P.F. 2006. Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze-thaw. *Theriogenology* 66: 1894–1900. [CrossRef] [Medline]
28. Purdy, P.H. and Graham, J.K. 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. *Cryobiology* 48: 36–45. [CrossRef] [Medline]
29. Purdy, P.H. and Graham, J.K. 2004. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. *Biol. Reprod*. 71: 522–527. [CrossRef] [Medline]
30. Rath, D., Bathgate, R., Rodriguez-Martinez, H., Roca, J., Strzezek, J., and Waberski, D. 2009. Recent advances in boar semen cryopreservation. *Soc. Reprod. Fertil. Suppl*. 66: 51–66. [Medline]
31. Santiani, A., Evangelista, S., Valdivia, M., Risopatrón, J., and Sánchez, R. 2013. Effect of the addition of two superoxide dismutase analogues (Tempo and Tempol) to alpaca semen extender for cryopreservation. *Theriogenology* 79: 842–846. [CrossRef] [Medline]
32. Serin, I., Aksoy, M., and Ceylan, A. 2011. Cholesterol-loaded cyclodextrin inhibits premature acrosomal reactions in liquid-stored rabbit spermatozoa. *Anim. Reprod. Sci*. 123: 106–111. [CrossRef] [Medline]
33. Shibahara, H., Obara, H., Ayustawati, Hirano, Y., Suzuki, T., Ohno, A., Takamizawa, S., and Suzuki, M. 2004. Prediction of pregnancy by intrauterine insemination using CASA estimates and strict criteria in patients with male factor infertility. *Int. J. Androl*. 27: 63–68. [CrossRef] [Medline]
34. Shimokawa, K., Oshiro, R., Yamanaka, K., Ashizawa, K., Ohta, S., and Tatemoto, H. 2012. Improvement of the post-thaw qualities of Okinawan native Agu pig sperm frozen in an extender supplemented with antiapoptotic PTD-FNK protein. *Theriogenology* 78: 1446–1455. [CrossRef] [Medline]
35. Yanni, A.E. 2004. The laboratory rabbit: an animal model of atherosclerosis research. *Lab. Anim*. 38: 246–256. [CrossRef] [Medline]