Korean Journal Of Agricultural Science

pISSN : 2466-2402
eISSN : 2466-2410

ANIMAL

Effect of Optixcell and Triladyl extenders on frozen-thawed sperm motilities and calving rates following artificial insemination in Hanwoo

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Abstract

In this study, we examined the effect of a liposome-based extender (Optixcell) and a tris-citric egg-yolk extender (Triladyl) on the frozen-thawed spermatozoa characteristics and the calving rate. The percentages for the total motility of the frozen-thawed spermatozoa were similar in the Optixcell and Triladyl groups. However, among the motile spermatozoa with a straight line velocity (VSL) ≥ 25 μm/sec, the curvilinear velocity (VCL, μm/sec), VSL (μm/sec), average path velocity (VAP, μm/sec), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), and plasma membrane integrity of the frozen-thawed spermatozoa for the Optixcell group were significantly higher than those for the Triladyl group. Furthermore, the calving rate in the Optixcell group (79.0%) was higher than that of the Triladyl group (62.8%). However, the acrosomal membrane integrity of the frozen-thawed spermatozoa in the Optixcell and Triladyl groups was not significantly different. These results indicate that semen freezing with Optixcell improved the motility and plasma membrane integrity of frozen-thawed spermatozoa and the calving rate of Hanwoo cows (native Korean cattle). In conclusion, our results suggest that semen freezing with the liposome-based extender Optixcell is more efficient than with the tris-citric egg-yolk extender Triladyl for improved offspring production.

Keywords: calving rate, Hanwoo, semen extender, sperm motility

Citation: Kang SS, Lee MS, Kim UH, Lee SD, Yang BC, Yang BS, Cho SR. 2019. Effect of Optixcell and Triladyl extenders on frozen-thawed sperm motilities and calving rates following artificial insemination in Hanwoo. Korean Journal of Agricultural Science. https://doi.org/10.7744/kjoas.20190009

DOI: https://doi.org/10.7744/kjoas.20190009

Received: March 28, 2018

Revised: February 7, 2019

Accepted: January 28, 2019

Introduction

Numerous semen extenders have been developed for cryopreservation of bull semen. Tris (hydroxymethyl) amino-methane (Tris), citric acid, sodium phosphate citric acid, and glycerol have been used as basic semen extenders (Vishwanath and Shannon, 2000). Bull semen collected by artificial vagina and collected semen diluted with various types of semen extenders. The diluted semen in semen extender undergoes cryopreservation process. Cryopreservation process can affect

to sperm detrimentally such as, cold shock, osmotic dehydration and plasma membrane alteration (Holt, 2000). To reduce detrimental effects of cryopreservation on sperm, egg yolk has been used widely as a cryoprotectant (Layek et al., 2016). However, egg yolk is an animal protein source that has a risk of contamination by bacteria or mycoplasma (Bousseau et al., 1998), and there is a risk of developing zoonotic disease, such as the influenza virus, from birds. Hence, the demand for other chemically defined alternative extenders is required. As substitutes for egg yolk, soybean lecithin, low density lipoprotein (LDL), and liposomes have been used as cryoprotectant semen extenders. The soybean lecithin extender improved freezability and fertility of sperm in buffalo (Akhter et al., 2012), ram (Fukui et al., 2008, de Paz et al., 2010), stallion (Papa et al., 2011), and goat (Vidal et al., 2013). The LDL obtained from egg yolk, which can protect plasma membrane integrity, improved frozen-thawed sperm motility in bull (Pace and Graham, 1974, Amirat et al., 2004, Akhter et al., 2011, Hu et al., 2011), canine (Bencharif et al., 2008), and stallion (Pillet et al., 2011). The addition of liposomes in semen extender improved plasma membrane integrity and motility of frozen-thawed spermatozoa in bull (Pillet et al., 2012, Kumar et al., 2015) and stallion (Ropke et al., 2011). Frozen-thawed semen in liposome-based semen extender improved conception rate in buffalo (Ansari et al., 2016) and calving rate in cow (Camus et al., 2016). Liposomes are widely used in various semen extenders because the structure and composition of liposome are already defined, and they are easy to manufacture for use as an additive in semen extenders. Regardless of the positive effects of liposomes on semen cryopreservation, there are few reports of field trials of liposome-based extenders. Especially, there are no report that pregnancy and calving rates after artificial insemination with frozen-thawed semen in liposome-based semen extender in Hanwoo.

Thus, in the present study, we compared calving rates following artificial insemination (AI) with semen treated with liposome-based or tris-citric egg-yolk-based semen extenders. For that investigation, Hanwoo (native Korean cattle) semen was frozen with a commercial liposome-based semen extender (Optixcell, IMV, France) or a commercial tris-citric egg-yolk-based extender (Triladyl, Minitube, Germany). In addition, we compared the motility, plasma membrane integrity, and acrosome integrity of Optixcell- and Triladyl-treated, frozen-thawed spermatozoa.

Materials and Methods

Ethics of animal experimentation

The treatment of cows used in this study was performed in accordance with guidelines (grade D) of the National Institute of Animal Science, Rural Development Administration, Jeonju, Korea, and was approved by the animal experiment and ethics committee of the National Institute of Animal Science.

Semen collection and cryopreservation

Semen was collected from 16 fertile Hanwoo bulls by using an artificial vagina (NFA-40, FHK, Japan). More than 80% of motile sperm in the ejaculate was used. Collected semen was diluted immediately with the same volume of Triladyl (Minitube, Germany) or Optixcell (IMV, France) extenders, and sperm concentration was adjusted to 40×10^6 cells/mL. Diluted semen was maintained at 4℃ for 4 to 6 h, loaded to 0.5 mL straws, and held at 9 cm from a liquid nitrogen (LN_2) surface for 14 min. Subsequently, straws were plunged into LN_2 and cryopreserved in a LN_2 tank until use for AI and evaluation of sperm motility by using a sperm class analyzer (Microptic, Barcelona, Spain).

In vivo fertility and calving

This study was conducted over a 5-year period (June 2009 to December 2010 and June 2014 to December 2016) at the Hanwoo Research Institute, National Institute of Animal Science, Pyeongchang, Korea. During that period, 765 Hanwoo cows underwent synchronized estrus cycles and were inseminated artificially. For that process, 1.9 g of a progesterone-supplying device (EAZI-BREED CIDR 1900, Zoetis, Belgium) was introduced to the vagina of Hanwoo cows for 10 days irrespective of estrus cycle. Ten days after introduction, the progesterone device was removed and 5 mL of PGF2α (Lutalyse, Zoetis, Belgium) was injected intramuscularly. All AI was conducted by one technician. The AI was accompanied by a 2 mL gonadotrophin-releasing hormone (GnRH; Fertagyl, Inervet International, Germany) intramuscular injection. This was followed 2.5 days later with a PGF2α injection, and a second AI was carried out 3.5 day after the PGF2α injection. Seventy to 80 days after the second AI, pregnancy diagnosis was conducted by performing rectal palpation.

Evaluation of sperm motility and motility parameters

Evaluation of sperm motility was performed as described previously report (Kang et al., 2015) with slight modification. Briefly, frozen-thawed semen was immersed into water at 37℃ for 40 sec, transferred to a 1.5 mL tube, and mixed well. Thawed semen (3 μL) was placed on four-chamber slides to a depth of 20 μm (Art. No. SC 20-01-04-B, Leja, Nieuw-Vennep, Netherlands) for counting. At least 1000 sperm in 4 to 6 fields in a slide chamber were divided into motile and dead sperm. The percentage of motile sperm and other sperm motility parameters were evaluated by using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, MicroOptic, Spain). The other sperm motility parameters evaluated were straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (Linearity, LIN = VSL/VCL × 100), straightness (STR), wobble (WOB), amplitude of lateral head (ALH) and flagellar beat cross frequency (BCF). For evaluation of sperm motility parameters, motile sperm with a VSL of ≥ 25 μm/sec were selected, as it has been reported that motile sperm with a VSL < 25 μm/sec will probably be unable to penetrate the oocyte (Aitken, 1985; Holt et al., 1985).

Evaluation of plasma membrane integrity of spermatozoa

Plasma membrane integrity of spermatozoa was examined as described in a previous study (Revell and Mrode, 1994) with slight modification. Briefly, frozen semen was immersed in water at 37℃ for 40 sec, transferred to a 1.5 mL tube, and mixed well. Frozen-thawed semen (30 μL) was mixed with 300 μL of pre-warmed hypotonic solution and incubated at 39℃ for 1 h. After incubation, 10 μL of the mixture was mounted on a slide glass, covered with a coverslip, and swollen and non-swollen spermatozoa were counted (Fig. 1). More than 200 spermatozoa were counted in five different fields at × 400 magnification by using a phase contrast microscope.

Evaluation of viability and acrosome integrity of spermatozoa

The viability and acrosome integrity of spermatozoa were evaluated as previously described (Kovacs and Foote, 1992) with slight modification. Briefly, frozen-thawed semen (20 μL) was mixed with 20 μL of 0.25% trypan blue staining solution, mounted on slide glass, and air dried. Specimens were fixed with a fixative solution for 5 min, washed with tap water, and air dried. The fixative solution comprised 86 mL of 1.0 N HCl, 14 mL of formaldehyde solution (35%), and 0.2 g of neutral red (50040, Sigma-Aldrich, USA). Fixed specimens were stained with 7.5% Giemsa solution for 10 to 12 h. A minimum of

200 spermatozoa were counted at × 1000 magnification by using a phase contrast microscope and were then divided into four groups: live with intact acrosome (LIA), live with damaged acrosome (LDA), dead with intact acrosome (DIA), and dead with damaged acrosome (DDA) as shown Fig. 2.

Statistical analysis

The calving rates of the two semen extender groups were compared by using Pearson's chi-square test. Mean motility, motility parameters, plasma membrane integrity, viability, and acrosome integrity of spermatozoa values were compared by

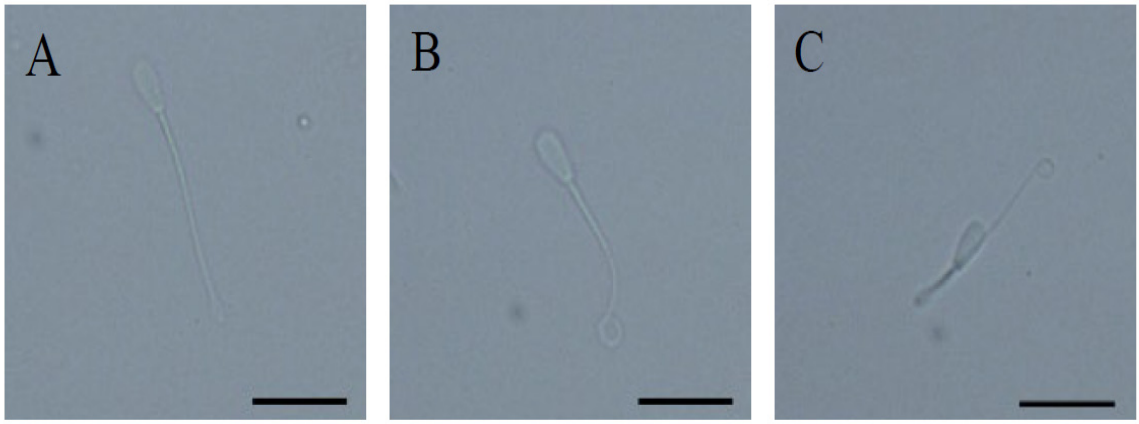

Fig. 1. Evaluation of plasma membrane integrity of spermatozoa. A, B, and C; swollen spermatozoa determined as plasma membrane integrity was normal. Scale bar: 20 μm.

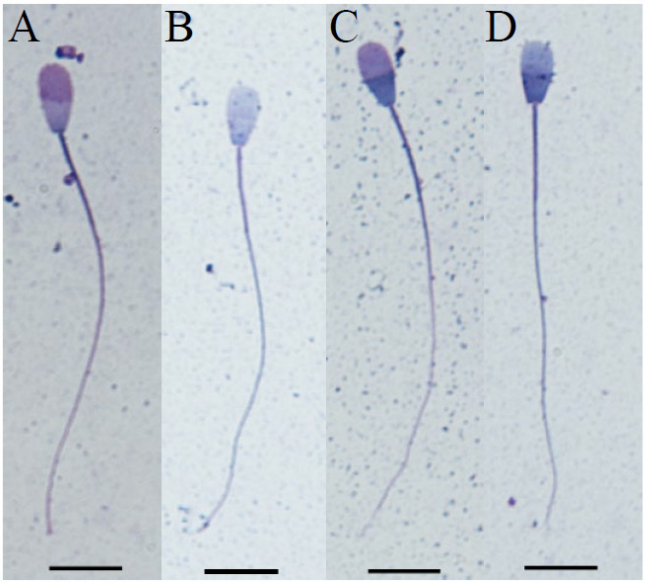

Fig. 2. Evaluation of viability and acrosomal membrane integrity. A, spermatozoa with live and intact acrosomal membranes; B, spermatozoa with live and damaged acrosomal membranes; C, spermatozoa with dead and intact acrosomal membranes; D, spermatozoa with dead and damaged acrosomal membranes. Scale bar: 20 μm.

performing one-way ANOVA followed by Tukey-Kramer's HSD test as a *post hoc* test. All analyses were performed by using JMP Pro (version 13.2, SAS Institute, Cary, USA).

Results

As shown in Table 1, calving rate in the Optixcell group (364/461, 79.0%) was significantly higher than that in the Triladyl group (191/304, 62.8%) ($p < 0.001$). There was no significant difference in gestation periods among Optixcell and Triladyl groups (284.1 ± 6.2 and 285.6 ± 9.0 days, respectively). There was no significant difference in percentage of total motile, LIN and STR among Optixcell and Triladyl groups. Among the motile sperm with VSL ≥ 25 μm/sec, VCL (μm/sec), VSL (μm/sec), VAP (μm/sec), ALH (μm/sec), and BCF (μm/sec) of the Optixcell group were significantly higher than those of the Triladyl group. However, WOB (%) of spermatozoa in the Optixcell group was significantly lower than that of spermatozoa in the Triladyl group (Table 2). Plasma membrane integrity of spermatozoa in the Optixcell group was significantly higher than that of spermatozoa in the Triladyl group (Table 3; $p < 0.05$). However, there were no significant differences in LIA, LDA, DIA, and DDA between the Optixcell and Triladyl groups (Table 4).

Table 1. The calving rate of Hanwoo cow after artificial insemination with Optixcell and Triladyl semen extenders.

Type of semen extender	No. of inseminated cows	No. of calved based on inseminated (%)	Days of gestation (mean ± SD)
Optixcell	461	364 (79.0)e	284.1 ± 6.2
Triladyl	304	191 (62.8)f	285.6 ± 9.0

e, f : Means in a column with different letters are significantly different ($p < 0.001$).

Table 2. Motility and motility parameters of frozen-thawed spermatozoa with Optixcell and Triladyl semen extenders.

Motility parameters	Optixcell	Triladyl
Total motile (%)	81.1 ± 16.5	77.3 ± 23.8
Motile with VSL ≥ 25 μm/sec	38.8 ± 15.8a	22.8 ± 10.5b
VCL (μm/sec)	127.8 ± 15.7e	87.7 ± 19.9f
VSL (μm/sec)	62.2 ± 8.7a	46.9 ± 12.0b
VAP (μm/sec)	80.0 ± 9.7c	59.0 ± 11.9d
LIN (%)	50.9 ± 4.5	56.8 ± 7.8
STR (%)	77.7 ± 2.4	79.5 ± 5.5
WOB (%)	64.6 ± 4.4a	70.3 ± 5.8b
ALH (μm/sec)	3.9 ± 0.7a	3.0 ± 0.7b
BCF (Hz)	14.6 ± 1.6c	11.1 ± 1.9d

VSL, straight line velocity; VCL, curvilinear velocity; VAP, average path velocity; LIN, linearity = VSL/VCL × 100; STR, straightness; WOB, wobble; ALH, amplitude of lateral head; BCF, flagellar beat cross frequency.
a, b : Means in a row with different letters are significantly different ($p < 0.05$).
c, d : Means in a row with different letters are significantly different ($p < 0.01$).
e, f : Means in a row with different letters are significantly different ($p < 0.001$).

Table 3. Plasma membrane integrity of frozen-thawed spermatozoa with Optixcell and Triladyl semen extenders.

Type of semen extender	% of spermatozoa with intact plasma membrane (mean ± SD)
Optixcell	60.0 ± 15.1a
Triladyl	40.9 ± 11.4b

Swollen spermatozoa evaluated at spermatozoa with intact plasma membrane.
a, b : Means in a column with different letters are significantly different ($p < 0.05$).

Table 4. Viability and acrosome integrity of frozen-thawed spermatozoa with Optixcell and Triladyl semen extenders.

Type of semen extender	% of (mean ± SD)			
	LIA	LDA	DIA	DDA
Optixcell	52.3 ± 15.2	0 ± 0	43.3 ± 12.8	4.4 ± 4.3
Triladyl	37.6 ± 22.3	0 ± 0	54.0 ± 19.0	8.4 ± 5.3

LIA, Live with intact acrosome; LDA, live with damaged acrosome; DIA, dead with intact acrosome; DDA, dead with damaged acrosome.

Discussion

The present study showed positive effects of a liposome-based semen extender (Optixcell) on plasma membrane integrity and motility of frozen-thawed bovine spermatozoa and on calving rate after AI compared to those obtained from a tris-citric egg-yolk semen extender (Triladyl). To our knowledge, the present study is the first comparative report on calving rates in Hanwoo cows after AI with Optixcell- or Triladyl-treated frozen semen. Plasma membrane integrity, motility, and acrosomal membrane integrity assessments have been used as indicators of bull fertility (Sellem et al., 2015; Utt, 2016). Dilution of spermatozoa with hypotonic solution induces swelling and tail bending in spermatozoa with intact plasma membrane due to the influx of water into the spermatozoa. The hypo-osmotic swelling test (HOST) can assess spermatozoa with an intact plasma membrane (Jeyendran et al., 1984), and a high percentage of HOST-positive spermatozoa has been related to the non-return rate in cow (Bacinoglu et al., 2008) and fertilization rate *in vitro* (Tartaglione and Ritta, 2004). In the present study, plasma membrane integrity of frozen-thawed spermatozoa with Optixcell treatment was higher than that with Triladyl treatment (60.0 ± 15.1 *vs.* 40.9 ± 11.4%, respectively). This result supports those in a previous report in which a liposome semen extender showed a higher percentage of plasma membrane integrity in frozen-thawed buffalo spermatozoa than that from a Triladyl extender (Ansari et al., 2016). We speculate that the spherical phospholipid liposome vesicle in the semen extender combines with the plasma membrane of spermatozoa (Anzar et al., 2002) to protect the plasma membrane from cold shock during cryopreservation (Quinn et al., 1980). It has been reported that motility and motility parameters of spermatozoa can be useful when determining effects on fertility (Santolaria et al., 2015), and those parameters have been correlated with bull fertility (Farrell et al., 1998). In the present study, we compared motility of frozen-thawed spermatozoa treated with two different semen extenders (Optixcell and Triladyl). The percentage of total motile sperm among frozen-thawed spermatozoa in the Optixcell group was similar to that in the Triladyl group. However, the percentage of motile spermatozoa with a VSL ≥ 25 μm/sec in the Optixcell group was higher than that in the Triladyl group. Previous studies reported that motile spermatozoa group with VSL ≥ 25 μm/sec can penetrate oocytes, whereas motile spermatozoa with a VSL < 25 μm/sec could not (Aitken et al., 1985; Holt et al., 1985). Thus, we suggest that the higher percentage of spermatozoa

with VSL ≥ 25μm/sec in Optixcell group would have higher fertilization success than that in the Triladyl group. In addition, VCL, VSL, VAP, ALH, and BCF of spermatozoa in the Optixcell group were significantly higher than those in the Triladyl group. It was previously reported that high VCL, VSL, ALH, and LIN of spermatozoa are highly related to fertility rate in bull (Al-Qarawi et al., 2002; Perumal et al., 2011). High motile group showed that high VSL, VAP, VCL, LIN, and BCF compared to those of low motile group (King et al., 2000). In human, ALH of spermatozoa in pregnant group was significantly higher than that of non-pregnant group. Increased ALH and decreased LIN were used as a predictor of hyperactivation correlated with low fertility in human (Fréour et al., 2010). However, in the present study, LIN of spermatozoa in Optixcell and Triladyl groups was similar. Thus, we speculated that spermatozoa in Optixcell group can move faster than spermatozoa in Triladyl group. The viability and acrosomal membrane integrity of frozen-thawed spermatozoa was not significantly different between the Optixcell and Triladyl groups, which coincides with results reported by Stewart et al. (2016) in which semen treated with Optixcell and Triladyl exhibited no different effects on viability and acrosomal membrane integrity of spermatozoa. These results suggested that Optixcell semen extender did not improved viability and acrosomal membrane integrity of frozen-thawed spermatozoa compared to Triladyl semen extender. Finally, the calving rate in the Optixcell group was significantly higher than that of the Triladyl group ($p < 0.001$). Based on our results, we suggest that, compared to the Triladyl-treated sperm, frozen-thawed spermatozoa in the Optixcell group has a higher fertilizing ability and, consequently, results in a higher calving rate in Hanwoo cows.

In conclusion, compared to Triladyl, the use of Optixcell for semen freezing has several merits, including protection of the plasma membrane and increased motility of spermatozoa. Furthermore, there was an improved calving rate in Hanwoo cows treated with Optixcell. Our results indicate that Optixcell is an effective substitute of tris-citric egg-yolk extenders.

Acknowledgments

This study was supported by a 2019 Postdoctoral Fellowship Program of the Hanwoo Research Institute, RDA, Republic of Korea. This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development project: "Development of improved technologies for preservation of Hanwoo bull semen and technology application", project PJ014325, RDA, Republic of Korea.

Conflict of interest

None of the authors have a conflict of interest to declare.

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