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# Analysis of epididymal sperm from Korean native bull (Hanwoo) aged at 8 and 15 months before freezing and after thawing

**Sung-Sik Kang[1,2], Sang-Rae Cho[1,2], Ui-Hyung Kim[2], Chang-Seok Park[2], Hyeong-Cheol Kim[2], Ki-Yong Chung[2], Seok-Dong Lee[2], Sun-Sik Jang[2], Gi-jun Jeon[2], Sidong Kim[3], Myeong-Suk Lee[2], Byoung-Chul Yang[2,*]**

*[1]Laboratory of Reproductive Physiology, Hanwoo Research Institute, Pyeongchang, Kangwondo, 232-950, Korea*
*[2]Hanwoo Research Institute of Animal Science, Rural Development Administration (RDA), Pyeongchang, 25340, Korea*
*[3]Animal Genetic & Breeding Division, Rural Development Administration (RDA), Cheonan, Chungcheongnamdo, 31000, Korea*

## ABSTRACT

The recovery of epididymal sperm in animals is considered as one of the important tools to preserve high value or endangered species. However, there are no appropriate castrating indicators such as months of age in bull, sperm morphology, and motility, particularly in young Korean native bull (Hanwoo). Therefore, this study aimed to investigate sperm number, morphology, and motility of sperm in the epididymis tail of young Hanwoo bulls at 8 and 15 months of age. After castration, epididymal tails were collected and minced with blades to recover sperm. In experiments 1 and 2, sperm number, morphology, and motility were examined. Total number of sperm and percentage of normal sperm from bulls at 8 months of age was lower than that of bulls at 15 months of age after collection ($P$<0.05). Percentage of abnormal head, tail, proximal cytoplasmic droplet, dead and damaged acrosome of sperm from bulls at 8 months of age were higher than those of bulls at 15 months of age ($P$<0.05). In experiment 3, sperm motility from bulls at 8 and 15 months of age were examined before freezing and after thawing. Frozen-thawed sperm at 8 months of age showed low total motility and motile sperm with ≥ 25 μm/sec compared to those at 15 months of age and commercially-used sperm ($P$<0.05). In conclusion, sperm derived from the epididymal tail of bulls at 8 months of age showed high abnormal morphology and poor motility, which are not adequate for AI and IVF. On the other hand, sperm derived from the epididymal tail of bulls at 15 months of age showed high normal morphology and motility.

(Key words: epididymis, Hanwoo, sperm, morphology, motility)

## Introduction

The technique of sperm recovery from epididymis is considered as one of the important tools in the rescue and preservation of high-value animals' gametes. Several authors demonstrated that recovery and cryopreservation of sperm from epididymis of testes after castrating are possible in diverse animals such as bull (Martins *et al*., 2007), boar (Kikuchi *et al.*, 1998), goat (Blash *et al*., 2000), ram (Abella *et al*., 2015), and stallion (Papa *et al.*, 2008). Previous authors had focused on only the recovery of sperm from epididymis of testes, irrespective of the fundamental requirements such as age, body weight, scrotal circumference, testicular size, sperm number, and motility.

In the beef farm industry, castration of bull is one of the most common livestock management practices (Coetzee *et al.*, 2010). In Holstein bull, the effect of castration on carcass and meat quality on age has been investigated (Devant *et al.*, 2012). In South Korea, steers are fattened, slaughtered, and classified according to carcass grade based on animal product grading service (Park *et al*., 2002). With high grade of carcass, a beef farm will have a stable or continuous increase in income. When the carcass grade was determined with the beef farm, the beef farm will try to produce similar grades of bulls after castrating. If the sperm derived from testes can be preserved, beef farmers can use the sperm to produce plenty of high potential and high quality bulls by artificial reproduction technology (ART), such

* Correspondence: Byoung-Chul Yang
Tel: +82-33-330-0651
E-mail: bcyang@korea.kr

as AI and embryo transfer after using IVF, with oocyte. In addition, if utilization of the sperm from castrated bulls' testes will be succeed in AI and IVF, it will cut down periods which need for progeny test of steers. Igboeli and Foote (1968) also demonstrated that sperm from epididymal tail of Angus and Holstein bulls at age 5 to 12 years showed 69% of non-return rate after AI. On the other hand, there were no appropriate castrating timing indicators at the young age of bulls to recover sperm which can be available to AI or IVF.

Testes of castrated steers have been discarded and investigation of sperm derived from testes in young Hanwoo bull was also not conducted, attributing to the inability to determine whether sperm will be recovered and available to AI or IVF. The sperm number, normal morphology, and progressive motile sperm recovered by ejaculation drastically increased according to the bull's development during puberty in Hereford, Angus, Red Poll and Brown Swiss (Lunstra and Echternkamp, 1982). Normal sperm percentage also increased according to testicular size (Foote, 1978) and scrotal circumference (Palasz *et al*., 1994) in bull after puberty. The spermatogenesis was completed by 8 months in Holstein bulls (Curtis and Amann, 1981). Therefore, it should be examined whether sperm can be recovered and if the recovered sperm have suitable morphology and motility for reproduction in bull. Thus, the present study was designed to examine sperm number, morphology, and motility of sperm in the epididymal tail of Hanwoo bulls at 8 months of age. Hanwoo bulls at 15 months were used in comparison with bulls at 8 months of age. In experiment 1, we confirmed body information of bulls and sperm number. In experiment 2, sperm morphology derived from the epididymal tail of bulls at 8 and 15 months of age were examined. In experiment 3, sperm motility before freezing and after thawing was examined using computer assisted sperm analysis (CASA) system.

## Materials and Methods

All animal procedures were performed in accordance with the guidelines for the ethical treatment of animals and were approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science (Pyeongchang, Gangwondo, Republic of Korea).

### Chemicals

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

### Recovery, freezing, and thawing of epididymal sperm

Testes of Hanwoo at 8 and 15 months of age were obtained from the Hanwoo Research Institute, RDA in Pyeongchang, Gangwondo, South Korea (8 and 15 months of age, n=5 and 5, respectively). The castration was carried out from December 2015 to January 2016. Before castration, body weight was recorded and scrotal circumference was measured with a scrotal measuring tape (Ideal instrument, Neogen Corporation, USA). Recovery of epididymal sperm from castrated bulls was performed as described in the previous study of Yang *et al.*, (2015). In brief, after castrating Korean native bulls, testicles and epididymis were transported to the laboratory in plastic bag with saline. One testis from each bull was selected randomly and used in the experiment. The sclerotic was removed and weighed; circumference, length, and width of testis were measured. After the dissection of epididymis, the weight and length of epididymis were also measured. The epididymal tail has a reserve of sperm, hence sperm was recovered in that area (Blash *et al*., 2000). About 2 cm of the epididymis tail was only used, cut, and minced using blades (No.21, AILEE, Korea) in a 100 mm dish (Falcon). Minced epididymal tail tissues were mixed with 4 to 8 ml of semen freezing medium (OptixCell, IMV Technologies, France) and sperms were recovered with a cell strainer (100 μm nylon mesh, Falcon). Sperm concentration was adjusted to $4 \times 10^7$ sperm/ml and the volume of semen at room temperature was recorded. Sperm dilution were preserved at 4°C for 8 to 12 h and loaded to 0.5 ml straw ($2 \times 10^7$ sperm/straw) using straw filling machine (T-10-05, FHK, Japan). These straws were cooled above 9 cm of liquid nitrogen surface for 14 min, dropped to liquid nitrogen ($LN_2$), and cryopreserved in $LN_2$ tank until use. To thaw frozen semen, frozen straws were immersed in water at 37°C for 40 sec and used in this study.

### Evaluation of sperm motility by CASA

The evaluation of sperm motility was performed as described previously by Kang *et al*. (2015) with slight modification. In brief, 2 μl of thawed semen was placed onto 4-chamber slides with a depth of 20 μm (Art. No. SC 20-01-08-B, Leja, Nieuw-Vennep, Netherlands) for counting.

At least 1000 sperms in 4 to 10 fields in a chamber were divided into motile and dead sperm. The percentage of motile sperm and other sperm motility parameters were evaluated using a 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 (LIN = VSL/VCL × 100), straightness (STR), wobble (WOB), flagellar beat cross frequency (BCF), and amplitude of lateral head (ALH). For evaluation of sperm motility parameters, motile sperm with a VSL of ≥ 25 μm/sec were selected, as it has been recognized that motile sperm having a VSL of less than 25 μm/sec will probably not able to penetrate the oocyte (Aitken, 1985; Holt *et al*., 1985).

### Preparation of sperm staining and fixing solutions

The staining and fixing solutions were prepared according to the previous study of Kovacs and Foote, (1992) with slight modification. Viability testing solution consisted of 0.25% trypan blue (Sigma-Aldrich, cat No. T6146) in 0.81% NaCl (w/v). Fixing solution consisted of 86 ml of 1N HCl, 14 ml of 1N formaldehyde solution (Sigma-Aldrich, cat No. 252549), and 0.2 g neutral red (Sigma-Aldrich, cat No. N4638). Acrosome testing solution consisted of 7.5% Giemsa solution (Merck, cat No. 109204100) in distilled water was prepared before use.

### Staining and fixing of sperm

Twenty μl of sperm dilution was mixed with pre-warmed 20 μl of viability solution. After which, 10 μl of mixed solution was mounted on a slide glass and smeared. Slides were air dried at room temperature, fixed with fixing solution for 5 min, washed with tap water, and air dried at room temperature. Slides were stained with acrosome testing solution for 12 to 18 h.

### Evaluation of sperm viability and morphology

Evaluation of viability and acrosome integrity of sperm was conducted according to previous studies (Kovacs and Foote, 1992; Serafini *et al*., 2014; Persson *et al*., 2006) with slight modification. In brief, more than 200 sperms in three slide glasses from each semen sample were counted by bright field microscopy at magnification 400× and evaluated for viability and acrosome integrity of sperm. Live sperm were not stained with trypan blue at posterior region (pale). Dead sperm were stained with trypan dark blue at posterior region. Normal acrosome of sperm was stained with Giemsa (violet). Abnormal or damaged acrosome of sperm was not stained with Giemsa (pale). In addition, abnormal head including macrocephalic, microcephalic, detatched, tailless; proximal cytoplasmic droplet, distal cytoplasmic droplet; abnormal midpiece including segmental aplasia, bent and double midpiece; and abnormal tail including bent, hairpin, and coiled tail sperm were assessed.

### Experimental design

In experiment 1, to obtain fundamental information on the bulls at 8 and 15 months of age, body weight, scrotal circumference, testicular size, epididymal size, and recovered sperm volume were recorded.

In experiment 2, to examine viability and morphology of sperm derived from epididymal tail, sperm were stained with 0.25% trypan blue and 7.5% Giemsa solution. Normal and abnormal sperm including defects of head, midpiece, and tail were examined before freezing and after thawing.

In experiment 3, to examine sperm motility and motility parameters, sperm recovered from epididymal tail at 8 and 15 months of age in bull were evaluated by CASA before freezing and after thawing. As control, commercial frozen Hanwoo (5 years old) semen was used.

### Statistical analysis

Body weight, scrotal circumference, testicular size (weight, circumference, length, and width), recovered sperm volume, and sperm morphological characteristics between groups of bulls at 8 and 15 months of age were compared using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer Honestly Significant Difference test for post hoc analysis. Sperm motility and motility parameters among the bulls at 8, 15 months, and the control were compared using one-way ANOVA. All analyses were performed using JMP Pro (version 10.0.2, SAS Institute, Cary, NC, USA).

## RESULTS

### Experiment 1

Body weight, scrotal circumference, testis (weight, circumference, length, width), epididymis (weight and length), and collected semen volume from the group of bulls at 8 months of age were significantly lower than those from bullas at 15 months of age (Table 1; $P<0.05$).

### Experiment 2

Viability and morphology in sperm samples derived from epididymal tail of bulls at 8 and 15 months of age before freezing and after thawing is shown in Table 2. Percentage of normal sperm or live sperm with intact acrosome (LIA) from bulls at 8 months of age were significantly lower than that of bulls at 15 months of age, irrespective of the before freezing and after freezing treatments ($P<0.05$). Percentages of abnormal head, tail, LIA with proximal cytoplasmic droplet, dead sperm with intact acrosome (DIA) and dead sperm with damaged acrosome (DDA) ($P<0.05$) of bulls at 8 months of age were significantly higher than those from bulls of 15 months of age.

### Experiment 3

As shown in Table 3, total motile sperm and motile sperm with $\geq$ 25 μm/sec from bulls at 8 months of age were significantly lower than those from bulls at 15 months of age ($P<0.05$).

## DISCUSSION

Sperm productivity, morphology, and motility of sperm in young Hanwoo bull were acquired in the present study. In Table 1, young bulls at 8 months of age showed 270 kg of body weight, 24.8 cm of scrotal circumference and 116.4 g of testis weight, and have few percentage of progressive motile of sperm (3.4%) and low number of normal sperm (13.8%). Previous study also supported our results that Holstein bulls at 8 months of age showed similar body weight (275 kg), scrotal circumference (26.8 cm) and testis weight (117g), and suggested sperm can be recovered from 8 months of age in bull due to the spermatogenesis being completed by 8 months (Curtis and Amann, 1981). These results means that scrotal circumference positively relates to normal sperm production (Palasz *et al*., 1994). In addition, Kastelic *et al*. (2001) also reported that scrotal circumference and sperm reserves in beef bulls were positively correlated. In the present study, we found out that young bulls at 8 months of age can reserve only a few numbers of immature sperm in the epididymal tail. Thus, we suspected that Hanwoo bull at 8 months of age can produce only a few numbers of sperms and we could not obtain enough number of sperms derived from the bull at 8 months, thus not satisfying the demand for the adult level of sperm productivity.

The bulls at 8 months of age produced low percentage of normal sperms compared to bulls at 15 months of age. In addition, thawed sperm of bulls at 15 months of age showed six times of live sperm with intact acrosome (LIA) compared to thawed sperm from bulls at 8 months of age. Recovered sperm at 8 months of age in bull showed high percentages of proximal droplet compared to bulls at 15 months of age (36.9 vs. 0.5%, respectively). Amann *et al.* (2000) reported that sperm with more than 30% of proximal droplet in semen have low fertilizing potential. Thus, we supposed that sperm derived from bulls at 8 months was immature and may have low fertilizing and fertility rate. In addition, thawed sperm from bulls at 8 months of age showed that dead sperm with intact acrosome (DIA) and dead sperm with damaged acrosome (DDA) were significantly increased compared to before freezing. However, DIA and DDA percentages of thawed sperm of bulls at 15 months of age did not increase compared to sperms before freezing. We supposed that the sperm of bulls at 8 months of age are easily affected by the freezing and thawing step and have low freezing resistance. Therefore, thawed semen of bulls at 8 months of age might be hard to apply for AI and IVF. However, sperm derived from epididymal tail of bulls at 15 months of age have positive possibility for application to AI or IVF because they have 61.9% of LIA.

Total motile sperm of bulls at 15 months of age was significantly higher than that of bulls at 8 months of age before freezing and after thawing. Furthermore, thawed motile sperm showed that sperm with VSL $\geq$ 25 μm/sec from bulls at 15 months of age were similar to thawed commercial sperm. These result means that thawed sperm derived from epididymal tail of bulls at 15 months of age may have fertilizing ability. In our previous study, Yang *et al.* (2015)

Table 1. Body weight, scrotal circumference, testicular size and recovered sperm volume derived from an epididymal tail of bulls at 8 and 15 months of age

| Months of age (bull=n) | Body weight (kg) | Scrotal circumference (cm) | Testis | | | | Epididymis | | Semen volume* (ml) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Weight (g) | Circumference (cm) | Length (cm) | Width (cm) | Weight (g) | Length (cm) | |
| 8 (5) | 270.2±34.0[a] | 24.8±2.5[a] | 116.4±28.0[a] | 12.6±1.7[a] | 11.7±0.8[a] | 5.0±0.4[a] | 12.6±2.4[a] | 13.5±1.7[a] | 8.4±6.8[a] |
| 15 (5) | 464.2±22.9[b] | 32.4±1.1[b] | 254.2±19.0[b] | 14.9±0.4[b] | 17.1±0.2[b] | 6.5±0.5[b] | 24.9±2.5[b] | 17.8±0.8[b] | 105.6±63.1[b] |

Bulls at 8 and 15 months of age were used for evaluation of body weight and scrotal circumference.
Evaluation of testes (weight, circumference, length, and width) and epididymis (weight and length) were obtained randomly from one testis of a pair of testes.
* Semen recovered from one side of epididymis of each bull. Sperm concentration was adjusted to 40 ×106 cells/ml and semen volume was recorded.
[a, b] Values (mean ± SD) with different letters differ significantly between bull at 8 and 15 months of age ($P$<0.05).

Table 2. Comparison of sperm viability and morphology in samples derived from epididymal tail from Hanwoo bulls at 8 and 15 months of age before freezing and after thawing

| Sperm morphology | | Age of months (bull = n) | | | |
|---|---|---|---|---|---|
| | | 8 (5) | | 15 (5) | |
| | | Before freezing | After frozen-thawing | Before freezing | After frozen-thawing |
| Normal | LIA | 13.8±8.6 [a] | 9.8±5.7 [a] | 70.4±7.4 [b] | 61.9±6.1 [b] |
| Abnormal | LDA | 0.2±0.3 | 0.1±0.2 | 0.4±0.3 | 4.5±9.5 |
| | DIA | 8.7±2.8 [a] | 47.3±12.2 [b] | 5.2±2.8 [a] | 8.7±4.1[a] |
| | DDA | 9.3±4.6 [a] | 22.8±9.8 [b] | 11.2±2.1 [ab] | 16.6±9.1 [ab] |
| | Head | 9.6±2.1 [b] | 5.3±2.7 [a] | 5.5±1.8 [a] | 2.1±1.5 [a] |
| | Midpiece | 4.6±1.6 | 1.7±1.5 | 3.1±3.0 | 2.0±1.6 |
| | Tail | 10.6±5.2 [b] | 6.3±2.9 [ab] | 2.0±1.3 [a] | 1.2±1.2 [a] |
| Proximal cytoplasmic droplet | LIA | 36.9±13.3 [b] | 0.8±1.4 [a] | 0.5±0.6 [a] | 0.2±0.3 [a] |
| | LDA | 0.1±0.2 | 0.3±0.6 | 0.0±0.0 | 0.0±0.0 |
| | DIA | 2.5±2.7 | 2.4±3.1 | 0.0±0.0 | 0.0±0.0 |
| | DDA | 0.8±0.6 | 1.0±1.3 | 0.0±0.0 | 0.2±0.4 |
| Distal cytoplasmic droplet | LIA | 3.4±3.6 | 0.9±1.2 | 1.6±2.0 | 2.1±3.0 |
| | LDA | 0.0±0.0 | 0.1±0.2 | 0.0±0.0 | 0.0±0.0 |
| | DIA | 0.4±0.3 | 0.5±0.2 | 0.1±0.2 | 0.1±0.2 |
| | DDA | 0.0±0.0 | 0.6±1.3 | 0.0±0.0 | 0.6±0.6 |

Live sperm with intact acrosome (LIA); live sperm with damaged acrosome (LDA); dead sperm with intact acrosome (DIA); dead sperm with damaged acrosome (DDA); abnormal head (macrocephalic, microcephalic, detatched, and tailless head); abnormal midpiece (segmental aplasia, bent, and double midpiece); abnormal tail (bent, hairpin, and coiled tail).
Proximal cytoplasmic droplet, remnants of cytoplasm under the plasma membrane in the neck region of the sperm; Distal cytoplasmic droplet, remnants of cytoplasm under the plasma membrane in the distance the neck region of the sperm.
[a, b] Values (means±SD) with different letters in a row differ significantly ($P$<0.05).

Table 3. Motility and motility parameters of sperm derived from epididymal tail of Hanwoo bulls at 8 and 15 months of age before freezing and after thawing

| Months of age (bull=n) | Treatment | Total motile (%) | Motile (%, VSL≥ 25 μm/sec) | VSL (μm/sec) | VCL (μm/sec) | VAP (μm/sec) | LIN (%) | STR (%) | WOB (%) | ALH (μm) | BCF (Hz) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 (5) | Before freezing | 29.0±14.1[a] | 3.4±3.7[a] | 38.4±2.1 | 71.9±18.6 | 47.2±6.0 | 60.4±14.9[b] | 82.9±9.1[b] | 71.1±10.4[b] | 2.5±0.7 | 9.3±2.9 |
| | After frozen-thawing | 26.4±8.2[a] | 2.6±1.8[a] | 44.2±3.4 | 86.0±24.1 | 55.3±7.3 | 58.5±10.7[b] | 80.8±5.8[ab] | 70.5±9.0[b] | 2.8±0.9 | 10.4±1.0 |
| 15 (5) | Before freezing | 82.7±9.5[b] | 22.8±12.1[b] | 35.4±13.3 | 99.6±33.6 | 53.2±17.6 | 38.4±10.3[a] | 65.6±13.0[a] | 55.4±6.7[a] | 3.6±1.1 | 9.6±2.9 |
| | After frozen-thawing | 70.4±15.9[b] | 20.3±12.3[b] | 43.0±5.9 | 98.7±37.7 | 59.5±15.7 | 53.1±10.7[ab] | 76.6±7.9[ab] | 67.7±7.0[ab] | 3.1±1.2 | 11.6±0.9 |
| Control (5) | After frozen-thawing | 41.2±14.6[a] | 13.8±4.9[ab] | 47.1±2.4 | 110.1±9.7 | 60.3±3.6 | 45.4±1.6[ab] | 78.7±1.3[ab] | 57.1±1.3[ab] | 4.1±0.3 | 10.6±0.5 |

Collected sperm in epididymal tail derived from one testis of a pair of testes in each bull at 8 and 15 months were used. As control, one kind of Hanwoo bull (commercial Hanwoo semen in Korea) was used (replicates=5).

[a,b] Values (mean ± SD) with different letters among groups in vertical differ significantly ($P<0.05$).

already reported that thawed sperm derived from epididymal tail at in one Hanwoo bull 13 months of age have similar motility, fertilizability, and blastocyst development compared to those of commercial thawed sperm. Brito *et al*. (2012) also demonstrated that mature sperm based on motility and normal morphology at 15 months of age were increased compared to 13 months of age in Angus × Charolias and Angus bulls. Therefore, we speculated that sperm derived from the epididymal tail of Hanwoo bulls at 15 months of age may have fertilizing ability due to them having more matured sperm compared to bulls at 13 months of age. However, further investigations by IVF or AI needs to confirm the fertilizing ability of sperm derived from the epididymal tail of bulls at 15 months of age.

In conclusion, sperm can be recovered from epididymis at 8 months of age in Hanwoo bull. However, percentage of sperm with normal morphology and motility at 8 months of age in Hanwoo bull was poor compared to that at 15 months of age. On the other hand, epididymal sperm at 15 months of age in Hanwoo bull showed similar percentage of sperm motility (VSL $\geq$ 25 μm/sec) before freezing and after frozen-thawing compared to frozen thawed commercial sperm.

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