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Comparison of Spermatozoa Recovery Methods on Cauda Epididymal Sperm of Hanwoo Bulls

Sung-Sik Kang, Ui-Hyung Kim, Min-Hyeong Jeon, Myung-Suk Lee and Sang-Rae Cho†

Hanwoo Research Institute, NIAS, RDA, Pyeongchang, Kangwondo, 25340 Republic of Korea

Abstract

In this study, two epididymal spermatozoa recovery methods in relation to sperm number, motility, viability and acrosome reaction were examined. Seven bulls were castrated and 7 testicles with epididymides were transferred to the laboratoy. Epididymis in each bull was randomly used for flushing and mincing methods with semen extender (Optixcell, IMV, France). The recovered spermatozoa with adjusted sperm concentration to 40 × 10^6 cells/mL was diluted with optixcell and cryopreserved. In experiment 1, the difference in the total number of spermatozoa using flushing and mincing methods was insignificant (2570.0 and 2505.2 × 10^6 cells/mL, respectively). For experiment 2, the percentage of motile spermatozoa and motility parameters between flushing and mincing methods were studied through the use of sperm class analyzer after frozen-thawing. The percentage of total motile sperm between flushing and mincing methods was almost the same with 89.5±12.8 and 91.4±7.9%, respectively. The same is the case with experiment 3 wherein the viability and acrosomal integrity of frozen-thawed epididymal spermatozoa by flushing and mincing was insignificantly different. The results from the study showed that both flushing and mincing methods can be used for epididymal spermatozoa recovery in bull.

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† Correspondence: Sang-Rae Cho (ORCID: 0000-0003-0209-6248)
Tel: +82-33-330-0625, Fax: +82-33-330-0660
E-mail: chosr@korea.kr

INTRODUCTION

The artificial insemination (AI) technology facilitates rapid improvement of livestock and beef industries (Foote, 2002). In the field, AI usually involves collection of bull semen by artificial vagina (AV) and electroejaculation (EJ) methods (Rego et al., 2015). The AV method requires training of bulls while EJ method which is used as an alternative does not need training of bulls (Jimenez-Rabadan et al., 2012). Animals with dead (Prieto et al., 2014) or erectile dysfunction (Kapoor et al., 2015) are not suitable for semen collection by EJ or AV methods. On the other hand, cauda epididymis has a role in storage of matured spermatozoa until ejaculation (Jones, 2004). Thus, spermatozoa recovery from cauda epididymis is used as an alternative of EJ or AV methods for gametes preservation of dead animals and animal sexual disorders. Numerous methods were utilized to recover epididymal spermatozoa namely cutting, floating, mincing, and flushing of epididymal spermatozoa for dead domestic and wild species (Fickel et al., 2007). In cutting method, used in bull (Martins et al., 2007) and goat (Jindal, 1984), isolated epididymis from testis was cut numerous times and spermatozoa was recovered by squeezing of epididymis. Floating method was applied in bull (Turri et al., 2012), canine (Yu and Leibo, 2002), feline (Thuwanut et al., 2010; Filliers et al., 2008), ram (Lone et al., 2011), and in stallion (Cary et al., 2004). In this method, sliced or incised epididymis was placed in recovery fluid in petri dish or tube for several minutes. Motile spermatozoa will swim out to fluid from tubules of cauda epididymis and fluid with spermatozoa will be recovered. For mincing method in bull (Kang et al., 2016), feline (Vernocchi et al., 2014), and stallion (Neuhauser et al., 2013), isolated cauda epididymis was minced finely and fluid with spermatozoa was immediately recovered. And lastly for flushing method, syringe with semen recovery fluid was connected to the vas deferens of the epididiymis of boar (Rath and Niemann, 1997), bull (Papa et al., 2015), ibex red deer (Santiago-Moreno et al., 2009), and rabbit (Brackett et al., 1978). Pressure was applied into epididymis and several incisions was made to cut tubules in cauda epidydimis thereby releasing the spermatozoa. Epididymal spermatozoa recovery methods affects sperm motility, sperm concentration, and quantity of debris and blood contamination. Thus, efficiency of recovery methods on epididymal spermatozoa were compared in several species, for example the cutting and mincing methods on sperm concentration and blood contamination on Iberian red deer (Martinez-Pastor et al., 2006). Influence of flushing and floating methods on sperm motility were also compared in bull (Turri et al., 2012) and stallion (Cary et al., 2004). No studies were done yet on the comparison of the two methods, thus this study examined the efficacy of flushing and mincing methods on epididymal spermatozoa recovery in bull. The spermatozoa of cauda epididymis was retrieved by flushing and mincing methods and was frozen using the semen freezing media. The motility and acrosomal membrane integrity of frozen-thawed spermatozoa were also examined.

MATERIALS AND METHODS

Ethics of animal experimentation

The cows used in this study were handled in accordance to the guidelines (grade D) of the National Institute of Animal Science (NIAS), Rural Development Administration, Cheonjoo, Republic of Korea. Procedures done were also approved by the animal experiment and ethics committee of the NIAS.

Castration and recovery of epididymis

Seven testicles with epididymides were castrated from seven Hanwoo bulls (mean±standard deviation, months of age = 15.1±0.2, scrotal circumference (cm) = 31.6±1.1, and body weight (kg) = 391.1±16.6). The castration of bulls was carried out until December 2017 at the Hanwoo Research Institute, NIAS, Pyeongchang, Republic of Korea. For general anesthesia of bull, 1.0 mL per 100 kg of xylazine hydrochloride (Rompun, Bayer, Republic of Korea) was injected into jugular vein of bull. After 5 to 15 minutes of injection, body, legs and head of bull were fixed with rope. And scrotal circumference was estimated with scrotal measuring tape and skin of testis was incised by scalpel. Testis with epididymides was recovered and surgical site was sutured. Twenty mL of dexamethasone disodium phosphate (Dexolone-20, SamyangAnifarm, Republic of Korea) and 20 mL of antibiotics (G.C.GPS-INJ., Green Cross Veterinary, Republic of Korea) were injected into the intramuscle. And testis with epididymides was transported to the laboratory within the first hour. In the laboratory, tunica albuginea of testicles was removed and testicular weight, length, width, and circumference were measured. Epididymis with vas deferens was isolated from testis and blood vessels on surface of cauda epididymis were removed. Random selection was done to determine if the epididymis will be processed by flushing or mincing method.

Spermatozoa recovery by flushing method

A 26 gauge needle connected with 10 mL syringe was inserted into the duct of vas deferens. The connecting part of the needle and vas deferens duct was fixed with forceps. About 12-15 incisions of cauda epididymis were made and pre-warmed semen freezing medium (OptixCell, IMV Technologies, France) was flushed in a 100 mm dish (Falcon).

Spermatozoa recovery by mincing method

The recovery of cauda epididymis spermatozoa by mincing adopted the procedure conducted by Kang et al., in 2016 with slight modification. Isolated cauda epididymis was minced using blades (No.21, AILEE, Korea) and surgical scissors in a 100 mm dish. Minced cauda epididymis tissues were incubated with 10 mL of semen freezing medium for 15 minutes at 25ºC. Sperm suspension was recovered by filtration using a cell strainer (100 µm nylon mesh, Falcon).

Semen freezing

Semen freezing was also performed by Kang et al., (2016) procedure with slight modification. Sperm concentration was adjusted to 40 × 10^6 cells/mL with semen freezing media and preserved at 4°C for 4 hours. Semen dilution was loaded to 0.5 mL straw, preserved at 3 cm from liquid nitrogen (LN_2) for 14 minutes, and plunged into LN_2.

Measurement of sperm motility

The measurement of sperm motility was done by also following a study of Kang et al., (2015). Semen was thawed at 37°C for 40 seconds and transferred to a 1.5 mL tube. Three µL of semen was placed onto 4-chamber slides with a depth of 20 µm (Art. No. SC-20-01-04-B, Leja, Nieuw-Vennep, Netherlands). At least 1000 sperm in 4-6 fields in a chamber were classified into motile and dead sperm. The percentage of motile sperm and motility parameters such as straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), linearity (LIN = VSL/VCL × 100, %), straightness (STR, %), wobble (WOB, %), flagellar beat cross frequency (BCF, Hz), and amplitude of lateral head (ALH, µm) were evaluated using CASA system (Sperm Class Analyzer, MicroOptic, Spain). Motile sperm with a VSL of ≥ 25 µm/sec were selected since having a VSL of less than 25 µm/sec will not be able to penetrate the oocyte according to Aitken (1985) and Holt et al., (1985).

Staining of frozen-thawed epididymal spermatozoa and evaluation of sperm viability and acrosomal membrane integrity

The staining and fixing solutions were prepared using the previous study of Kovacs and Foote, (1992) with minimal modification. Viability testing solution consisted of 0.25% trypan blue (Sigma-Aldrich, cat No. T6146) in 0.81% NaCl (w/v) while 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). Meanwhile, acrosome testing solution consisted of 7.5% Giemsa solution (Merck, cat No. 109204100) in distilled water. Twenty µl of spermatozoa dilution was mixed with pre-warmed 20 µl of viability solution while another ten µl of mixed solution was mounted on a slide glass and smeared. Slides were air dried, fixed with fixing solution for 5 minutes, washed with tap water, air dried at RT, and stained with acrosome testing solution for 12 hours. For the evaluation of sperm viability, morphology, and acrosome integrity of sperm, previous study of Kovacs and Foote (1992) was used. From each semen sample, more than 200 sperms in a slide glasses were counted by bright field microscopy at magnification 400×. The viability and acrosome integrity of sperm were also evaluated. Unlike live sperm which were not stained, dead sperm were stained with trypan dark blue at posterior region. On the other hand, normal acrosome of sperm was stained with Giemsa (violet) while abnormal or damaged acrosome was not stained.

STATISTICAL ANALYSIS

Testis characters, recovered sperm concentration and total number of spermatozoa, percentage of motile, motility parameters, viability and acrosomal integrity of spermatozoa among flushing and mincing groups were compared using one-way ANOVA, followed by the Duncan test for post hoc analysis. All analyses were performed using SAS (statistical analysis system for software version 9.2).

RESULTS AND DISCUSSION

Numerous studies were done on epididymal spermatozoa recovery methods from cauda epididymis in wild and domestic animals. However, comparison of flushing and mincing methods

Table 1. Testis characteristics and recovered spermatozoa concentration using flushing and mincing methods

Recovery methods (Rep.)	Testis Characteristics (Mean ± SD)				Sperm Recovered		
	Weight (g)	Length (cm)	Width (cm)	Circumference (cm)	Vol. (mL)	Sperm conc. ($\times 10^6$cells/mL)	Total No. sperm ($\times 10^6$cells)
Flushing (7)	221.0±12.0	15.9±0.9	5.7±0.3	15.0±1.2	63.3±28.0	40.6±1.0	2570±28.0
Mincing (7)	216.6±15.2	16.0±0.9	5.7±0.3	14.9±1.1	61.7±23.8	40.6±1.5	2505.2±35.7

for spermatozoa recovery of bull were not yet studied. Thus, in this study, the efficiency of flushing and mincing methods were compared using the following factors; recovered sperm number and motility, viability and acrosomal integrity of frozen-thawed spermatozoa. As shown in Table 1, testis characteristics were similar among flushing and mincing methods. Testicle size and scrotal circumference were positively correlated to sperm production in bull (Palasz et al., 1994) and ram (Elmaz et al., 2007). Results showed that similar number of sperm was recovered by flushing and mincing methods which is 2570.0 × 10^6 and 2505.2 × 10^6 cells, respectively. Thus, it can be concluded that condition of epididymal spermatozoa from cauda epididymis was similar and testis characteristics and method used will not influence the number of spermatozoa (Turri et al., 2012).

Table 2 and Table 3 also showed that values on total motility and motility parameters and viability and acrosomal membrane integrity of frozen-thawed spermatozoa using flushing and mincing methods were insignificantly different. However, a study of Hori et al., (2015), demonstrated that mincing methods have demerits of blood contamination from blood vessels in the surface of cauda epididymis and mixing of tissues from cauda epididymis. The blood contamination in epididymal spermatozoa dilution can induce increase in acrosome reaction and decrease in motility and viability (Martinez-Pastor et al., 2006). In this study, the indifference in the values may be due to clear removal of blood vessels on the surface of cauda epididymis. Meanwhile, a study of Martinez-Pastor et al., (2006) and Cary et al., (2004) found out that flushing method's use in the recovery of cauda epididymal spermatozoa in stallion is without blood contamination and tissue debris. Therefore, it is recommended that flushing method be used by well-trained practitioners since connecting and making incisions between needle and vas deferens are time consuming time and require technical skills. On the other hand, mincing methods is more appropriate for beginners due to easier collection of epididymal spermatozoa.

Several researches have been done to compare the type of semen extenders used for epididymal spermatozoa collection. Lopes et al., (2015) and Krishnakumar et al., (2011) differentiated the effect of Andromed and Tris egg yolk glycerol extender on bull epididymal spermatozoa. On the other hand, Neuhauser et al., (2018) compared stallion epididymal sperm motility using extenders such as skim milk, defined milk protein, egg yolk and caseinate extenders. In this study, only one semen extender (Optixcell) was utilized for epididymal spermatozoa collection

Table 2. Motility and motility parameters of frozen-thawed epididymal spermatozoa by flushing and mincing methods

Recovery method (Rep.)	Flushing (35)	Mincing (35)
% of total motile sperm	89.5±12.8	91.4±7.9
% of motile sperm (VSL≥25 μm/s)	40.9±10.0	37.3±9.3
VCL (μm/s)	135.8±23.1	131.7±19.7
VSL (μm/s)	50.4±3.7	48.6±4.1
VAP (μm/s)	76.5±9.1	74.2±8.0
LIN (%)	43.9±7.6	43.6±6.5
STR (%)	68.4±6.0	68.1±5.0
WOB (%)	61.6±5.2	61.5±4.8
ALH (μm)	4.5±0.9	4.4±0.8
BCF (Hz)	10.9±1.3	10.5±1.2

Seven testes were used in both flushing and mincing groups. Five replicates of sperm motility and motility parameters in each bull were evaluated. Mean ± SD.

Table 3. Viability and acrosomal integrity of frozen-thawed epididymal spermatozoa by flushing and mincing methods

Recovery method (Rep.)	LIA	LDA	DIA	DDA
Flushing (35)	71.7±6.3	0±0	23.9±3.8	4.4±3.3
Mincing (35)	72.4±7.3	0.2±0.4	23.8±6.5	3.6±2.9

Seven testes were used in both flushing and mincing groups. Five replicates in each bull were evaluated. Mean ± SD. Live spermatozoa with intact acrosome (LIA), live spermatozoa with damaged acrosome (LDA), dead spermatozoa with intact acrosome (DIA), and dead spermatozoa with damaged acrosome (DDA).

thus there is no report on the effect of semen extenders on epididymal spermatozoa motility in Hanwoo bull. Therefore, it is recommended for future studies to examine the effect of semen extenders on increasing the recovered epididymal spermatozoa quality in Hanwoo bull. In conclusion, the results demonstrated that both of flushing and mincing methods are appropriate for recovery of epididymal spermatozoa.

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CONFLICT OF INTEREST

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

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