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Motility, Fertilizability and Subsequent Embryonic Development of Frozen-thawed Spermatozoa derived from Epididymis in Hanwoo

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ABSTRACT

The aim of the study was to investigate the ability of sperm derived from the epididymis in regard to sperm motility, sperm penetration to oocyte and subsequent development of the embryo. Frozen-thawed sperm from epididymis showed similar percentage of motile sperm (VSL ≥ 25 μm/sec) as compared to that of commercial sperm (control). Sperm penetration of frozen-thawed epididymal and commercial sperm was not significantly different. Moreover, cleavage and blastocyst rates were similar in both epididymal and control. Sperm derived from the epididymis also showed fertilizability and subsequent embryonic development

(Key words : epididymis, Hanwoo, sperm)

INTRODUCTION

The unexpected loss of genetically valuable animals, as well as the difficulty in collecting semen from wild species lead to an increase in the use of artificial reproductive techniques, since it proved to be one of the unique possibilities in preserving the genetic material of the animals (Kaabi *et al.,* 2003).

In general, semen collection in the domestic animal industry has played an important role in animal reproduction. Semen from domestic animals is at present routinely collected by artificial vagina (Jimenez-Rabadan *et al.,* 2012; Sylla *et al.,* 2015). Also, semen collection from epididymis has been used as an important alternative tool, particularly on abnormal animals including those with joint disorder (Persson *et al.,* 2007), herpes virus infection on penis (Nandi *et al.,* 2009), poor sex drive (Perry *et al.,* 1991) and poor libido (Hoflack *et al.,,* 2006). In such cases, efforts to avoid total loss of genetic material can be achieved using reproductive assisted techniques (Martins *et al.,* 2007).

In the field, semen collection derived from epididymis can be an option for the preservation of spermatozoa of high-value animals and of endangered species. Semen collection methods employed to harvest the epididymal sperm in cattle are cutting method (Kaabi *et al.,* 2003), retrograde flushing (Bruemmer 2006) and float-up method (Santiago-Moreno *et al.,* 2009). It is possible that semen samples will be available on artificial insemination, *in vitro production* of embryo, and the juvenile selection of animals. Previous studies have shown satisfactory results with regards to the efficacy and potential of epididymal spermatozoa *in vitro* and *in vivo* fertilization using artificial insemination, *in vitro* fertilization (IVF) of oocyte and intracytoplasmic sperm injection with satisfactory results (Martins *et al.,* 2007; Monteiro *et al.,* 2011; Ringleb *et al.,* 2011; Santiago-Moreno *et al.,* 2006). Although there are few reports on the recovery of spermatozoa and cryopreservation in Hanwoo (Yoon *et al.,* 2015), no report has been made yet related to IVF using frozen-thawed sperm derived from the epididymal spermatozoa in Hanwoo. Therefore, the quality of sperm from epididymis should be investigated whether or not the sperm having fertilizability and subsequent embryonic development. Firstly, we examined sperm motility using computer assisted sperm analysis (CASA) system after frozen-thawed. Secondly, sperm penetration ability to oocyte and subsequent embryonic development were examined.

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MATERIALS AND METHODS

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

1. Chemicals

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

2. Recovery of Epididymal Sperm from a Castrated Bull and Cryopreservation

Native Korean Bull (Hanwoo) testes were obtained from the Hanwoo Research Institute, Rural Development Administration Pyeongchang, Gangwondo, South Korea. After castrating a Korean native bull at 13 months of age, testicles and epididymides were transported to the laboratory in saline. The sclerotic was removed and only the epididymis was recovered.

About 2 cm of the epididymis tail was dissected and mined using blades (No.21, AILEE, Korea) in a 100 mm dish (Falcon). Minced epididymis tail tissues were mixed with 4 ml of semen freezing medium (OptixCell, IMV Technologies, France) and sperm were recovered using a cell strainer (100 μm nylon mesh, Falcon). A total of 40 ml freezing medium was added to the sperm at room temperature. Sperm in freezing medium were preserved at 4℃ for 4 h and loaded to 0.5 ml straw (2 × 10^7 sperm/straw). These straws were cooled above 9 cm of liquid nitrogen (LN2) surface for 14min and cryopreserved in LN_2 tank until needed.

3. Evaluation of Sperm Motility and Motility Parameters by CASA

The evaluation of sperm motility was performed as described previously by Kang *et al.,* (2015) with a modification. Two μl of frozen-thawed semen was placed onto 8-chamber slides with a depth 20 μm (Art. No. SC 20-01-08-B, Leja, Nieuw-Vennep, Netherlands) for counting. Sperm in 3 fields or replicates with at least 800 spermatozoa per field were placed in a chamber and 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 are probably not able to penetrate the oocyte (Aitken 1985; Holt *et al.,* 1985).

4. *In Vitro* Maturation and Fertilization

The method for *in vitro* maturation (IVM) of bovine oocytes was performed as previously described by Rho *et al.,* (2007) with minor modification. Briefly, the bovine ovaries were transported from a local slaughterhouse to the laboratory in saline. Cumulus oocyte complexes (COCs) were aspirated from follicles measuring 2 to 6 mm in diameter and 10 to 13 COCs were cultured for 22 to 24 h in a 50 μl droplet of IVM medium under mineral oil (Sigma) at 39℃ in a humidified atmosphere of 5% CO_2 in air. The maturation medium consisted of TCM-199 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 10 μg/ml follicle-stimulating hormone (FSH; from porcin epituitary), 10 μg/ml luteal hormone (LH; Sigma), 10 ng/ml epithelial growth factor (EGF; Sigma) and 50 μg/ml gentamicin (Sigma).

Similarly, the method for *in vitro* fertilization (IVF) was performed as previously described by Rho *et al.,* (2007) with minor modification. Briefly, as control, commercial frozen-thawed semen from one kind of Hanwoo bull (KPN) was layered on a Percoll (Sigma) gradient (45 and 90%) and centrifuged at 750 × g for 15 min. To recover motile sperm from epididymis of a castrated bull (13 months of age), frozen-thawed semen was layered on a Percoll gradient (45 and 60%) and centrifuged at 750 × g for 15 min. The supernatant was discarded and the sperm pellet was resuspended in IVF-Tyrode albumin lactate phosphate (TALP) medium with 3 mg/ml fatty acid free bovine serum albumin (BSA). Matured COCs were co-incubated with motile sperm for 18 to 20 h in droplets (10 to 13 COCs / 50 μl) of IVF-TALP medium supplemented with 20 μg/ml heparin at 39℃ in a humidified atmosphere of 5% CO_2 in air. The final sperm concentration was adjusted to 2 × 106 cells/ml.

5. Examination of Spermatozoon Penetration after IVF

Examination of spermatozoon penetration after IVF was performed as described previously by Kang *et al.,* (2015). After

co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing. Ten to eleven presumptive zygotes of each experiment group were fixed with ethanol: acetic acid at a ratio of 3:1 and stained with 1% aceto-orcein solution as described previously Takahashi and First (1992). Oocytes having an enlarged sperm head(s) or male pronucleus(ei) were defined as penetrated by sperm, and the following categories of oocytes penetrated by sperm were recorded: 1) oocytes with male and female pronuclei with a corresponding sperm tail (2PN), 2) oocytes with more than two enlarged sperm heads or male pronuclei (polyspermy), and 3) oocytes other than 2PN and polyspermy, such as oocytes with an enlarged sperm head and anaphase II/telophase II chromosome or female pronucleus, or oocytes with a male pronucleus and telophase II chromosome (others).

6. *In Vitro* Culture of Presumptive Zygotes and Evaluation of Embryo Development

In vitro culture (IVC) was conducted as described previously Kang *et al.,* (2015) with minor modification. After co-incubation with sperm, presumptive zygotes freed from cumulus cells by vortexing were washed 3 times and cultured for 192 h in a 45 μl droplet using a modified synthetic oviduct fluid, which contained 1 mM glutamine, 12 essential amino acids, 7 non-essential amino acids and 10 μg/ml insulin and was supplemented with 5 mM glycine, 5 mM taurine and 1 mM glucose Takahashi and Kanagawa, 1998), and added 3 mg/ml fatty acid free BSA instead of polyvinyl alcohol at 39℃ under 5% CO_2, 5% O_2 and 90% N_2. Cleavage and blastocyst developmental rates were assessed after 48 h and 192 h of IVF, respectively.

7. Experimental Design

In experiment 1, to examine sperm motility and sperm motility parameters, frozen-thawed sperm from epididymis was evaluated immediately after thawing.

In experiment 2, to examine sperm penetration ability to oocytes, matured COCs were co-incubated with frozen-thawed sperm from epididymis for 18 h.

In experiment 3, to evaluate blastocyst developmental rate of oocytes fertilized with frozen-thawed sperm from epididymis, presumptive zygotes were cultured for 192 h.

8. Statistical Analysis

Sperm penetration rates (total penetration, 2PN and polyspermy rates) between groups were compared using the Chi-square test. The cleavage and blastocyst developmental rate, sperm motility and sperm motility parameters between groups were also compared using one-way ANOVA. followed by the Tukey-Kramer HSD (Honestly Significant Difference) test for post hoc analysis. All analyses were performed using JMP Pro (version 10.0.2, SAS Institute, Cary, NC).

RESULTS

Experiment 1

The percentage of total motile sperm derived from the epididymis was significantly lower than that of control ($P<0.01$). In terms of the percentage of motile sperms with VSL $\geq$25 μm/sec, no significant difference was found between those sourced from the epididymis and that of the control. Percentages of VSL, VAP, LIN, WOB and BCF of sperms from the epididymis were significantly lower than those of the control ($P<0.01$). However, percentage of ALH in sperm obtained from epididymis was significantly higher than the control ($P<0.01$).

Experiment 2

The percentages of total penetration, 2PN and polyspermy were similar in both epididymal sperm and the control.

Experiment 3

The percentages of cleavage, blastocyst and blastocyst based on cleaved oocytes in the epididymal sperm were found not to be significantly different with the control.

DISCUSSION

Recent studies have demonstrated that epididymal spermatozoa have been viable for useful tools to rescue genetic material by the unexpected loss of the animal (Turri *et al.,* 2012). The importance of the technology in cattle breeding, selection programs and preservation of endangered species is because of its advantage in the recovery and cryopreservation of epididymal spermatozoa and *in vitro* embryo production (Martins *et al.,* 2007). The reduced sperm and poor semen quality of immature bulls, including the Hanwoo line, puberty are in adequate to produce results leading to poor reproductive performance. To pass breeding soundness evaluation in bulls, age of bull should be overcome 12～16 months of age (Arteaga

et al., 2001; Higdon *et al.,* 2000; Kennedy *et al.,* 2002). For this reason, semen collection from epididymal spermatozoa has the distinct economic advantage of shortening the breeding period.

In this study, the spermatozoa collected from the cauda epididymides of Hanwoo showed 76.5% motility just before freezing (not shown in this study). This is in agreement with the results observed by Turri *et al.,* (2012) on sperm motility (71.6%) from the cauda epididymides of bull. After frozen-thawing, the percentage of total motile sperm and sperm motility parameters (VSL, VAP, LIN, WOB, ALH and BCF) of epididymis sperm were found to be significantly lower ($P<0.01$) than that of control as shown in Table 1. However, percentage of motile sperm with a VSL $\geq$ 25 μm/sec was observed to be similar in both epididymis derived and the control.

Sperm motility is one of the most important features associated with semen fertilizing capacity (Sellem *et al.,* 2015). For many years, it has been recognized as fundamental in sperm transport and fertilization of the oocyte inside the female reproductive tract (Januskauskas *et al.,* 1999; Verstegen *et al.,* 2002) and IVF in bovine (Sapanidou *et al.,* 2015). Previous studies reported that motile sperm with a VSL $\geq$ 25 μm/sec can move forward and meet oocytes in droplet (Aitken 1985; Holt *et al.,* 1985; Kang *et al.,* 2015).

Sperm penetration rate in oocytes was similar between epididymal sperm and the control as shown Table 2. It means that epididymal sperm have similar fertilizing ability compared with the control. In addition, blastocyst developmental competence with epididymal sperm was similar with that of control as shown Table 3. These results suggest that it is possible to embryo production using cryopreserved epididymal sperm in Hanwoo. If these embryos are transferred to recipients, it would be possible to produce offspring.

Indeed, the current study provides the useful tools for recovery of epididymal spermatozoa, cryopreservation and embryo production to preserve high-value genetic materials.

In conclusion, this study was able to provide a useful tool in the recovery and cryopreservation showed that sperm derived from epididymis can be cryopreserved. Frozen thawed sperm derived from epididymis has an ability to penetrate oocytes and subsequent blastocyst developmental competence *in vitro*. The values of sperm characteristics and function from Hanwoo epididymis have not been extensively investigated,

Table 2. Sperm penetration rate with bull epididymides sperm at 18 h after IVF

Sperm	No. of oocytes (replicates)	Percentages of			
		Total penetration	2PN	Poly	Others*
Epididymal	88 (7)	86.4	75.6	12.5	2.3
Control	87 (7)	74.7	66.7	5.7	3.5

Epididymal sperm from one bull at 13 months of age and one kind of Hanwoo bull (KPN; control) were used.
2PN: two pronuclei, Poly: penetrated with more than two sperm.
* Others: an enlarged sperm head with anaphase II/telophase II chromosome or a male pronucleus was observed.

Table 3. Blastocyst developmental rate of oocytes fertilized with epididymal sperm from a bull

Sperm	No. of oocytes (replicates)	Percentages of		
		Cleavage	Blastocyst	Blastocyst/ cleaved
Epididymal	150 (7)	80.1±6.5	25.9±6.4	32.0±5.8
Control	120 (5)	70.6±9.4	20.2±4.3	29.1±7.7

Epididymal sperm from one bull at 13 months of age and kind of Hanwoo bull (KPN; control) were used.

Table 1. Sperm motility and sperm motility parameters of epididymal from a bull

Sperm (replicates)	Total motile (%)	Motile (%, VSL ≥25 μm/sec)	VCL (μm/sec)	VSL (μm/sec)	VAP (μm/sec)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
Epididymal (6)	67.3±6.2[a]	21.7± 4.3	88.4±6.2	41.0±1.3[a]	51.6 ±2.3[a]	52.8±3.0[a]	80.6±1.9	64.4±2.2[a]	3.0±0.3[a]	12.4±0.5[a]
Control (5)	87.7±6.2[b]	23.8±10.1	82.6±1.9	49.6±1.6[b]	60.4±1.8[b]	61.2±1.2[b]	81.7±1.4	74.5±1.7[b]	2.4±0.1[b]	13.6±0.4[b]

Epididymal sperm from one bull at 13 months of age and control (commercial sperm) from one bull were used.
[a,b] Values (means±standard deviation (S.D.)) with different letters differ significantly between epididymal sperm and control (commercial sperm) ($P<0.01$).

which still remained. Further studies are needed to demonstrate whether the sperm will be produce offspring by AI or not.

REFERENCES

Aitken RJ. 1985. Diagnostic value of the zona-free hamster oocyte penetration test and sperm movement characteristics in oligozoospermia. Int. J. Androl. 8(5):348-356.

Arteaga A, Baracaldo M and Barth AD. 2001. The proportion of beef bulls in western Canada with mature spermiograms at 11 to 15 months of age. Can. Vet. J. 42(10):783-787.

Bruemmer JE. 2006. Collection and freezing of epididymal stallion sperm. Vet. Clin. North. A,. Equine. Pract. 22(3): 677-682.

Higdon III HL, Spitzer JC, Hopkins FM and Bridges WC Jr. 2000. Outcomes of breeding soundness evaluation of 2898 yearling bulls subjected to different classification systems. Theriogenology 53(6):1321-1332.

Hoflack G, Van Soom A, Maes D, de Kruif A, Opsomer G and Duchateau L. 2006. Breeding soundness and libido examination of Belgian Blue and Holstein Friesian artificial insemination bulls in Belgium and The Netherlands. Theriogenology 66(2):207-216.

Holt WV, Moore HD and Hillier SG. 1985. Computer-assisted measurement of sperm swimming speed in human semen: correlation of results with *in vitro* fertilization assays. Fertil. Steril. 44(1):112-119.

Januskauskas A, Gil J, Soderquist L, Haard MG, Haard MC, Johannisson A and Rodriguez-Martinez H. 1999. Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bull semen used for artificial insemination in Sweden. Theriogenology 52(4):641-658.

Jimenez-Rabadan P, Ramon M, Garcia-Alvarez O, Maroto-Morales A, del Olmo E, Perez-Guzman MD, Bisbal A, Fernandez-Santos MR, Garde JJ and Soler AJ. 2012. Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtiberica buck ejaculates. Anim. Reprod. Sci. 132(1-2):88-95.

Kaabi M, Paz P, Alvarez M, Anel E, Boixo JC, Rouissi H, Herraez P and Anel L. 2003. Effect of epididymis handling conditions on the quality of ram spermatozoa recovered postmortem. Theriogenology 60(7):1249-1259.

Kang SS, Koyama K, Huang W, Yang Y, Yanagawa Y, Takahashi Y and Nagano M. 2015. Addition of D-penicillamine, hypotaurine, and epinephrine (PHE) mixture to IVF medium maintains motility and longevity of bovine sperm and enhances stable production of blastocysts *in vitro*. The Journal of reproduction and development 61(2):99-105.

Kang SS, Koyama K, Huang W, Yang Y, Yanagawa Y, Takahashi Y and Nagano M. 2015. Addition of D-penicillamine, hypotaurine, and epinephrine (PHE) mixture to IVF medium maintains motility and longevity of bovine sperm and enhances stable production of blastocysts *in vitro*. The Journal of reproduction and development 61(2):99-105.

Martins CF, Rumpf R, Pereira DC and Dode MN. 2007. Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses *in vitro* embryo production. Anim. Reprod. Sci. 101(3-4):326-331.

Monteiro GA, Papa FO, Zahn FS, Dellaqua JA, Jr., Melo CM, Maziero RR, Avanzi BR, Alvarenga MA and Guasti PN. 2011. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. Anim. Reprod. Sci. 127(3-4):197-201.

Nandi S, Kumar M, Manohar M and Chauhan RS. 2009. Bovine herpes virus infections in cattle. Animal health research reviews/Conference of Research Workers in Animal Diseases 10(1):85-98.

Perry VE, Chenoweth PJ, Post TB and Munro RK. 1991. Patterns of development of gonads, sex-drive and hormonal responses in tropical beef bulls. Theriogenology 35(2):473-486.

Persson Y, Soderquist L and Ekman S. 2007. Joint disorder; a contributory cause to reproductive failure in beef bulls? Acta Vet. Scand. 49:31.

Rho GJ, Kim DS, Son WJ, Cho SR, Kim JG, and Choe SY. 2007. Influence of *in vitro* oxygen concentrations on preimplantation embryo development, gene expression and production of Hanwoo calves following embryo transfer. Mol. Reprod. Dev. 74(4):486-496.

Ringleb J, Waurich R, Wibbelt G, Streich WJ and Jewgenow K. 2011. Prolonged storage of epididymal spermatozoa does not affect their capacity to fertilise *in vitro*-matured domestic cat (Felis catus) oocytes when using ICSI. Reprod. Fertil. Dev. 23(6):818-825.

Santiago-Moreno J, Astorga RJ, Luque I, Coloma MA, Toledano-Diaz A, Pulido-Pastor A, Gomez-Guillamon F, Salas-

Vega R and Lopez-Sebastian A. 2009. Influence of recovery method and microbial contamination on the response to freezing-thawing in ibex (*Capra pyrenaica*) epididymal spermatozoa. Cryobiology 59(3):357-362.

Santiago-Moreno J, Toledano-Diaz A, Pulido-Pastor A, Gomez-Brunet A and Lopez-Sebastian A. 2006. Birth of live Spanish ibex (*Capra pyrenaica* hispanica) derived from artificial insemination with epididymal spermatozoa retrieved after death. Theriogenology 66(2):283-291.

Sapanidou V, Taitzoglou I, Tsakmakidis I, Kourtzelis I, Fletouris D, Theodoridis A, Zervos I and Tsantarliotou M. 2015. Antioxidant effect of crocin on bovine sperm quality and *in vitro* fertilization. Theriogenology 84(8):1273-1282.

Sellem E, Broekhuijse ML, Chevrier L, Camugli S, Schmitt E, Schibler L and Koenen EP. 2015. Use of combinations of *in vitro* quality assessments to predict fertility of bovine semen. Theriogenology 84(9):1447-1454 e1445.

Sylla L, Palombi C, Stradaioli G, Vagniluca A and Monaci M. 2015. Effect of semen collection by transrectal massage of accessory sexual glands or artificial vagina on the outcome of breeding soundness examinations of Italian yearling beef bulls. Theriogenology 83(5):779-785.

Takahashi Y and First NL. 1992. *in vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 37(5):963-978.

Takahashi Y and Kanagawa H. 1998. Effects of glutamine, glycine and taurine on the development of *in vitro* fertilized bovine zygotes in a chemically defined medium. J. Vet. Med. Sci. 60(4):433-437.

Turri F, Madeddu M, Gliozzi TM, Gandini G and Pizzi F. 2012. Influence of recovery methods and extenders on bull epididymal spermatozoa quality. Reprod. Domest. Anim. 47(5):712-717.

Verstegen J, Iguer-Ouada M and Onclin K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57(1):149-179.

Yoon SJ, Kwon WS, Rahman MS, Lee JS and Pang MG. 2015. A novel approach to identifying physical markers of cryodamage in bull spermatozoa. PLoS One 10(5):e0126232.

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