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Original Article

Pregnancy rate in Hanwoo cows after timed artificial insemination using different sperm concentrations

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ABSTRACT

Background: Sperm quality and the number of sperm introduced into the uterus during artificial insemination (AI) are pivotal factors influencing pregnancy outcomes. However, there have been no reports on the relationship between sperm concentration at AI and sperm quality in Hanwoo cattle. In this study, we examined sperm quality and pregnancy rates after AI using sperm inseminated at different concentrations.

Methods: We evaluated the motility, viability, and acrosomal membrane integrity of sperm at different concentrations (10, 15, 18, and 20 million sperm/straw) in 0.5-mL straws. Subsequently, we compared the pregnancy rates after AI with different sperm concentrations.

Results: After freeze-thawing, sperm at the assessed concentrations showed similar viability and acrosomal membrane integrity. After AI, cattle in the 10 million group had significantly lower pregnancy rates compared to those in the 18 and 20 million groups. Conversely, there were no statistically significant variances observed between cattle in the 10 and 15 million groups.

Conclusions: Sperm at concentrations of 10, 15, 18 and 20 million per straw exhibited comparable motility, viability, and acrosomal membrane integrity. However, a concentration of at least 18 million sperm per straw is required to achieve a consistent rate of pregnancy rate in Hanwoo cattle after AI.

Keywords: artificial insemination, Hanwoo, pregnancy rate, sperm concentration, sperm quality

INTRODUCTION

Natural mating, artificial insemination (AI), and estrus synchronization with hormone treatment have been used to reproduce cows (Baruselli et al., 2018). In Hanwoo, the number of AI procedures per pregnancy was 1.5 ± 0.3 times (Yang et al., 2017) and age at first AI for Hanwoo cattle is 13.9 ± 1.0 months (Kang et al., 2021). The pregnancy rate is affected by both the nutritional status of the cows and the quantity and quality of sperm injected during AI. In Hanwoo cattle, AI is used to facilitate reproduction in almost all cows, with reported rate of offspring production of between 64% and 79% (Kang et al., 2019). In this context, the detection of estrus and appropriate timing of AI prior to ovulation in the ovary are important for obtaining successful pregnancies and offspring production (Sales et al., 2011; Richardson et al., 2017).

However, the conception rate of cows may differ depending in the number of sperm introduced into the uterus (Mohanty et al., 2018), as well as on the type of semen (liquid vs. freeze-thawed semen) used (Richardson et al., 2017). In Korea, the Hanwoo Bull Center (Hanwoo Improvement Center, NH, Seosan, Korea) selected elite Hanwoo bulls across the nation and produced ~10 million frozen straws per Hanwoo bull lifespan. AI is primarily performed using freeze-thawed semen, and for Korean cattle, frozen semen produced at the Hanwoo Improvement Center for AI is commonly used, with frozen Hanwoo semen typically being cryopreserved in liquid nitrogen and supplied at a concentration of approximately 18 million spermatozoa per 0.5-mL straws (Kang et al., 2020). The criteria for producing frozen sperm require the use of samples with vitality of 70% or higher, a survival rate of 60% or higher, and a malformation rate of 15% or less, following the collection of fresh semen. Only sperm with vitality of at least 40% after freezing and thawing is utilized.

In a typical AI program, in which 10 to 30 million sperm are introduced into the uterus, the pregnancy rate achieved is approximately 60% to 70% (Stevenson et al., 2009). It has, however, been established that the rate of pregnancy differs depending on the number of sperm introduced into cows.

The findings of a previous study, in which the authors evaluated the effects of different numbers of sperm (8, 12, and 16 million) introduced into the uterus of dairy cows on the rate pregnancy after AI, revealed that the 16 million group had a significantly higher rate of pregnancy (69.2%) compared with the 65.7% and 66.8% rates achieved in cows inseminated with 8 and 12 million sperm, respectively. In a further comparison, conception rates of 31.3% and 44.9% were obtained for cows inseminated with 2 and 15 million sperm, respectively (Andersson et al., 2004).

Consistent with these observations, a further comparison of AI using 12, 15, and 18 million sperm, revealed that compared with cows in the 12 and 15 million groups, there were significantly higher rates on conception in cows inseminated with 18 million sperm (Kommisrud et al., 1996). Numerous groups have reported that although there may be differences depending on the breed of cow and the frozen-thawed semen used, conception tends to be influence to a greater extent by the concentration of the frozen-thawed sperm, with a reduced sperm quality being associated with reduced rates of conception (Wiltbank and Parish, 1986).

In addition, sperm viability and acrosomal integrity, which are important factors for evaluating sperm quality, have been established to have a notable influence on conception after AI (Januskauskas et al., 1999; Kathiravan et al., 2011). To the best of our knowledge, however, there have to date been no reports regarding the association among the number of introduced sperm on the viability and acrosomal membrane integrity of frozen-thawed spermatozoa in the AI of Hanwoo cows.

In this study, we analyzed the sperm quality and rate of pregnancy rate in Hanwoo cattle after AI with different concentrations of frozen-thawed spermatozoa. In experiment 1, we collected Hanwoo semen from 10 bulls and prepared frozen semen at concentrations of 10, 15, 18, and 20 million spermatozoa per 0.5-mL straws, using which, we compared the motility, viability, and acrosomal membrane integrity of spermatozoa after freeze-thawing. In Experiment 2, following estrus synchronization, we conducted AI using semen containing different numbers of freeze-thawed spermatozoa, and subsequently determined pregnancy rates based on the detection of pregnancy-associated glycoproteins using a pregnancy test kit.

MATERIALS AND METHODS

Semen collection and semen freezing procedure

Using an artificial vagina, semen was collected from 10 Hanwoo bulls aged 14-18 months at the Hanwoo Research Institute in Pyeongchang from June 2020 to April 2021. Having collected the semen, the volume, color, pH, and sperm motility were evaluated to ensure the selection of normal semen. Sperm showing greater than 80% motility were used for subsequent freezing. The collected semen was diluted with semen freezing media (Optixcell; IMV imaging, France) and the concentrations of sperm per 0.5-mL straws were adjusted to 10, 15, 18, or 20 million. Following dilution, the diluted semen was stored at 4℃ for 3 to 4 h, after which the pre-cooled semen was loaded into 0.5-mL straws and sealed with polyvinyl alcohol powder. The loaded straw were then maintained at a height of 3 cm above the surface of liquid nitrogen for 14 min, after which, the cooled straw was immersed in liquid nitrogen and cryopreserved in a liquid nitrogen tank. Sperm in frozen-thawed semen at a concentration of 10 million per 0.5-mL straw were used to evaluate viability and acrosomal membrane integrity of spermatozoa, and frozen semen in 0.25-mL straws were used for AI.

Evaluation of frozen-thawed spermatozoa motility

Sperm motility was assessed based on computer-assisted sperm analysis (Microoptic, Spain). Frozen straws were thawed in water at 37℃ for 40 s and transferred to 1.5-mL microtubes. Aliquots (3 μL) of the thawed semen were introduced into slide chambers (SC 20-01-04-B; Leja, Nieuw-Vennep, Netherlands) and more than 1,000 spermatozoa in four to six fields of view were assessed for determination of sperm motility based on the following parameters: total motility (%), progressive motility (%), slow progressive motility (%), non-progressive motility (%), immotile (%), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN = VSL/VCL, %), straightness (STR = VSL/VAP, %), amplitude of the lateral head (ALH, μm/s), and flagellar beat cross frequency (BCF, Hz).

Evaluation of the viability and acrosomal membrane integrity of frozen-thawed spermatozoa

Samples of frozen-thawed semen (20 μL) were mixed with 20 μL of 0.25% trypan blue solution, and 10 μL of the stained semen was placed on a glass slide, smeared, and dried. Glass slides were immersed in a fixative solution for 5 min, washed with tap water, and dried. The slides were then immersed in a 7.5% Giemsa solution for 12 h, washed with tap water, and dried. Sperm viability and acrosomal membrane integrity were evaluated using a microscope at ×400 magnification. Spermatozoa with purple-stained acrosomes and non-staining of the posterior head were classified as live spermatozoa with intact acrosomes; those with unstained acrosomes and posterior heads were classified as live spermatozoa with damaged acrosomes; those with blue- or purple-stained acrosomes and dark purple posterior heads were classified as dead spermatozoa with intact acrosomes; and those with unstained acrosomes and dark purple posterior heads were classified as dead spermatozoa with damaged acrosomes (Kang et al., 2020).

Synchronization of estrus in Hanwoo cows and artificial insemination

Feed was provided to maintain a body condition score of 3.0 to 3.5. In brief, cows were fed 3.5 kg of concentrate feed, while roughage consisted of a mixture of hay or straw, with a feeding amount ranging from 4.5 to 6.0 kg. Water was freely available for ad libitum consumption. A total of 209 Hanwoo cows were used for the synchronization estrus and AI by Kang et al. (2019). The average age was 29.9 ± 12.0 months (ranging from 14 to 56), and the average parity was 1.5 ± 1.2 (ranging from 0 to 5), comprising 53 heifers, 64 primiparous, and 92 multiparous Hanwoo cows. Irrespective of the estrous cycle, a controlled internal drug release device contained with 1.9 g of progesterone (CIDR; Zoetis, New Zealand) was introduced into the vagina at 09:00, and 2 mL of gonadotropin-releasing hormone (GnRH; Fertagyl, GmbH, Germany) was injected into the muscle around the neck of the cow. Seven days after CIDR insertion, the CIDR device was removed at 09:00, 5 mL of 25 mg PGF2a (Lutalyse, Zoetis, Belgium) was injected, and estrus detection patches (Estrotect; Rockway Inc., Spring Valley, WI, USA) were applied approximately midway between the hip and tail.

Two days after CIDR removal, 2.0 mL of GnRH was injected, and ovulation was induced. AI was performed at 18:00 on the day of GnRH injection and at 09:00 on the following day. For AI, we used frozen-thawed semen samples at concentrations of 10, 15, 18, and 20 spermatozoa

per 0.5-mL straws.

Prior to performing AI, frozen straws were thawed in water at 37℃ for 40 s and adapted to an AI gun with a sheath covered by a cover sleeve. AI was performed by three well-trained technicians. The mating combination of numerous frozen semen samples from 10 bulls and Hanwoo cows was planned according to the mating system of the Hanwoo Research Institute.

Assessment of pregnancy after artificial insemination

After 28 to 30 days after AI, the cows were assessed for pregnancy using a Rapid Visual Pregnancy Kit (IDEXX, Switzerland), which detects pregnancy-associated glycoproteins.

Briefly, 1.0 mL samples of blood collected from the jugular vein of the cows were placed in vacutainers containing EDTA. Blood samples were transferred to the laboratory within 30 min of collection. Samples of negative control solution, positive control solution, and collected blood (all 100 μL) were added to each well of antibody-coated plates, followed by three drops of detection solution, and the plates were then incubated for 7 min. Having subsequently removed the diluents from plate wells, the wells were washed three times with distilled water, and after removing the final wash, three drops of the conjugate solution were added to wells and the plates were incubated for a further 7 min. Thereafter, diluents in each well were again removed, followed by three washes with water, and the subsequent addition of three drops of TMB substrate solution. After 7 min, the reaction was terminated by the addition of three drops of stop solution into each well, and the resulting color reactions were recorded to determine the pregnancy of cows. Well contents with a blue or dark blue coloration identical to that observed in the positive control wells was taken to be indicative of pregnancy, whereas a light blue coloration or clear contents comparable with the negative control wells was assumed to indicate an absence of conception. After 90 days post-AI, pregnancy was confirmed by rectal palpation and transrectal ultrasound (Easi-Scan equipped with a linear probe 4.5 to 8.5 MHz, IMV imaging, France). Cows were considered non-pregnant when no pregnancy was detected by ultrasound examination, despite pregnancy being confirmed at 30 days post-insemination.

Statistical analysis

Sperm motility, viability, and acrosomal membrane integrity were compared using a one-way ANOVA with a post hoc Duncan's test (SAS, Ver. 9.4; SAS Institute, Inc.). Differences in the pregnancies of cow inseminated with different sperm numbers at AI were analyzed using the chi-square test. Statistical significance was set at $p < 0.05$.

Table 1. Comparison of frozen-thawed sperm motility for different numbers of sperm in straws

No. spermatozoa/straw (replicates)	10 million (15)	15 million (15)	18 million (15)	20 million (15)
Total motile (%)	78.3 ± 5.3^{b}	82.2 ± 4.4^{a}	83.3 ± 5.3^{a}	85.0 ± 6.8^{a}
Fast progressive (%)	36.1 ± 5.5^{a}	35.0 ± 2.9^{ab}	31.9 ± 5.1^{bc}	28.3 ± 5.7^{c}
Slow progressive (%)	20.2 ± 3.9^{b}	23.0 ± 3.0^{ab}	23.6 ± 3.7^{ab}	25.4 ± 8.7^{a}
Non-progressive (%)	22.0 ± 2.9^{c}	24.2 ± 2.0^{c}	27.7 ± 2.7^{b}	31.3 ± 3.9^{a}
Immotile (%)	21.7 ± 5.3^{a}	17.8 ± 4.4^{b}	16.7 ± 5.3^{b}	15.0 ± 6.8^{b}
VCL (μm/s)	90.7 ± 8.7^{a}	81.8 ± 4.4^{b}	77.8 ± 6.1^{b}	77.6 ± 8.9^{b}
VSL (μm/s)	39.1 ± 3.1^{a}	34.0 ± 3.2^{b}	31.7 ± 4.1^{bc}	30.4 ± 3.8^{c}
VAP (μm/s)	53.3 ± 4.4^{a}	47.6 ± 3.6^{b}	45.4 ± 4.3^{b}	44.9 ± 3.8^{b}
LIN (%)	43.4 ± 4.2^{a}	41.5 ± 2.2^{ab}	40.7 ± 3.2^{ab}	39.4 ± 5.6^{b}
STR (%)	73.5 ± 4.0^{a}	71.3 ± 2.1^{ab}	69.6 ± 3.3^{bc}	67.6 ± 5.7^{c}
WOB (%)	58.9 ± 2.8	58.2 ± 1.7	58.4 ± 2.2	58.1 ± 3.5
ALH (μm)	3.3 ± 0.4	3.2 ± 0.2	3.1 ± 0.2	3.1 ± 0.4
BCF (Hz)	17.0 ± 1.1^{a}	15.4 ± 1.4^{b}	15.3 ± 1.4^{b}	15.4 ± 1.6^{b}
Hyperactive (%)	16.0 ± 3.1^{a}	14.7 ± 1.9^{a}	12.0 ± 2.8^{b}	10.4 ± 3.2^{b}

Values are presented as the means ± standard deviation (SD). ^{a,b}Values (mean ± SD) in rows denoted by different superscript lower-case letters differ significantly among groups ($p < 0.05$). In total, 15 freeze-thawed straws derived from five bulls (F, G, H, I, and J) were used to evaluate sperm motility and motility parameters. Three straws from each bull were used in the experiment. Total motile (%), Progressive motile (%), Slow progressive (%), Non-progressive (%), Immotile (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN = VSL/VCL, %), straightness (STR = VSL/VAP, %), amplitude of lateral head (ALH, μm/s), and flagellar beat cross frequency (BCF, Hz), and hyperactivity (%).

Table 2. The viability and acrosomal membrane integrity of frozen-thawed spermatozoa at different concentrations in straws

No. sperm/straw	Replicate	LIA	LDA	DIA	DDA
10 million	15	63.8 ± 10.7	0 ± 0	34.1 ± 10.4	2.1 ± 1.3
15 million	15	67.7 ± 5.5	0.1 ± 0.2	29.5 ± 4.8	2.7 ± 1.9
18 million	15	71.7 ± 6.8	0.1 ± 0.2	26.8 ± 6.4	1.5 ± 0.9
20 million	15	70.1 ± 8.6	0.1 ± 0.3	28.3 ± 8.4	1.6 ± 1.1

Frozen-thawed semen was collected from five bulls (F, G, H, I, and J), and three straws per bull were used to evaluate viability and acrosomal membrane integrity. LIA, live spermatozoa with intact acrosomes; LDA, live spermatozoa with damaged acrosomes; DIA, dead spermatozoa with intact acrosomes; DDA, dead spermatozoa with damaged acrosomes.

Table 3. Pregnancy rate Hanwoo cows after artificial insemination using different concentrations of sperm

Sperm concentration/straw	No. of cowsinseminated	No. of pregnant cows at diagnosis	
		Day 30 (%)	Day 90 (%)
10 million	63	28 (44.4)[b]	25 (39.7)[b]
15 million	51	32 (62.8)[ab]	30 (58.8)[ab]
18 million	46	36 (78.3)[a]	34 (73.9)[a]
20 million	49	35 (71.4)[a]	33 (67.3)[a]
	209	131 (62.7)	122 (58.4)

[a,b]Values in columns denoted by different superscript lower-case letters differ significantly among groups ($p < 0.05$). The cows were combined according to the mating program of the Hanwoo Research Institute. Frozen-thawed semen derived from 10 bulls (A-J) was used for AI. Pregnancy was confirmed through blood testing 30 days after AI and further confirmed by rectal palpation and ultrasound examination at 90 days post-insemination. Cows were considered non-pregnant due to the absence of pregnancy detected by ultrasound examination 90 days after AI, despite the presence of pregnancy at 30 days post-insemination.

RESULTS

The results presented Table 1 reveal that the percentage of total motile sperm in the 10 million group was significantly lower than that in the 15, 18, and 20 million groups (78.3% ± 5.3%, 82.2% ± 4.4%, 83.3% ± 5.3%, and 85.0% ± 6.8%, respectively, $p < 0.05$). Contrastingly, the percentage of sperm in the 10 million group with fast progressing motility was significantly higher than that of sperm in the 18 and 20 million groups (36.1% ± 5.5%, 31.9% ± 5.1%, and 28.3% ± 5.7%, respectively, $p < 0.05$). In addition, values for the VCL, VSL, and VAP of sperm in the 10 million group were all significantly higher than those of sperm in the 15, 18, and 20 million groups ($p < 0.05$), and the values of LIN of sperm in the 10 million group were significantly higher than those of sperm in the 20 million group. Furthermore, sperm in the 10 and 15 million groups had significantly higher STR values than sperm in the 18 and 20 million groups, respectively, whereas sperm in the 10 million group had significantly higher BCF values than sperm in the 15, 18, and 20 million groups ($p < 0.05$), and the percentage hyperactivity of sperm in the 10 and 15 million groups was significantly higher than that of sperm in the 18 and 20 million groups ($p < 0.05$). As shown in Table 2, there were no significant differences among sperm in the different concentration groups with respect to viability or acrosomal membrane integrity after freeze-thawing.

In terms of pregnancy rates at 90 days after AI, whereas we detected was no significant difference between cows inseminated with 10 and 15 million sperm, pregnancy among cows in the 10 million sperm group was found to be significantly lower than that in cows inseminated with 18 and 20 million sperm (10, 15, 18, and 20 million sperm groups: 39.7%, 58.8%, 73.9%, and 67.3%, respectively; $p < 0.05$) (Table 3).

DISCUSSION

In this study, we investigated the effects of different sperm counts on the rates of pregnancy among Hanwoo cows after AI. Similar previous studies have provided evidence to indicate that the rate of pregnancy in artificially inseminated cow differs according to the concentration of the inseminated sperm. To the best of our knowledge, however, this is the first study that in addition to determining pregnancy rates has also sought to assess the effects of sperm concentration on the motility, viability, and acrosomal membrane integrity of freeze-thawed sperm. Accordingly, we believe that our findings in this study will be of particular relevance with respect to the production of frozen Hanwoo semen.

As shown in Table 1, we recorded total motility values of between 78% and 85% for sperm in frozen-thawed semen at four different sperm concentrations, thereby indicating that concentration per se had no significant effect on the motility of sperm. It has previously been demonstrated that increases in sperm VCL, VSL, and VAP contribute to improving the rate of pregnancy in cows inseminated with frozen-thawed semen containing 20 million sperm per straw (Kang et al., 2019), and consistently, AI using sperm

with high VCL, VSL, and VAP levels has been found to enhance to rate of pregnancy in Jersey cows (Perumal et al., 2011). In the present study, we found that compared with cows inseminated with semen at sperm concentrations of 15, 18, and 20 million per straw, there was a notably lower rate of pregnancy in cows inseminated with sperm at a concentration of 10 million per straw, irrespective of the high values VCL, VSL, and VAP in the 10 million sperm group. On the basis of these findings, we speculate that compared with semen concentrations of 15, 18, and 20 million sperm, insemination with fewer sperm is insufficient to achieve pregnancy via AI.

As shown in Table 2, there were no significant differences among the different sperm concentrations with respect to sperm viability or acrosome membrane integrity, which is consistent with the findings of Januskauskas et al. (1996), who observed no significant differences in the motility or viability of sperm at concentrations of 10 and 15 million per straw, and also those of Pickett et al. (1964), who detected no significant differences in the motility or viability of sperm at concentrations of 20 and 30 million. Consequently, we speculate that post-thawing, there would be differences in the quality of sperm at loads of between 10 and 30 million sperm per straw. The rate of pregnancy in artificially inseminated cows has been established to be dependent on both the quality and number of inseminated sperm, and although we found that frozen-thawed sperm at concentrations of 10, 18, and 20 million sperm per straw groups had similar viabilities, the rate of pregnancy in cows inseminated with a concentration of 10 million sperm per straw was notably lower than that in the other assessed groups. Similar findings have been reported by Almquist (1975), who obtained significantly higher rates of pregnancy in cows inseminated with semen at a concentration of 20 million sperm per straw compared with those inseminated at concentrations of 10 and 15 million.

Following the study conducted by Harstine et al. (2018), we also assessed the effect of different freeze-thawed sperm concentrations on pregnancy rate after AI at concentrations of 1.5, 3, 6, 12, and 24 million sperm per straw, and accordingly recorded significantly higher rates of pregnancy among cow inseminated at a concentration of 24 million sperm per straw than in those receiving insemination at concentration of 1.5, 3, and 6 million.

The Hanwoo Improvement Center (NH, Seosan, Korea) produces Korean Proven Bull Numbers (KPN). Traditionally, egg yolk has been used as an additive in the freezing solution for KPN semen production. However, there are reports suggesting that the quality of sperm and post-thaw vitality may improve when using Optixcell instead of deteriorating, as reported with Triladyl. In this study, Optixcell semen diluent was utilized for semen freezing. Optixcell semen diluent utilizes liposomes as cryoprotectants, while Triladyl semen diluent employs egg yolk as its base. Previous studies have compared Triladyl, which is egg yolk-based, with Optixcell, which utilizes liposomes, for freezing bovine semen. Optixcell semen diluent may lead to potential enhancements in post-thaw sperm motility (Kumar et al., 2015; Layek et al., 2016), plasma membrane integrity (Kang et al., 2019), and pregnancy rates (Camus et al., 2016; Kang et al., 2019) compared to Triladyl semen diluent.

When using different numbers of sperm in straws for AI, it is possible to adjust the number of sperm according to the ability of bull semen to pregnancy of a cow. In this regard, it has previously been established that between approximately 20 and 25 million sperm can injected into a straw for freezing without reducing the subsequent rate of pregnancy (Harstine et al., 2018). However, at concentrations of less than 20 million, it has been found that introducing semen into the uterine horn during AI can contribute to enhancing the rate of pregnancy by approximately 5% to 10% (Andersson et al., 2004). Consequently, an adequate concentration of semen above a certain threshold is required to achieve pregnancy via AI.

CONCLUSION

In this study, we investigated the effects of different concentrations of sperm (10, 15, 18, and 20 million sperm/straw) obtained from 10 bulls on the quality of frozen-thawed sperm and the rates of pregnancy achieved following artificial insemination (AI). We accordingly found that freeze-thawed sperm at different concentrations were characterized by similar viability and acrosome membrane integrity, and were generally of a similar overall quality However, compared with cows inseminated with semen at sperm concentrations of 18 and 20 million sperm per straw, those receiving 10 million sperm were characterized by a significantly lower rate of successful conception. Although pregnancy rates among cow in

the 10 million group were found to be lower than those among the 15 million group cows, the difference was not significant. Taken together, our results suggest that a semen concentration of at least 18 million sperm per straw is required to attain a consistent pregnancy rate through AI.

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