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Sex ratio and conception rates of fresh/vitrified embryos at different developmental stages by ovum pick up in Hanwoo cows

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Abstract

Embryo transfer plays a crucial role in enhancing the breeding value of livestock; it has been applied in Hanwoo cattle, which is a popular breed for beef production in Korea. Both *in vivo*-derived (IVD) and *in vitro*-produced (IVP) embryos are used for this purpose; however, IVP embryos have been preferred recently owing to advancements in ovum pick-up (OPU) technology and genomic selection. Despite technological advancements, comprehensive data on large-scale OPU/IVEP/embryo transfer in Hanwoo cows are lacking. In this study, 16 elite Hanwoo donor cows were selected on the basis of specific criteria. Oocytes were retrieved from 241 cows using OPU. The collected cumulus-oocyte complexes (COCs) were matured, fertilized, and cultured *in vitro* to produce transferable embryos. Embryos were classified according to their developmental stage and then transferred to 675 recipient cows. A total of 3,317 COCs were collected, with an average of 13.76 COCs per cow. The number of transferable embryos produced per cow was 3.7. Hanwoo OPU-derived IVP embryos exhibited a higher production yield than the global average, indicating a stable IVEP environment. Both fresh and frozen IVP embryos yielded similar conception rates; hence, the use of vitrified-thawed embryos in transfer plans feasible. However, frozen-thawed embryos at Stage 7 had a lower conception rate than those at earlier stages. There was no significant difference between the conception rates of sexually mature heifers and postpartum cows used as recipients. The male-to-female offspring ratio increased as the developmental stage progressed. Seasonal effects on conception rates were not observed; however, higher abortion rates and a higher proportion of male offspring were observed during winter. This study provides valuable data for the Korean embryo transfer industry, enabling more strategic growth of the domestic Hanwoo embryo industry.

Keywords: Embryo transfer, *In vitro*-produced (IVP) embryos, Ovum pick-up (OPU), Frozen-thawed embryos, Conception rate, Sex ratio

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Ethics approval and consent to participate
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INTRODUCTION

Reproductive performance in cattle breeding is of paramount importance, as it directly influences the efficiency and profitability of livestock operations. In particular, the Hanwoo cattle, renowned for their beef production in Korea, require meticulous breeding strategies to enhance their genetic potential. Embryo transfer (ET) aims to improve the breeding value of livestock more rapidly than artificial insemination [1]. Cattle is a highly popular breed for beef production, and continuous efforts have been made to enhance the meat quality, quantity, and thickness of the preferred beef parts [2]. Embryos for transfer are obtained either *in vivo* or *in vitro* and ET either as fresh or frozen embryos. Numerous studies have been conducted to identify the factors that influence the efficiency and conception rate of this technique [3]. In the field of embryo transfer, both *in vivo*-derived (IVD) and *in vitro*-produced (IVP) embryos are utilized to achieve the same purpose through different methods. Recently, the embryo transfer industry has experienced significant changes in the production and consumption of IVD and IVP embryos, particularly those derived from ovum pick-up (OPU). According to the data from the Embryo Technology Newsletter of the International Embryo Technology Society (IETS) published in 2021, OPU-IVP embryos are produced approximately 3.47 times more than IVD embryos (1,166,034:313,780). The drastic increase in the consumption of IVP embryos resulted in the largest increase in embryo production since 2003 [4]. Moreover, the survival and conception rates of frozen IVP embryos have improved due to the rapid increase in their production since 2015 [4]. Until 2018, IVD embryos were the preferred choice for embryo transfer in Korea because of their stable conception rates, and embryo transfer plans were developed and executed according to the production cycle of each farm [5].

Currently, the embryo transfer industry has seen significant advancements in the utilization of IVP embryos, mainly due to the implementation of OPU technology [6]. The integration of genomic technology, which allows shorter reproductive intervals and more accurate selection, has sparked growing interest in the application of *in vitro* embryo production (IVEP) for commercial purposes. Consequently, the OPU/IVEP program is actively conducting numerous studies on assessing the quality of cumulus–oocyte complexes (COCs) obtained through OPU and enhancing the efficiency of IVEP for a more effective application of this technology [7–10].

Recent advancements in OPU technology and genomic selection have shifted the preference towards IVP embryos. Before 2010, the conception rate of IVP embryos lagged behind that of IVD embryos. However, as the culture environment and freezing technology improved, the conception rate of IVP embryos has become similar to that of IVD embryos [11–14]. Consequently, the use of OPU in Korea has significantly increased to meet the increasing demand for IVP embryos [4]. However, despite the wide application of OPU/IVEP, comprehensive data on large-scale OPU/IVEP/embryo transfer are insufficient [15]. At present, a more efficient method of producing and transferring IVP embryos should be applied to reduce the imbalance between the growing demand for embryo transfer in Hanwoo cattle and the insufficient supply of high-capacity embryos, similar to the global trend in both in the embryo production and transfer [16].

The aim of this study was to investigate the conception and abortion rates and the offspring sex ratio that resulted from the transfer of fresh and frozen IVP embryos in Hanwoo cattle. To ensure reliable results, a sufficient number of recipient cows were utilized to minimize confounding variables that may have arisen from the production of IVP embryos in different laboratories. The findings of this study will serve as valuable data for further improvements/advancements in the bovine embryo transfer, especially in Hanwoo cattle and the Korean embryo transfer industry.

MATERIALS AND METHODS

Selection of donor cows for commercial utilization in the embryo transfer industry

Specific criteria, including body weight exceeding 550 kg, sirloin cross-section of at least 130 cm^2, and a marbling score of 1++ (meat quality index 9) or higher based on outstanding slaughter performance, were referred to in selecting Hanwoo donor cows. Additional evaluations were conducted through ultrasound examination of the reproductive tract to identify the cows with normal ovarian cycles in this elite group. The 16 elite Hanwoo donor cows that obtained negative results for four key disease tests were selected for OPU; this final selection step was conducted to ensure the health and suitability of donor cows for embryo transfer programs. The OPU procedure took place in 10 sessions from April to June and 6 sessions from October to November each year (2019–2022). During the OPU sessions, oocytes were collected from the selected donor cows to facilitate embryo transfer. The OPU sessions were carefully planned and executed to ensure that only viable oocytes were collected for the successful embryo transfer in Hanwoo cows.

Procedures for oocyte retrieval under ultrasound guidance

In this study, oocyte retrieval procedures were performed on Hanwoo cows under ultrasound guidance. Before each procedure, the cows were placed in a frame to ensure restraint and their rectums were cleared of feces. Proper hygiene was maintained by thoroughly cleaning their external genitals with 70% ethanol. To ensure the safety and comfort of the human researchers and animal test subjects, epidural anesthesia (2% lidocaine hydrochloride, Lidovet, Bravet, Brazil) was administered to the donor cows. A migration-type scanner transducer (BLUE, Draminski Tech, Olsztyn, Poland) assembled into a vaginal handle with a stainless-steel needle guide (20 G; 0.9 × 50 mm, Terumo Europe, Leuven, Belgium) was used to facilitate oocyte retrieval. All follicles ≥2 mm were carefully aspirated. A 20-G disposable hypodermic needle (Agulha com Rosca injetada-20 g, Watanabe Tecnologia Aplicada, Cravinhos, Brazil) was used for follicular puncture. Throughout the retrieval of oocytes from both ovaries of the donor cows, negative pressure for aspiration was maintained between 38 and 52 mmHg using a vacuum pump (BV-003, Watanabe Tecnologia Aplicada). BO-IVF (IVF Bioscience, Cornwall, UK) was used as the perfusate for aspiration. Successful recovery of the follicular contents was achieved using a 120-cm-long tube with a 1.1 mm inner diameter (Watanabe Tecnologia Aplicada). The meticulous and controlled oocyte retrieval process ensured the acquisition of viable oocytes for further use.

In vitro maturation, fertilization, and culture of embryos from oocytes

The selection process focused on the COCs with more than three layers of cumulus cells and an evenly distributed cytoplasm. For *in vitro* maturation, the COCs were cultured in 450 μL of TCM-199 medium for 22 h. The medium consisted of 0.005 AU/mL follicle-stimulating hormones (FSH; F2293, Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (FBS; GIB16000-044, Thermo Fisher Scientific, Waltham, MA, USA), 1 μg/mL 17β-estradiol (E4389; Sigma-Aldrich), and 100 μM cysteamine (M6500; Sigma-Aldrich). The cultures were meticulously maintained in a humidified atmosphere with 5% CO_2 at 38.5℃.

Subsequently, the Percoll gradient technique was employed to purify spermatozoa from thawed semen straws. The spermatozoa were purified through density-gradient centrifugation on a Percoll discontinuous gradient (45%–90%) at 1,500 rounds per min (rpm) for 15 min. The Percoll density gradient was prepared by layering 1 mL of 45% Percoll solution onto 1 mL of 90% Percoll solution in a 15-mL conical tube. After centrifugation, the pellet was washed twice with capacitation Tyrode's medium base, albumin, lactate, and pyruvate (TALP) and centrifuged for 5 min at 1,500

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rpm. The motile spermatozoa from the pellet were carefully added to the droplets containing mature oocytes. The oocytes were inseminated on Day 0 with 1–2 × 10^6 spermatozoa/mL for 18 h in an IVF-TALP medium (NO-100, Nutricell, Sao Paulo, Brazil) with mineral oil. All oocytes were carefully maintained in a humidified atmosphere with 5% CO_2 at 38.5℃.

Following successful fertilization, the oocytes were denuded and cultured in a two-step chemically defined culture medium; the oocytes were cultured for 5 days in the early stage medium and 2 days in the later-stage medium. The oocytes in both media were maintained at 38.5℃ in an atmosphere with 5% O_2, 5% CO_2, and 90% N_2. This controlled environment allowed embryo development during the subsequent stages of the study.

Blastocyst vitrification and warming procedure

Blastocyst vitrification was conducted on the 7th day, following the established protocols. The entire procedure was conducted in a clean room with a temperature of 32℃, and a heated surface at 37.5℃ was used to ensure optimal conditions. During vitrification and warming, the embryos were handled using a holding medium (HM) consisting of TCM199 (Gibco, Billings, MT, USA) with HEPES and 20% fetal calf serum (FCS).

For vitrification, the blastocysts were initially exposed to a solution of 10% ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO) (vitrification solution 1; VS1) for 3 min. Subsequently, they were transferred to a well containing a solution of 20% EG + 20% DMSO + 0.5 M sucrose (VS2) for 45 s. The loaded blastocysts were then placed into a cryotop device containing 0.2 μL of VS2 and immediately submerged in liquid nitrogen for storage.

For the warming process, the pulled end of a straw was directly immersed in 1.2 mL of 0.25 M sucrose in HM. After 5 min, the blastocysts were transferred to 0.15 M sucrose medium in HM for an additional 5 min. Afterwards, they were washed twice with HM solution. After the warming process, the blastocysts were washed with the later-stage culture medium and transferred to a well containing the same medium. These careful steps were taken to ensure successful vitrification and subsequent warming of the blastocysts for further use in the study.

Embryo transfer

The Hanwoo recipients, with an average age of 47.3 ± 1.82 months and an average parity of 2.7 ± 0.13, exhibited an average body condition score (BCS) of 3.2 ± 0.3 on a scale ranging from 1 to 5, where 1 indicates very thin and 5 indicates very fat. The embryos were classified according to the IETS standard and their developmental stage and grade on Days 7 and 8 [17]. Depending on the number of recipients and transferable embryos available on Day 7, fresh or frozen-thawed embryos at IETS Stages 4–7 and Grade 1 were selected for transfer. The recipient cows selected for embryo transfer were either nulliparous heifers, with a normal estrous cycle and aged 14–24 months, or multiparous cows at 60–90 days postpartum. Fresh (n = 366) and frozen-thawed embryos (n = 309) derived from OPU were transferred to a total of 675 recipient cows. To ensure a safe calving process the following year, the majority (86.2%) of the transferred embryos were transferred between the months of May and November, which were preferred by farmers. To induce the emergence of a new follicular wave regardless of the estrous cycle, 1.9 g of progesterone (EAZI-BREED™ CIDR®, InterAg, Hamilton, New Zealand) was intravaginally inserted, and 2.0 mg of intramuscular estradiol benzoate (Esrone, Samyang Anipharm, Seoul, Korea) was intramuscularly injected simultaneously on Day 0. On Day 6, CIDR was removed, and 500 μg of prostaglandin F2α (PGF2α, Synchromate, Pfizer, Manhattan, NY, USA) and 300 IU of pregnant mare serum gonadotropin (PMSG, Merck & Co., Rahway, NJ, USA) were intramuscularly injected. Estrus was detected 2.5 days after PGF2α and PMSG injections; 200 mg of GnRH (Fertagyl®, Merck &

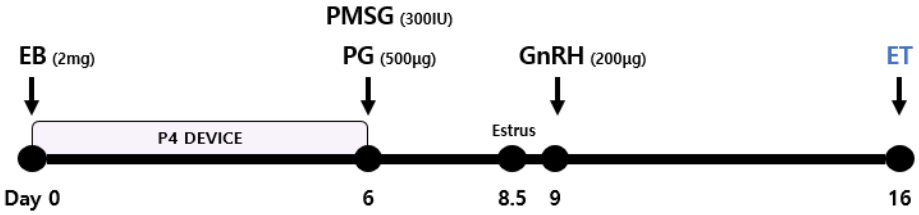

Fig. 1. Estrus synchronization schedule. Estrus synchronization schedule of recipient cow for embryo transfer. PMSG, pregnant mare's serum gonadotropin; EB, estradiol benzoate; GnRH, gonadotropin hormone-releasing hormone; ET, embryo transfer.

Co.) was intramuscularly injected 12 h after estrus detection (Fig. 1). Non-surgical embryo transfer was performed 7.5 days after estrus detection, following the administration of epidural anesthesia with 5 mL of 2% lidocaine. A fresh or frozen-thawed IVP embryo was transferred using a sterile 133-mm straw (IMV Technologies, L'Aigle, France) into the uterine horn with the presence of the corpus luteum, as confirmed by rectal palpation and ultrasonography.

Pregnancy detection and sex investigation

To diagnose pregnancy, cows aged 40–50 days after the embryo transfer underwent ultrasonography. Data on the delivery and sex of the offspring were collected from delivery records 1 year after ET. The conception rate was defined as the number of pregnancies per ET and the parturition rate was defined as the number of calves per ET. These parameters were used to evaluate the success and effectiveness of the embryo transfer program in Hanwoo cows.

The conception and parturition rates were compared between fresh and frozen-thawed embryos and between nulliparous and multiparous Hanwoo cow recipients. The conception and parturition rates at developmental stages 4, 5, 6, and 7 were analyzed. Seasonal variations on the conception and parturition rates following the transfer of IVP embryos in Hanwoo cows were also analyzed, and the seasonal sex ratios of Hanwoo calves born after IVP embryo transfer were examined.

Statistical analysis

Data were analyzed using chi-square test, and differences among groups were considered significant at $p < 0.05$.

RESULTS

Oocyte retrieval through ovum pick-up and production of transferable embryo

OPU was highly efficient in oocyte retrieval; a total of 3,317 COCs were collected from 241 open cows, and an average of 13.8 COCs were retrieved per head (Table 1). IVEP yielded 890 transferable embryos, with an average of 3.7 embryos per head (Table 1). This indicates a successful and productive outcome in generating transferable embryos suitable for subsequent embryo transfer procedures.

Conception and parturition rates after the transfer of fresh and frozen-thawed embryos from *in vitro*-produced embryos

The conception rates of fresh and frozen-thawed embryos were 50.6 and 51.8%, respectively (Table 2); there was no significant difference between the conception rates of the two embryo conditions

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Table 1. Hanwoo OPU recovered oocytes and transferable embryos

No. of donor cow	No. of recovered COCs (/donor)	No. of transferred embryos (/donor)
241	3,317 (13.8)	890 (3.7)

OPU, ovum pick-up; COCs, cumulus–oocyte complexes.

Table 2. Pregnancy and delivery rate of fresh and frozen embryos

Embryo status	No. of transferred embryos	No. of recipient (%)	
		Pregnant	Delivered
Fresh	366	185 (50.6)	158 (43.2)
Frozen	309	160 (51.8)	137 (44.3)

$P = 0.987115$.

Statistical significance established at $p < 0.05$.

($p = 0.987115$). Regarding parturition rates, 43.2% of fresh embryos and 44.3% of frozen-thawed embryos resulted in successful deliveries (Table 2). Similar to the conception rates, the parturition rates did not significantly differ between fresh and frozen-thawed embryos, indicating comparable success in achieving delivery for both conditions.

Conception and parturition rates at different developmental stages after *in vitro*-produced embryo transfer

The analysis of the conception and parturition rates at different developmental stages revealed no significant difference in conception and parturition rates, while interesting trends observed as the developmental stages progressed. The conception rates of Stages 4 and 5 embryos were 52.9% and 51.9%, respectively. Table 3 shows that as the embryos progressed to Stage 6, the conception rate declined to 49.3%; at Stage 7, it decreased further to 49.1%. This trend indicates that the conception rate decreased as the developmental stage advanced. Parturition rates followed a similar pattern. The parturition rates of Stages 4 and 5 embryos were 45.9% and 46.8%, respectively. At Stage 6, the parturition rate declined to 41.7% (88/211), and at Stage 7, it further declined to 40.1% (80/222). Among the four stages, the lowest parturition rate was recorded at Stage 5, while the lowest was at Stage 7 (Table 3). Despite these observed trends, statistical analysis revealed no significant difference among the conception and parturition rates at different stages of embryo development ($p = 0.2255$, Table 3). Upon further examination of fresh embryos, Stages 5, 6, and 7 embryos exhibited relatively similar conception rates (50.6%–50.9%). However, Stage 4 embryos had a slightly lower conception rate (48.6%). In contrast, the conception rates of frozen-thawed embryos displayed a declining trend as the developmental stage progressed. The highest conception rate was observed at Stage 4 (62.0%), while the lowest was at Stage 7 (46.8%). The parturition rate exhibited a similar tendency as the conception rate for both fresh and frozen-thawed embryos (Fig. 2).

Seasonal conception and parturition rates after *in vitro*-produced embryo transfer

Interesting trends were observed on the effects of different seasons on the success rate of embryo transfer. During spring, the conception rate for embryo transfer was 43.0%. As the season transitioned to summer, the conception rate notably increased to 52.5%. A relatively high conception rate at 51.9% was also observed during fall; however, it decreased to 42.9% during winter. The highest conception rate was observed in summer; lower conception rates were observed in spring and winter (Table 4). The parturition rate followed a similar trend across the different seasons. The highest parturition rate (46.8%) was recorded in summer, indicating successful

Table 3. Pregnancy and delivery rate of Hanwoo IVP fertilized eggs by stage

Embryo stage	No. of transferred embryos	No. of recipient (%)	
		Pregnant	Delivered
4	85	45 (52.9)	39 (45.9)
5	158	82 (51.9)	74 (46.8)
6	211	104 (49.3)	88 (41.7)
7	222	109 (49.1)	89 (40.1)

P = 0.2255.

Statistical significance established at $p < 0.05$.

IVP, *in vitro*-produced.

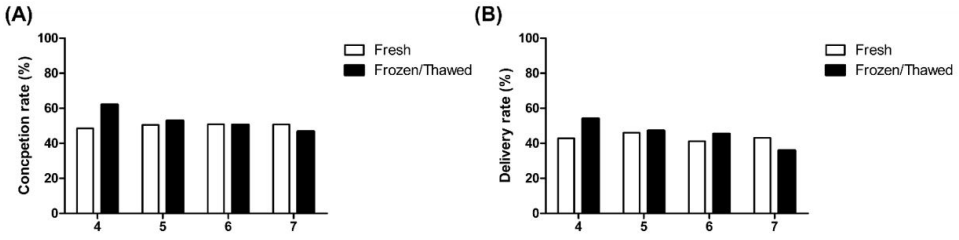

Fig. 2. Conception rate and delivery rate by stage. (A) The conception rate of embryo transfer by stage of fresh and frozen/thawed embryos in Hanwoo cows. *P* = 0.651216. Statistical significance established at $p < 0.05$. (B) The delivery rate of embryo transfer by stage of fresh and frozen/thawed embryos in Hanwoo cows. *P*= 0.554952. Statistical significance established at $p < 0.05$.

Table 4. Seasonal conception and delivery rates of Hanwoo IVP fertilized embryos

Season	No. of transferred embryos	No. of recipient (%)	
		Pregnant	Delivered
Spring	62	26 (41.9)	18 (29.6)
Summer	528	277 (52.5)	247 (46.8)
Autumn	54	28 (51.9)	21 (38.9)
Winter	28	12 (42.9)	7 (25.0)

P = 0.66695.

Statistical significance established at $p < 0.05$.

IVP, *in vitro*-produced.

pregnancies and deliveries, followed by those in fall (38.9%) and spring (30.7%). The lowest parturition rate (25.0%) was recorded in winter. Although seasonal variation in conception and parturition rates observed, statistical analysis revealed no significant differences in the conception and parturition rates by season (Table 4).

Comparison of conception and parturition rates between nulliparous and multiparous recipient

Interesting insights can be gleaned on the effect of age on conception and parturition rates. The conception rate of nulliparous individuals was 45.7%, while that of multiparous individuals was higher at 54.2%. However, there was no significant difference between the two groups, suggesting that age at ET alone may not be a major contributing factor to conception success in Hanwoo cattle (Table 5). Furthermore, the parturition rate of the nulliparous recipients was 40.2%, while that of the multiparous recipients was slightly higher at 45.6%. Similar to the conception rate, the

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Table 5. Comparison of conception rates by recipient cow status of Hanwoo

Recipient cow	No. of recipient		
	Transferred	Pregnant (%)	Delivered (%)
Nulliparous	127	58 (45.7)	51 (40.2)
Multiparous	502	272 (54.2)	229 (45.6)

$P = 0.837463$.

Statistical significance established at $p < 0.05$.

parturition rates of the two groups did not significantly differ, indicating that the age at ET may not be a determining factor for successful parturition in Hanwoo recipients (Table 5).

Sex ratio of calves after *in vitro*-produced embryo transfer by developmental stage

The distribution of male and female calves across different developmental stages revealed interesting patterns, indicating a potential relationship between developmental stage and sex determination. At Stage 4 of embryo development, the male-to-female ratio was 2.1:7.8, with thirty-three male (21.4%) and six female calves (78.6%) born. At Stage 5, the male-to-female ratio became 4.5:5.4, with 33 male (45.2%) and 40 female calves (54.8%) born. At Stage 6, the increasing trend of male-to-female ratio continued, with 52 male (57.1%) and 39 female calves (42.9%) born. Finally, at Stage 7, the male-to-female ratio further increased to 6.8:3.1, with 61 male (68.5%) and 28 female calves (31.5%) born (Fig. 3). Fig. 3 clearly shows that the sex ratio became more biased towards males as the developmental stage progressed.

Seasonal sex ratio of calves after *in vitro*-produced embryo transfer

The male-to-female ratio varied across seasons; the sex ratio became more biased towards in later seasons. The male-to-female ratio was equal in spring with 10 male (50.0 %) and 10 female (50.0 %) calves. During summer, the male-to-female ratio remained slightly male-biased, with 128 male (51.8%) and 119 female calves (48.2%) born. In fall, the male-to-female ratio exhibited a further increase in male bias, with 12 male calves (57.1%) and 9 female calves (42.9%). Finally, in winter, the male-to-female ratio showed a highly male-biased distribution with five male (71.4 %) and two female (28.6 %) calves born (Fig. 4).

DISCUSSION

We conducted this study to assess the current status of embryo production and transfer in Hanwoo

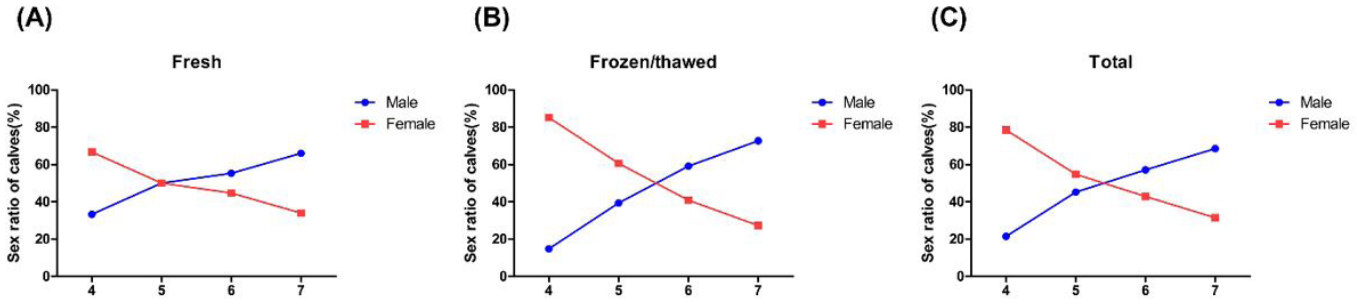

Fig. 3. IVP embryo status (fresh vs. frozen/thawed) and sex ratio by stage. (A) Sex ratio of calves using fresh embryos. (B) Sex ratio of calves using frozen/thawed embryos. (C) Result of sex ratio of total calves. IVP, *in vitro*-produced.

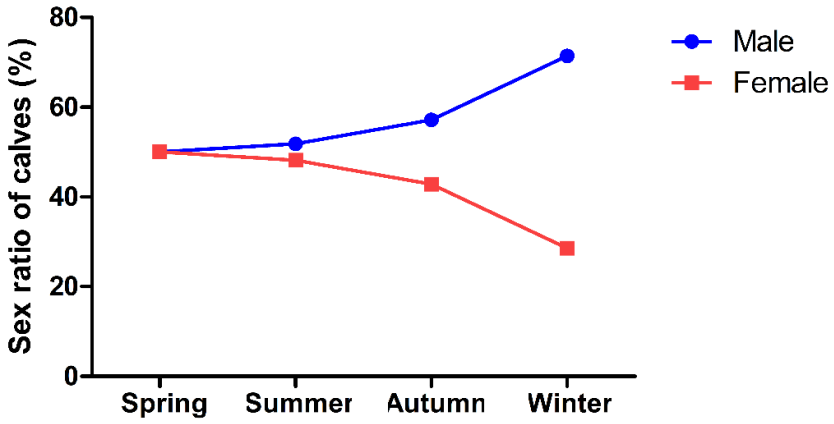

Fig. 4. The sex ratio of Hanwoo calves by season.

cows by repeatedly collecting oocytes from 16 high-capacity Hanwoo donor cows. The resulting embryos were then transferred to 675 recipient cows. The results we obtained by examining the distinction among recipient cows and embryos used in the *in vitro* fertilization (IVF) process, focusing on conception rates and number of successful births, are discussed in the succeeding paragraphs.

Compared with the IETS statistics, the COCs recovery rate (13.8) recorded in this study is approximately 4.6 less than the global average COCs recovery rate (18.4); however, we recorded more transferable embryos per head (3.7) than the global average (2.7) [4]. Our findings highlight the significance of OPU in facilitating oocyte collection and subsequent embryo production. Furthermore, our findings demonstrate the potential of OPU and IVEP techniques for enhancing genetic advancement in cattle breeding through efficient reproduction and embryo transfer procedures.

In *Bos indicus*, an average of 18–25 COCs per head were recovered using OPU [9,18]. On average, *B. indicus* has more follicular waves and follicles larger than 5 mm than *B. taurus*. Moreover, *B. indicus* has a higher COCs recovery rate than *B. taurus* [19]. This explains the lower average COCs recovery rate observed in this study because Hanwoo cows (*B. taurus coreanae*) were the animal subjects in this study. Efficient transferable embryo production can be attributed to a stable laboratory environment.

To determine which age group of recipient Hanwoo cows would yield better conception rates after embryo transfer, we selected sexually mature heifers and postpartum cows aged between 60 and 90 days. Generally, sexually mature heifers are considered more suitable for embryo transfer because they experience less nutritional stress, uterine damage, and other reproductive issues than cows with a history of three or more calvings, which may have reduced fertility [20,21]. In dairy cows, a comparison between sexually mature heifers and cows that had calved revealed slightly higher conception rates among sexually mature heifers; however, the difference between the two groups was not statistically significant [22]. Among the Hanwoo recipients in this study, multiparous cows had higher conception rates and exhibited a higher rate of embryonic loss than nulliparous cows. However, the differences between the two age groups were not statistically significant. Further research with larger sample sizes is warranted to gain a deeper understanding of the various factors affecting reproductive outcomes in Hanwoo cattle recipients. The findings of such research will aid in the development of effective breeding strategies and management practices to optimize the reproductive performance of this valuable cattle breed.

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In our previous study on IVD embryo transfer in Hanwoo cattle, we found that the conception rate of fresh embryos was higher than that of frozen embryos; however, the difference was not statistically significant [5]. Additionally, the abortion rate of frozen-thawed embryos was 3% higher than that of fresh embryos (21.1% vs. 18.2%). In this study, however, the conception rates of fresh (50.6%) and frozen-thawed (51.8%) embryos after IVP embryo transfer were almost similar. The abortion rates were also comparable (14.6% vs. 14.9%). Moreover, there were no significant differences in the conception and abortion rates between the two embryo conditions. Such a finding is consistent with a study of conception rates in fresh and frozen thawed embryos from IVD embryos by Hasler et al. [23], but contrary to other research findings [24,25]. We speculate that the use of FBS-free culture media and the rapid freezing technique positively influenced the survival rate of embryos during the freezing process. This implies that using frozen-thawed embryos is as effective as using fresh embryos in achieving successful conception and parturition rates. Moreover, using frozen-thawed embryos is more advantageous in preserving and storing embryos for future cattle breeding programs.

Several studies have shown that the stage of embryonic development does not affect conception rates [26,27]. However, other studies reported higher conception rates at Stages 5 and 6 than at Stages 4 and 7 [28]. Putney et al. observed the lowest conception rate at Stage 4; they also reported that the conception rate increases as the embryonic development stage progresses [29]. In this study, the conception rate decreased from 52.9% to 49.1% as the embryos developed from Stage 4 to 7; however, there was no significant difference among the stages. Such a findings confirms that the stage of embryonic development does not significantly affect conception rates [26,27].

However, the embryonic development stage affects conception rates when frozen-thawed embryos are transferred. In fresh IVP embryos, the average conception rate for all developmental stages was 51.1%, indicating similar conception rates across all stages. However, the conception rates of frozen-thawed embryos varied with the developmental stages; as the developmental stages progressed, the conception rates decreased. Similarly, the conception rate of frozen-thawed IVD embryos decreased as the developmental stages progressed [5]. This trend was similar for both IVP and frozen-thawed embryos, indicating a consistent pattern across the different developmental stages. However, the conception rate observed at Stage 7 is noteworthy. The conception rate of IVD embryos subjected to slow-freezing at Stage 7 significantly decreased by over 20% compared with that at Stage 6 embryos (47.8% vs. 20.0%). In contrast, the conception rate of vitrified IVP embryos at Stage 7 slightly decreased by 4% compared with that at Stage 6 (50.5% vs. 46.7%). In general, fresh embryo transfer yielded better conception rates. However, the conception rates of frozen-thawed embryos vary widely depending on the laboratories involved in embryo production [30]. Overall, we demonstrated that the conception and parturition rates of Hanwoo cows can be influenced by the developmental stage of embryos after IVP. However, no statistically significant differences were found, indicating that all analyzed stages remain viable options for successful embryo transfer in Hanwoo cow breeding programs.

Slow freezing and vitrification are the most commonly used procedures for embryo cryopreservation. Both cryopreservation methods prevent ice crystal formation, oxidative stress, osmotic shock, and cytotoxicity of cryoprotectants [31–33]. Embryos cryopreserved by vitrification have higher conception rates after embryo transfer than embryos cryopreserved by slow freezing [34–36]. In this study, the conception rates of frozen-thawed embryos obtained through vitrification were consistently over 50% at all developmental stages, except at Stage 6 (46.7%). However, the abortion rate at Stage 7 was nearly double that at Stages 4, 5, and 6. Although it has been confirmed that more high-quality IVP embryos can be produced and preserved now than in the past, it is recommended to freeze embryos up to Stage 6 when selecting embryos for freezing

and preservation. Vitrification is believed to operate more effectively because of its core principles of creating smaller and fewer ice crystals.

To preserve embryos by freezing, the quality and developmental stage of the embryos are critical factors to consider. The classification of IVP embryos relies on their developmental speed, which has been shown to be correlated with higher conception and hatching rates after warming [37,38]. Lower-quality IVP embryos exhibit reduced freezing resistance compared with *in vivo* embryos [34]. Thus, strict morphological selection is a crucial factor for successful conception rates after IVP embryo transfer using freezing protocols, and developmental speed can also be a determining factor for classifying such embryos [35,36]. In addition to the kinetics of development, male and female embryos differ in their metabolism, gene expression, and stress responses. Leme et al. [39] indicated a clear relationship between cryotolerance and embryo quality, which can be evaluated based on the developmental speed. Therefore, selecting embryos of the highest quality for freezing may induce a bias towards male embryos. In this study, the proportion of male IVP frozen embryos increased as the developmental stages progressed. Such findings are consistent with other studies claiming that male embryos develop faster than female embryos in IVEP, and male embryos have better morphology and higher vitrification survival rates [40]. A noticeable disparity was observed, with a considerably lower number of female embryos successfully advancing from the morula/early blastocyst stage to more advanced developmental stages [41,42]. These discrepancies in the developmental progress between male and female embryos could potentially reflect the phenomena that naturally occur *in vivo*, offering adaptability to embryo selection during early pregnancy [43–45]. However, Larson et al. [41] reported that more female embryos reached the morula and blastocysts stages on D6. This study demonstrated a notable relationship between the developmental stage of embryos and the male-to-female ratio of calves born through IVP embryo transfer in Hanwoo cows. The sex ratio progressively favored males as the embryos advanced in development. This suggests the presence of sexual dimorphism and varying blastocyst tolerance between the sexes. This finding is consistent with that of Pegoraro et al. [46], who similarly observed a higher proportion of male than female embryos during co-culture with feeder cells. In addition, Mittwoch [47] demonstrated that XY embryos tend to exhibit faster growth than XX embryos *in vitro*. Furthermore, male blastocysts were predominant over female blastocysts when the embryos were cultured singly *in vitro* [48]. Additionally, the male-to-female ratio exhibited a distinct difference between fresh and frozen embryos. These findings contribute to our understanding of sex determination in bovine reproduction and may have implications for the breeding strategies appropriate for Hanwoo cows. Further research is required to elucidate the mechanisms underlying sex determination and the influence of freezing on sex ratios.

One of the most critical effects of heat stress in the livestock industry is the decline in the reproductive performance of cows. Heat stress caused by elevated body temperature can disrupt the function of the ovaries and uterus, leading to early embryonic death [45,49–51]. The increase in the body temperature of recipient cows may have various effects on hormone secretion, embryonic development, and other aspects related to pregnancy [52]. Oocytes are sensitive to various stressors before conception, and early embryonic loss mainly occurs during the preimplantation stage. While the blastocyst stage is more developed and less sensitive to temperatures approximately 40℃–42℃ than the 1–8 cell-stage embryos, a lower number of cells—lower-stage or larger code oocytes—has a greater impact on conception [53]. Therefore, embryo transfer that bypasses the heat-sensitive stages (oocyte maturation, fertilization, and early embryo stages) is the most promising technique for improving the low conception rates caused by artificial insemination during summer [54]. In this study, a stable conception rate of approximately 50% was observed during summer and fall, which is consistent with the findings of Hasler et al. [23]. The average summer temperature in

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Korea is below 30℃, and temperatures approximately 10℃ lower than those used in heat stress experiments (above 40℃) do not significantly affect embryo transfer [55]. Moreover, during spring and winter, a conception rate of approximately 40% was observed, which was approximately 10% lower than the conception rates during summer or fall. The abortion rate during winter was 41.7%, which was 1.5–3.8 times higher than that in other seasons. These findings suggest that seasonal variations influence the conception and parturition rates in Hanwoo cows following IVP embryo transfer. The higher conception and parturition rates observed during summer indicate that this period may be more favorable for successful embryo transfer and reproduction in cattle breeding programs. However, statistical analysis did not show any significant differences, indicating that successful embryo transfer and parturition are possible throughout the year. However, further research is required to elucidate the underlying factors contributing to these seasonal differences and optimize reproductive strategies for cattle breeding programs.

The sex ratio varied seasonally; the male-to-female ratios observed in spring and summer were similar. According to Roche, the climate during the month before embryo transfer can influence the sex ratio, and higher temperatures during that period result in a higher probability of male calves being born [56]. However, other research findings indicate that the reproductive season does not influence the sex ratio of the offspring of cows [57]. To assess the statistical significance and reliability of the observed differences in sex ratios among the seasons, it is important to note that the number of samples for each season was relatively small. Owing to this limitation, it is challenging to arrive at definitive conclusions and infer statistically significant differences between the male-to-female ratios in different seasons.

In conclusion, we validated that Hanwoo OPU-derived IVP embryos have a production yield 1.4 times higher than the global average, establishing a stable IVEP environment. There was no significant difference in conception rates based on the age of the recipient cows for embryo transfer, although a slightly higher abortion rate was observed in cows aged 60 months or older. This finding confirms that there is no significant age-related difference in embryo transfer success in Hanwoo cattle. Additionally, there was no difference in conception rates between fresh IVP and vitrified-thawed embryos, allowing for the routine use of vitrified-thawed embryos in embryo transfer plans. Furthermore, as the developmental stage of the embryos increased, the likelihood of having male offspring also increased. Although conception rates did not vary by season, higher resorption rates were observed during winter. Additionally, during fall and winter, a higher proportion of male offspring was born than female offspring. The findings of our study contribute to the more effective and strategic growth of the domestic Hanwoo embryo industry in Korea.

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