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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Improved pregnancy rate and sex ratio in fresh/frozen in vivo derived embryo transfer of Hanwoo ( <i>Bos taurus coreanae</i> ) cows
Running Title (within 10 words)	Pregnancy rate and sex ratio in fresh/frozen IVD embryo transfer
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## Abstract

This study aimed to assess the effects of embryonic developmental stage, quality grade, and fresh or frozen/thawed conditions on the pregnancy rate and sex ratio of live offspring in Hanwoo (*Bos taurus coreanae*) cows. The quality and developmental stage of *in vivo*-derived (IVD) transferred embryos were evaluated using the standard criteria of the International Embryo Technology Society. The recipient cows were synchronized using conventional (estradiol benzoate and progesterone) protocols before embryo transfer. Embryos were transferred to 297 cows, and pregnancy was monitored for 60–70 days after embryo transfer. The pregnancy rates of fresh and frozen/thawed embryos were 56.90% and 52.49%, respectively. Pregnancy rates varied according to embryo quality (56.18% for grade 1 vs. 36.67% for grade 2). Pregnancy rates also varied by developmental stage and cryopreservation (67.86% vs. 63.49% for stage 4-1, 64.00% vs. 54.72% for 5-1, and 50.00% vs. 47.83% for 6-1, in fresh embryos vs. frozen/thawed embryos, respectively). For stage 7-1, the pregnancy rates were 72.73% for fresh embryos and 20.00% for frozen/thawed embryos. In 66 fresh embryos, the sex ratio of live offspring was 5:5, whereas it was 4:6 for frozen/thawed embryos among the 95 frozen/thawed embryos. The miscarriage rate was approximately 3% higher for frozen/thawed embryos than for fresh embryos. Seasonal fertility rates were 55% and 57% for fresh and frozen/thawed embryos in spring, 63.08% and 53.57% in summer, and 68.42% and 66.67% in autumn, respectively, with the highest rates observed in autumn (72.73% vs. 27.27%). The current data revealed no significant differences in pregnancy rates between fresh and frozen/thawed IVD embryos. However, there was a lower pregnancy rate with advanced-stage frozen/thawed embryos (stage 7-1). The current study provides comprehensive results for the better optimization of embryo transfer in Hanwoo cattle to obtain the desired fertility rate, pregnancy rate, and sex ratio of calves. These results provide important insights into the factors that influence the viability and success of IVD embryo transfer in Hanwoo cows and may have practical applications for improving breeding programs and reducing production costs.

**Keywords:** Hanwoo; embryo transfer; *in vivo* derived embryo; pregnancy rate; miscarriage rate

## Introduction

Embryo transfer in cattle can accelerate the rate of genetic improvement compared to artificial insemination, particularly through the breeding of high-value female donors [1, 2]. In Hanwoo cattle (*Bos taurus coreanae*), embryo transfer is performed for commercial purposes to enhance the quality and quantity of beef meat, with males typically raised for fattening and females for breeding [3, 4]. With the accelerating development in embryo transfer technologies, understanding their impact on pregnancy rates and the male-female sex ratio remains an important area of research to improve the livestock industry.

Numerous factors can influence the success of embryo transfer, with the quality and developmental capacity of the embryos being the most critical. Achieving a high-quality embryo, technically skilled embryo transferers, and specifically managing the recipient can greatly increase the chances of successful pregnancy to around 70–80% [5, 6]. The sex ratio of the offspring can also be affected by various characteristics of the embryo, including developmental rate, freezing resistance, and the ability to maintain pregnancy after embryo transfer [7].

The commercial availability of frozen/thawed embryo technology began in the early 1980s, and a significant improvement was achieved in the 1990s using ethylene glycol instead of glycerol as a cryoprotectant [8, 9]. This allowed for the transplantation of thawed embryos under field conditions instead of depending on the laboratory microscope. However, the freezing process exposes embryos to various stresses that can negatively affect their viability and, consequently, the pregnancy rate [10, 11]. The developmental stage and quality of the embryo also affect pregnancy rates. It is important to use frozen/thawed embryos for efficient breeding programs and timed parturition in farms and to consider the developmental stage of the embryos when selecting embryos for transfer. Embryo survival after transfer is a critical factor in the productivity and profitability of cattle breeding. Premature embryo death and abortion can lower pregnancy rates and increase calving-to-pregnancy intervals, leading to higher production costs and financial losses [12–14]. Therefore, it is necessary to monitor pregnancy rates in relation to the conditions and developmental stages of the embryos.

In Angus beef cows, the pregnancy rates of *in vivo*-derived (IVD) fresh embryos were higher than those of frozen/thawed embryos (53% vs. 44%, respectively). However, there was no significant difference between grades 1 and 2 (57% and 56% respectively) [1]. In Holstein cows, there was no difference in pregnancy rates between stages 4 to 7 and grades 1 and 2; however, in another study [15], cows with grade-1 embryos had a higher pregnancy rate than cows with grade-2 embryos, with no significant difference in pregnancy rates among different stages of grade-1 embryos. Heat shock (40°C)-exposed sperm were found to decrease embryonic development and the male ratio [16, 17]. The effect of temperature during semen collection on sex ratio during embryo production should also be considered. Moreover, male embryos show rapid development and strong freezing resistance compared with female embryos [18]. Additionally, midsummer heat stress reduces feed intake, increases the negative energy

balance, induces changes in ovarian follicular dynamics, reduces the estrus detection rate, and changes fallopian tube function, leading to fertilization failure and early embryo death [19]. However, on the other hand, the pregnancy rate with embryo transfer is higher than artificial insemination under these stressful conditions [20]. Conversely, frozen/thawed embryos are more sensitive to heat stress and develop slower when exposed to heat than fresh embryos [21]. During a heat wave in Korea in August 2018, a temperature of 41°C was recorded in Hongcheon-gun, Gangwon-do, South Korea. In dairy cows, the artificial insemination rates are 55.6% in the summer and 87.8% in the cool season.

Therefore, it is necessary to study the differences in pregnancy rates and sex ratios according to developmental stage, quality grade, and fresh and frozen/thawed IVD embryos in Hanwoo cows to improve the efficiency and success of breeding programs. Additionally, the effect of ambient temperature on embryo transfer is an important consideration for maximizing pregnancy rates and minimizing early embryo death.

## Materials and Methods

### Preparing donor cows

Hanwoo heifers raised in cattle breeding farms in Hongcheon-gun, Gangwon-do, South Korea with a carcass weight of 500 kg or more, backfat thickness of 120 cm<sup>2</sup> or more, marbling score of 1 + + or more, and 60 days since calving underwent rectal examinations. Heifers with healthy reproductive organs and negative test results for the four major diseases (Leukosis, Brucellosis, John's D., and Tuberculosis) were selected (68 cows).

### Estrus synchronization & superovulation of donor cows

To induce corpus luteum regression, 2 mL of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>; cloprostenol 250 µg/mL, Synchronate, Pfizer, New York, NY, USA) was injected intramuscularly. To induce the emergence of new follicles, an intravaginal device containing 1.9 g of progesterone (EAZIBREED™ CIDR®, InterAg, Hamilton, New Zealand) was inserted into the vagina. After 8 days, 1 mL (2 mg/mL, Esrone, Samyang Anipharma Co., Seoul, South Korea) was injected intramuscularly. From day 5 after controlled internal drug release (CIDR) device insertion, 300 mg of follicle stimulating hormone (FSH; Folltropin V, Bioniche Animal Health Canada, Belleville, ON, Canada) was intramuscularly injected eight times at 12-h intervals as a reduction method, and 2 mL Synchronate was administered intramuscularly on days 6–7 of FSH administration. The CIDR was removed in the 7<sup>th</sup> round. Estrus was checked approximately 12 h after the final administration of FSH, and artificial insemination was performed twice, 60–72 h after the administration of Synchronate (Figure 1A).

### Embryo collection & evaluation

On day 7.5 after estrus detection, 5 mL of 2% lidocaine (Lidocaine HCl Hydrate; Daihan, Seoul, South Korea) was administered to induce epidural anesthesia. Both uterine horns were flushed with flushing media (BIOLIFE Advantage Complete Flush Media; Agtech Inc., Manhattan, KS, USA) warmed to body temperature. The embryos were recovered by perfusion through a 3-way catheter (IMV; L'Aigle, France). The collected perfusate was filtered using an Em-con filter (Agtech Inc.) and transferred to a 100-mL Petri dish. Embryos were examined under a stereomicroscope at 40× magnification according to the International Embryo Technology Society standards [22]. They were visually evaluated and transferred to a holding medium (BIOLIFE Holding and Transfer Medium, Agtech Inc.) on a warm plate at 37.5°C. In the case of fresh embryo transfer, embryos were transferred within 5 h after quality grade 1–2, stage 4–7, and freezing was started within 1 h after testing quality grade 1 and stage 4–7 freezing.

### **Embryo freezing and thawing**

Embryos were washed thrice with holding medium (BIOLIFE Holding and Transfer Medium, Agtech Inc.) and immersed in freezing medium (BIOLIFE Freeze medium ethylene glycol with sucrose, Agtech Inc.) for 5 min. After equilibration, the embryo was loaded into a 0.25-mL plastic straw, and the straw was placed in a portable embryo freezer (CL8800i; CryoLogic, Blackburn, Australia) containing liquid nitrogen. After a 2-min exposure to -6°C, the seeding zone was inoculated using a cotton swab dipped in liquid nitrogen. When the temperature dropped to -32°C at a rate of -0.3°C/min, the straw was immediately immersed in liquid nitrogen and stored (Figure 2).

To thaw frozen embryos, the straw was taken out from the liquid nitrogen and was left at room temperature for about 10 s, slowly immersed in a 35°C water bath for 10 s, and then kept for an additional 10 s. The straw was removed from the water bath, dried on the outer surface, and transplanted.

### **Embryo transfer**

In total, 297 recipient cows were selected from 20 Hanwoo cow farms in Hongcheon-gun, Gangwon-do, South Korea. Of these recipient cows, 116 received fresh embryos and 181 received frozen IVD embryos. Estradiol benzoate (1 mL) was injected intramuscularly. On day 8 after CIDR insertion, 2 mL of PGF<sub>2α</sub> (Synchromate) was intramuscularly injected while the CIDR was removed. Estradiol benzoate (0.5 mL) was intramuscularly injected approximately 24 h after Synchromate administration and heat was confirmed approximately 12 h thereafter (Figure 1B). At 7.5 days after the discovery of estrus, the ovaries were palpated, and 5 mL of 2% lidocaine was applied for epidural anesthesia to the donor cow with the corpus luteum, and then an embryo transfer syringe (IMV) was used on the uterine horn in the direction of the corpus luteum. Fresh and frozen embryos were transferred.

### **Pregnancy detection & sex investigation**

For the diagnosis of pregnancy, individuals who did not undergo re-estrus after embryo transfer were confirmed by rectal palpation between 60 and 70 days. Data were collected by searching delivery records based on the records of transplanted one year after transplanted to investigate the delivery and sex of live births. The pregnancy rate was defined as the number of pregnancies divided by the number of recipients, and the delivery rate was defined as the number of calves divided by the number of recipients.

### **Statistical analysis**

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

## **Results**

### **Pregnancy rates after transfer of fresh and frozen embryos**

We transferred 116 fresh and 181 frozen embryos to 297 recipient cows. Pregnancy rates were compared between fresh and frozen embryos. The fresh embryos had pregnancy rates of 56.90% (66/116), and the frozen embryos had a lower pregnancy rate of 52.49% (95/181) (Figure 3). However, there was no significant difference in pregnancy rates between fresh and frozen embryos ( $p = 0.4$ ).

### **Quality grades 1 and 2 transfer pregnancy rates in fresh and frozen embryos**

Pregnancy rates were examined according to the quality grades of both fresh and frozen embryos. Among all transferred embryos, the pregnancy rate was higher in grade-1 than in grade-2 embryos. Furthermore, there was a significant increase in the pregnancy rate of fresh embryos for both quality grades, with a 9% increase in grade 1 and a 13% increase in grade 2 ( $p < 0.05$ ) (Figure 4).

### **Pregnancy rates of quality grade-1 fresh and frozen embryos by stage**

We then investigated how the embryo stage affects pregnancy rates under both fresh and frozen conditions. Initially, we found that fresh stage-7 embryos exhibited the highest pregnancy rates. Surprisingly, there were no significant differences in pregnancy rates across the different stages, and the overall rates remained stable and comparable to those of quality grade-1 embryos. However, our analysis of frozen embryos revealed a notable decline in fertility as the stage progressed from 4 to 7. Specifically, when examining pregnancy rates by stage in frozen embryos, the percentages were 63.5% for 4-1, 54.7% for 5-1, 47.8% for 6-1, and 20.0% for 7-1 ( $p < 0.05$ ) (Figure 5). Remarkably, frozen embryos at stage 7-1 displayed the lowest rates of fertilization compared to the other groups, and this decrease was significant compared to fresh embryos at stage 7-1 ( $p < 0.05$ ) (Figure 5).

### **Sex ratio and miscarriage rate of fresh and frozen embryos**

We then examined the outcomes of fresh and frozen embryo transfers in a cohort of pregnant cows. Among 66 pregnancies resulting from fresh embryo transfer, 12 (18.18%) resulted in miscarriages. Within this group, there were 27 female (50.00%) and 27 male (50.00%) calves with an equal male-to-female ratio of 1:1. In contrast, among the 95 pregnancies resulting from frozen embryo transfer, 20 (20.05%) resulted in miscarriage. The resulting offspring consisted of 29 female (38.67 %) and 46 male (61.33%) calves with a male-to-female ratio of 6:4. The miscarriage rate was approximately 3% higher in pregnancies involving frozen embryos than in those involving fresh embryos. Additionally, frozen embryos exhibited a 2% higher miscarriage rate and a higher percentage of female births than fresh embryos. However, it is important to note that these observed differences were not statistically significant (Table 1).

**Table 1.** Sex ratio of transferred embryos in Hanwoo cows

	No. of recipient		Sex ratio of calves	
	Pregnant	Aborted (%)	Male	Female
Fresh	66	12 (18.1)	27/54 (50.0)	27/54 (50.0)
Frozen	95	20 (21.1)	46/75 (61.3)	29/75 (38.7)

$p = 0.200115$ . Statistical significance established at  $p < 0.05$ .

#### Sex ratio and miscarriage rate by stage in grade 1 embryos

We investigated the sex ratio and miscarriage rate based on the stage of grade-1 embryos. The male-to-female ratio varied across the different stages, with ratios of 4.5:5.5 in stage 4, 3.2:6.8 in stage 5, 5.2:4.7 in stage 6, and 2.9:7.1 in stage 7. The miscarriage rates for stages 4–7 were 28.8%, 24.4%, 3.9%, and 36.4%, respectively. Notably, stage 6 exhibited a relatively low miscarriage rate; however, no significant difference was observed between stages (Table 2).

**Table 2.** Sex ratio of transferred embryos by embryo stage in Hanwoo cows

Stage	No. of recipients		Sex ratio of calves	
	Pregnant	Aborted (%)	Male	Female
4-1	59	17 (28.8)	19/42 (45.4)	23/42 (54.8)
5-1	45	11 (24.4)	11/34 (32.43)	23/34 (67.7)
6-1	35	1 (3.9)	18/34 (52.9)	16/34 (47.1)
7-1	11	4 (36.4)	2/7 (28.6)	5/7 (71.4)

$p = 0.3$ . Statistical significance established at  $p < 0.05$ .



### Pregnancy rate, sex ratio, and miscarriage rate of fresh and frozen embryos by season

We conducted a comprehensive study to examine the impact of season on the pregnancy rate, miscarriage rate, and sex ratio of both fresh and frozen embryos. From February to April, nine embryos were transferred, resulting in three pregnancies with a pregnancy rate of 33.3%. Between May and August, out of 194 embryos transferred, 108 embryos were successfully conceived, yielding a pregnancy rate of 55.7%. Similarly, from September to November, 47 of the 89 transfers resulted in pregnancy, yielding a pregnancy rate of 52.8%. Finally, from December to January, three out of five transfers led to embryo pregnancy, resulting in a pregnancy rate of 60.0%. When comparing the pregnancy rates of fresh and frozen embryos across different seasons, the following percentages were observed: 42.9% for fresh embryos vs. 0.0% for frozen embryos in spring, 59.8% for fresh embryos vs. 52.7% for frozen embryos in summer, 51.9% for fresh embryos vs. 53.2% for frozen embryos in autumn, and 60.0% for frozen embryos in winter. Notably, frozen embryos displayed higher fertility rates in autumn and winter than fresh embryos. The following male-to-female ratios were observed in different seasons: 6.7:3.3 in spring, 4.0:6.0 in summer, 5.5:4.5 in autumn, and 3.3:6.7 in winter. These ratios suggested a slight predominance of female births in spring and autumn, whereas summer and winter exhibited relatively balanced sex ratios (Table 3).

**Table 3.** Sex ratio of transferred embryos by season in Hanwoo cows

	No. of recipients		Sex ratio of calves			
	Pregnant	Aborted (%)	Fresh	Frozen	Male	Female
Spring (February to April)	3/9 (33.3)	0 (0.0)	3/7 (42.9)	0/2 (0.0)	2/3 (66.7)	1/3 (33.3)
Summer (May to August)	108/194 (55.67)	16 (14.8)	49/82 (59.8)	59/112 (52.7)	37/92 (40.2)	55/92 (59.8)
Autumn (September to November)	47/89 (52.8)	16 (34.0)	14/27 (51.9)	33/62 (53.2)	14/31 (54.8)	14/31 (45.2)
Winter (December to January)	3/5 (60.0)	0 (0.0)	NA	3/5 (60.0)	1/3 (33.3)	2/3 (66.7)

NA; not applicable.

## Discussion

This study investigated the impact of seasonal transfer on the pregnancy rate, miscarriage rate, and sex ratio in recipient cows using fresh and frozen embryos. The comparison was based on the stage and quality grade of the IVD embryos. The recipient cows were synchronized using conventional synchronization methods [23]. While no significant difference was observed, the pregnancy rate of fresh embryos exceeded that of frozen embryos by over 4%, and the miscarriage rate was 2% higher for frozen embryos. These results are consistent with those of Ferraz et al. [24] who demonstrated a higher pregnancy rate with fresh embryos than with frozen embryos. This suggests that freezing affects the pregnancy rate of IVD embryos [25]. However, the present study revealed that frozen embryos transferred using the current freezing protocol exhibited pregnancy rates similar to fresh embryos.

Evaluation of embryo quality is based on visual assessment, which, although subjective, serves as a predictor of embryo viability [26]. Grades 1–4 are assigned based on the characteristics of the zona pellucida, cytoplasmic regularity, and viability, with grades 1 and 2 considered suitable for embryo transfer [27]. This is consistent with previous findings reported by Phillips and Jahnke [27] and Erdem et al. [15], who observed a higher pregnancy rate with grade-1 than with grade-2 embryos. However, another study [1] reported no significant differences between grade-1 and grade-2 embryos. In this study, fresh embryos demonstrated pregnancy rates of 9% and 13% in both grades, respectively. Notably, grade-2 embryos exhibited a pregnancy rate of less than 40%, representing a difference of approximately 25% compared with grade-1 embryos. For grade-2 embryos, prioritizing the transfer of fresh embryos over freezing is recommended. However, for grade-1 embryos, the results indicated that the pregnancy rate was not significantly affected by the convenience of using fresh or frozen embryos. Therefore, the findings of this study in Hanwoo cows coincide with those of Holstein embryos, in which grade-1 and -2 transfers achieve the highest rates of pregnancy [28].

To investigate the impact of fresh and frozen embryos on the pregnancy rate based on embryo stage, we observed that the pregnancy rate may vary according to embryo stage. The positioning of the embryo within the female genital tract corresponds to its developmental stage, ranging from the morula to the late blastocyst stage on days 6–8 of fertilization [29, 30]. Given that the synchronization protocol utilized in this experiment involved transferring embryos 7.5 days after estrus detection, the pregnancy rate was expected to be highest when embryos were transferred at stages 6 to 7. Surprisingly, the highest pregnancy rate was observed in patients with stage-4 embryos (64.8%). As the stages progressed from 4 to 7, the pregnancy rate gradually decreased by 7%, 9%, and 6%, respectively, with stage 7 demonstrating the lowest pregnancy rate of 42.3%. In the case of fresh embryo transfer, stage 7 exhibited the highest pregnancy rate at 72.3%, whereas stage 6 exhibited the lowest rate at 50%. Nevertheless, the overall pregnancy rates exceeded 50% across all stages, indicating stable pregnancy rates, regardless of whether stages 4–7 were used during fresh embryo transfer [31–33].

Pregnancy rate is also influenced by the freshness of the embryo and the stage of development [34]. In our study, there was a significant drop of approximately 10% in the pregnancy rate from stages 4 to 7. This suggests that the developmental capacity of embryos is more susceptible to damage caused by freezing. Slow freezing during cryopreservation leads to a decrease in viability due to the formation of ice crystals and the physical destruction of organelles caused by the presence of free water inside the cells [35, 36]. As the embryo develops and progresses to the blastocyst stage, the size of the blastocyst cavity and the amount of water within it increases, thereby increasing the likelihood of damage during freezing. While no significant difference in pregnancy rates between fresh and frozen embryos was observed at stage 4, the difference in pregnancy rates became more pronounced as the embryos progressed to stage 7. Therefore, to achieve a stable pregnancy rate after embryo cryopreservation, it is advisable to select and freeze embryos at stages 4–5.

Pregnancy rates based on developmental stages of fresh and frozen embryos have not been extensively investigated in previous studies. Erdem et al. [15] reported that stages 5 and 6 exhibited the highest pregnancy rates, which aligns with the findings of Hasler [37] who observed the highest pregnancy rates at stage 5. However, contrasting results were reported by Putney et al. [38] and Ferraz et al. [24], who reported higher pregnancy rates in stage 7 and lower rates in stage 5. Vieira et al. [39] reported that the developmental stage of the embryo did not significantly affect pregnancy rates. The results of the present study differ from those of previous studies, suggesting that pregnancy rates during embryo transfer vary according to the developmental stage of embryos in different countries and breeds. These variations highlight the importance of considering specific factors such as geographical location and breed when interpreting and comparing pregnancy rate outcomes across studies [40, 41].

Pre-selection of calf sex in dairy and beef cattle breeding offers significant economic advantages and has a profound impact on management strategies. To investigate the potential influence of embryo developmental stage on the sex ratio of live Hanwoo cows, we conducted a follow-up observation of the sexes based on embryo stage. In fresh embryos, a 1:1 male:female ratio was observed, whereas in frozen embryos, the male:female ratio was 3:2, indicating a relatively higher proportion of males (approximately 20%). However, no significant differences were observed between the groups.

Determining offspring sex from embryos has been a subject of great interest, with several studies attempting to establish associations between embryonic sexes and morphological and temporal assessments, leading to conflicting results. Early bovine studies suggested that early embryos were more likely to be male, implying a potentially faster developmental rate of male embryos compared to female embryos [42, 43]. However, other studies have found no significant effect of embryo sex on the timing of bovine embryo division [44–46]. Consequently, investigators have used morphological and temporal evaluations to identify potential developmental differences between male and female bovine embryos. However, the effect of embryo sex on the timing of division remains controversial. Previous studies by

292 Holm et al., Sugimura et al., and Magata did not find a relationship between developmental dynamics and  
293 bovine embryo sex [47-49].

294 Monthly embryo transfer records were reanalyzed to investigate the impact of temperature and  
295 other environmental factors on the pregnancy rates of frozen and fresh embryos. Lee et al. [28]  
296 highlighted the negative effects of cumulative heat stress during the hot summer season in Korea,  
297 particularly from September to early October, resulting in poor embryo grades. In the present study,  
298 embryo transfers were rarely performed between December and February due to local business practices.

299 In Korea, spring is considered as February to April, summer as May to August, autumn as  
300 September to November, and winter as December to January [17]. In this study, over 65% of embryo  
301 transfers took place between March and August, as farmers preferred to deliver calves during the warm  
302 season following hot summers. Fresh embryos had a higher pregnancy rate during summer, whereas  
303 frozen embryos exhibited a higher pregnancy rate during autumn.

304 Ferraz et al. [24] reported a negative correlation among temperature, humidity, and pregnancy  
305 rates. Heat shock-induced aging of sperm at 40°C results in decreased embryo development and male sex  
306 ratios [16]. Heat stress during midsummer reduces feed intake, increases negative energy balance, alters  
307 ovarian follicular dynamics, decreases the estrus detection rate, and affects fallopian tube function,  
308 leading to fertilization failure and early embryo mortality [19]. Consequently, embryo transfer shows  
309 higher pregnancy rates than pregnancies by artificial insemination [20]. However, frozen embryos display  
310 increased sensitivity to heat stress and slower development under heat exposure than fresh embryos [21].  
311 Although the temperature used for embryo exposure in this study was 41°C, it should be noted that an  
312 actual heatwave in Hongcheon-gun, Gangwon-do, South Korea recorded a temperature of 41°C in early  
313 August 2018. In dairy cows, the artificial insemination rate was 55.6% in summer and 87.8% in the cool  
314 season [50]. Although embryos can be influenced by seasonal ambient temperatures [51, 52], no  
315 significant differences were observed in this study because of the use of an embryo warmer to maintain  
316 optimal conditions until immediately before transfer. Furthermore, 93% of the actual embryo transfers  
317 occurred after mid-October during the fall. This schedule allowed sufficient recovery from heat stress,  
318 thereby contributing to a stable pregnancy rate. Male-to-female ratios for embryo transfer were 6.7:3.3 in  
319 spring, 4.0:6.0 in the summer, 5.5:4.5 in the autumn, and 3.3:6.7 in the winter. It can be concluded that  
320 there was no significant effect on the male-to-female ratio during the summer and autumn.

321 In conclusion, the comprehensive findings of this study provide valuable insights into the  
322 optimization of embryos in Hanwoo cows. By exploring the factors that influence the viability and  
323 success of IVD embryo transfer in Hanwoo cows, this study contributes to enhancing the desired fertility  
324 rate, pregnancy rate, and sex ratio of the resulting calves. The determinants identified to achieve optimal  
325 outcomes in Hanwoo cattle reproductive management have significant implications for animal breeding.  
326 Moreover, the results of this study offer potential solutions to improve the efficiency and effectiveness of

embryo transfer techniques in Hanwoo cattle, thereby benefiting the overall productivity and sustainability of the Hanwoo cattle industry.

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ACCEPTED

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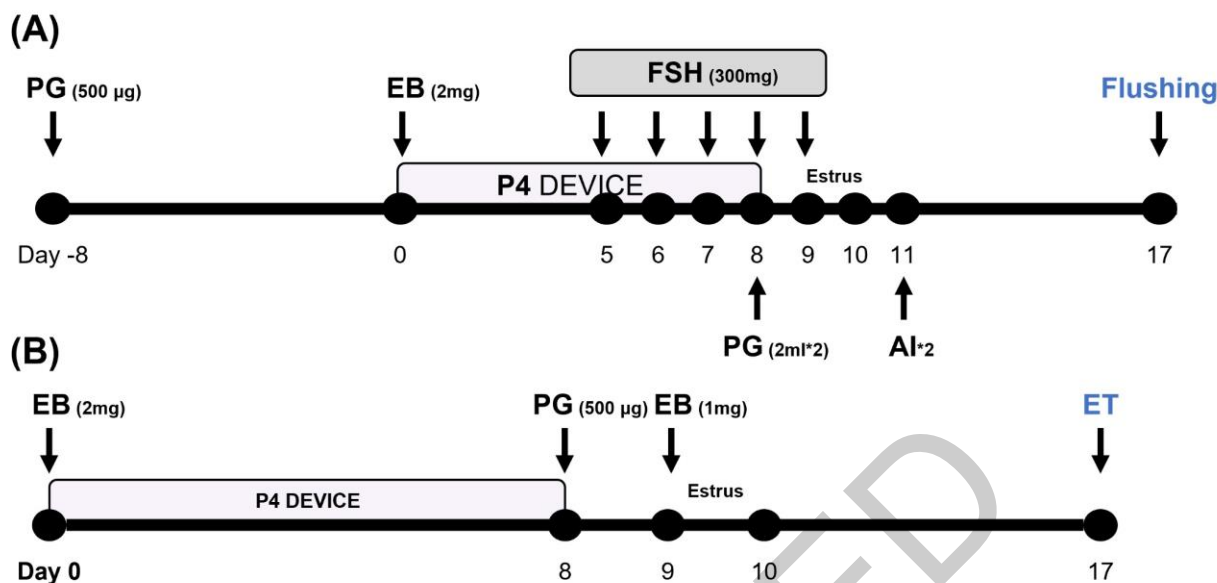
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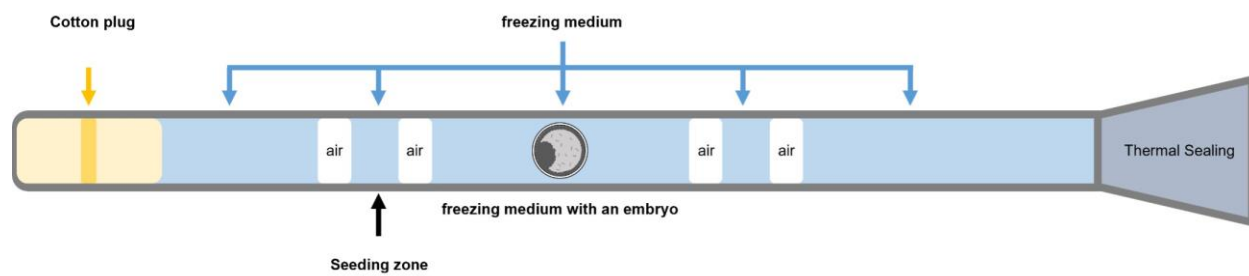
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Figures

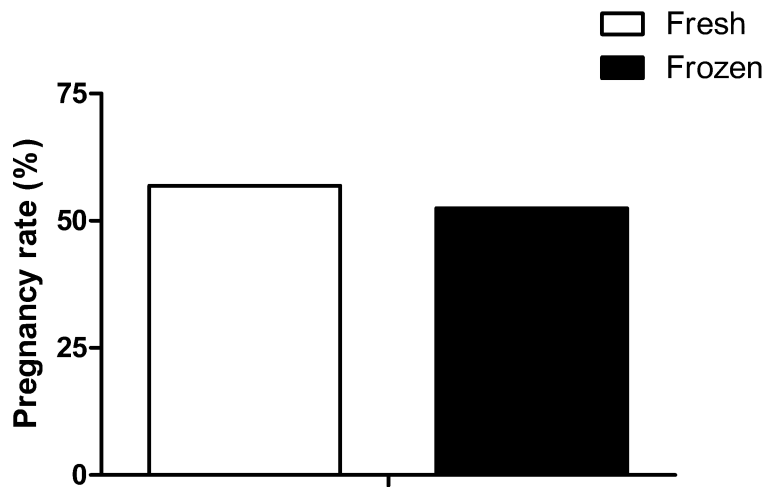


**Figure 1.** (A) Estrus Synchronization Schedule for Donor Cow Preparation. (B) Estrus synchronization schedule of recipient cow for embryo transfer.

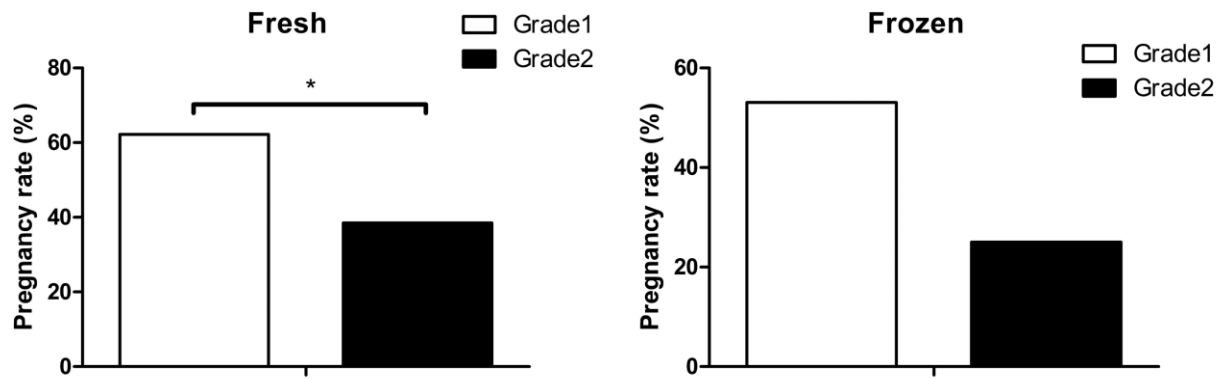


**Figure 2.** Image of embryo cryopreservation straw.

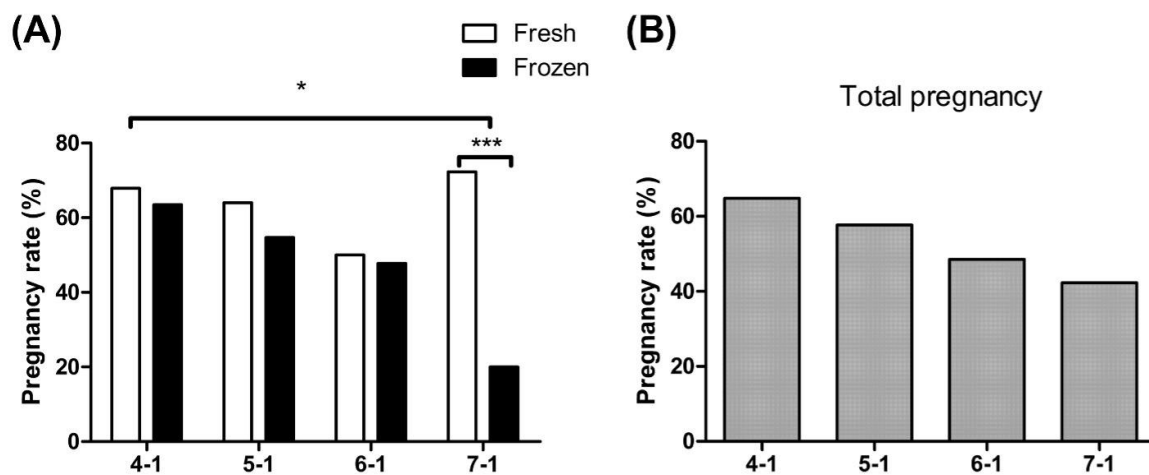
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**Figure 3.** Pregnancy rate of embryo transfer using fresh and frozen embryos in Hanwoo cows.  $p = 0.456705$ .



**Figure 4.** Total pregnancy rate of embryo transfer using grade-1 and -2 embryos in Hanwoo cows. (A) Pregnancy rate according to fresh embryo grade.  $p = 0.03$ . (B) Pregnancy rate according to frozen embryo grade.  $p = 0.26$ . (C) Total pregnancy rate according to embryo quality.  $p = 0.04$ .



**Figure 5.** (A) The pregnancy rate of embryo transfer by stage of fresh and frozen embryos in Hanwoo cows. (B) Total pregnancy rate. Fresh embryos,  $p = 0.46$ ; frozen embryos,  $p = 0.019$ ; total pregnancy rate,  $p = 0.08$ . Fresh vs. frozen embryo transfer according to stage 7-1,  $p < 0.00001$ . \* indicates significant difference between stage 7-1 and other codes. \*\*\* indicates significant difference within groups.