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Effect of Timed Artificial Insemination Protocols on the Pregnancy Rate Per Insemination and Pregnancy Loss in Dairy Cows and Korean Native Cattle under Heat Stress

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Abstract : We aimed to determine the effect of timed artificial insemination (TAI) protocols on the pregnancy rate per insemination and pregnancy loss compared with AI performed at detected estrus in dairy cows and Korean Hanwoo cattle under heat stress. In dairy cattle, 1,250 sets of data that underwent AI during heat stress (temperature-humidity index ≥ 72) were categorized according to their TAI protocols or as controls: 1) $PGF_{2\alpha}$-36 h-estradiol benzoate (EB)-36 h-TAI (PG-EB group, n = 113); 2) GnRH-7 days-$PGF_{2\alpha}$-56 h-GnRH-16 h-TAI (Ovsynch group, n = 455); or 3) GnRH-6 days-Ovsynch (G6G group, n = 136). The remaining cows underwent AI at detected estrus (AIDE group, n = 546). The probability of pregnancy per AI 45 days after AI was higher ($P < 0.01$) in the PG-EB (odds ratio [OR]: 1.68), Ovsynch (OR: 1.48), and G6G (OR: 1.79) groups than in the AIDE group. However, the prevalence of pregnancy loss between 30 and 45 days after AI did not differ among the groups. In Hanwoo cattle, 617 sets of data inseminated artificially under heat stress were categorized into AIDE (n = 281), PG-EB (n = 194), and combined Ovsynch or G6G (n = 142) groups. The probability of pregnancy per AI 45 days after AI and the prevalence of pregnancy loss between 30 and 45 days after AI did not differ among the groups. Thus, implementation of a TAI protocol (PG-EB, Ovsynch, or G6G) in dairy cows under heat stress improves the pregnancy rate per AI *versus* AIDE, whereas there is no beneficial effect of TAI on the pregnancy rate of Hanwoo cattle under heat stress.

***Key words* :** heat stress, timed artificial insemination, pregnancy rate, pregnancy loss, cattle.

Introduction

Global warming has made heat stress a serious threat to the milk production, health, welfare, and reproduction of cattle (6,16,21,47). Previous publications have emphasized the deleterious impacts of heat stress on fertility (12,29), which is more serious during heat stress in lactating dairy cows because of the larger amount of heat produced during lactation than in non-lactating dairy and beef cattle. However, beef cattle can also be affected by heat stress, particularly when kept in feedlots (27). To date, there have been few publications regarding the effects of heat stress on the reproductive performance of beef cattle.

Heat stress may disturb endocrine status, including by reducing estradiol and progesterone concentrations (7,24), and may alter follicular development and disturb ovulation (19,49). In addition, impairments in oocyte and embryo viability under conditions of heat stress (14,41) reduce the likelihood of fertilization (22,40) and increase embryonic mortality (17,32) in cattle. Therefore, measures to counteract heat stress may be required to avoid economic loss due to impairments in reproductive performance in cattle.

Heat stress occurs when an animal fails to dissipate an adequate quantity of heat to maintain dermal balance. Because the thermoregulation in cows is affected by air temperature and relative humidity (RH), and records of these can usually be obtained from a meteorological station, the majority of studies of heat stress have focused mainly on the effects of air temperature and RH in livestock (33,47). Thus, temperature-humidity index (THI) might represent an indicator of the combined effects of air temperature and RH, and might be useful for assessment of the impact of heat stress. In previous studies, THIs 72 or 73 were chosen as thresholds, on the basis of the relationship between mean or maximum THI on the day of insemination and the resulting pregnancy rate (8,28,42).

Strategies that have been designed to mitigate the adverse effect of heat stress on reproductive performance have included the use of shade, fens, or evaporative cooling (4,15,44). However, because these efforts to reduce the magnitude of heat stress do not fully resolve the condition, alternative approaches to counteract the adverse effects of heat stress on reproductive performance might be required, such as hormonal treatment (12,50). The synchronization of ovulation, Ovsynch, which involves the administration of gonadotropin-releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), has been widely used to control reproductive management in cattle (31). Moreover, G6G, which involves the administration of GnRH, followed by Ovsynch 6 days later, yields a higher pregnancy rate per artificial insemination (AI) than Ovsynch, because of a larger ovulatory response to the first GnRH of Ovsynch (5). In addition, a $PGF_{2\alpha}$-based timed AI (TAI) pro-

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tocol, in which estradiol benzoate (EB) is administered 36 or 56 h after $PGF_{2\alpha}$ injection (PG-EB protocol), is also used to induce synchronized ovulation for TAI (25). The implementation of TAI protocols, such as Ovsynch (10) or Double-Ovsynch (13), in cows suffering from heat stress has been shown to improve fertility, although other studies showed no effect of TAI protocols (Ovsynch or its modification) on the pregnancy rate under conditions of heat stress (3,11). Therefore, more extensive research regarding the effects of TAI protocols on the fertility of heat-stressed cattle might be required.

In Korea, the management of heat stress has become a bigger challenge because of the change of climate from temperate to sub-tropical and the large increases in milk production per cow, which involves greater metabolic activity. For these reasons, lactating dairy cows suffer from severe heat stress and show poorer reproductive performance, which justifies more extensive research regarding appropriate methods to counteract this loss of fertility. In addition, the effects of the implementation of TAI protocols on reproductive outcomes have not been documented in Hanwoo cattle that are subjected to heat stress. Therefore, the objective of this retrospective study was to evaluate the effect of TAI protocols (PG-EB, Ovsynch, or G6G) on the pregnancy rate per AI and the incidence of pregnancy loss, in compared with AI performed at detected estrus in dairy cows and Hanwoo cattle under heat stress.

Materials and Methods

Animal and management

The retrospective study was performed in 10 dairy and 13 Hanwoo farms located in Seasan city, Chungnam province. Each dairy farm contained 35 to 126 milking cows. The cows were maintained in loose housing systems and fed a total mixed ration, which comprised brewers' grain, alfalfa hay, cotton seed, beet pulp, corn silage, tall fescue, timothy hay, minerals, and vitamins. Drinking water was provided *ad libitum*. All the barns in which this study was conducted were equipped with fans and shade during the hot months of the year (May to October). The mean milk yields for the farms were 10,203-11,192 kg per cow per year. All the cows that had calved were milked twice daily.

Each Hanwoo farm contained 29 to 79 cattle, which were maintained in indoor feedlots and fed concentrate, rice straw, minerals, and vitamins. Drinking water was provided *ad libitum*. All the feedlots included the study were provided with fans and shade during the hot months.

Hanwoo heifers were artificially inseminated at 12 months of age for the first time. The voluntary waiting periods between calving and the first AI were 30 days and 40 days for Hanwoo and dairy cows, respectively. In addition to estrus detection, TAI programs were employed, which are described in detail below. Pregnancy diagnosis was performed by transrectal ultrasonography 30 and 45 days after AI. Pregnancy loss was diagnosed when there was no embryonic heartbeat, a lack of positive signs of pregnancy in a cow that had been previously diagnosed as being pregnant, or there were signs of embryo degeneration (37).

Calculation of THI and case definition

All data were obtained at 36º74-96′ N latitude and 126º22-57′ E longitude. Meteorological data were collected between 2015 and 2019, which included the daily ambient temperature and RH, from the Korea Meteorological Administration (46). The monthly mean ambient temperature varied from −1.48ºC in January to 26.0ºC in August, while the RH ranged between 69.7% in April to 87.1% in August during the study period.

The daily maximum THI were calculated during study period (2015 to 2019) as in a previous study (18) using the following equation:

$$\text{Maximum THI} = (0.8 \times \text{maximum temperature} + [\text{minimum RH } (\%)/100] \times [\text{maximum temperature} - 14.4] + 46.4)$$

The definitions of the peri- or postpartum disorders that were used in the present study were similar to those described in previous publications (9,43). Dystocia was defined as calving requiring some or significant force, or cesarean section. Retained placenta was defined as the retention of the fetal membranes for longer than 24 h. Ketosis was diagnosed in the presence of the following clinical signs: anorexia, depression, and the odor of acetone on the breath. Abomasal displacement was diagnosed by a "ping" sound during abdominal auscultation by veterinarians in the research team. Clinical endometritis was diagnosed on the basis of the presence of a visible mucopurulent vaginal discharge and/or rectal palpation and ultrasonography 4 weeks after calving.

Study design

In the first part of the study, 1,250 sets of data (inseminations of 442 primiparous and 808 multiparous cows) from 507 dairy cows inseminated artificially under condition of heat stress (THI ≥72) were categorized according to which of the three TAI protocols they underwent or as controls. TAI was performed as follows: 1) An injection of 500 µg of a $PGF_{2\alpha}$ analog, cloprostenol sodium (Estrumate, MSD Animal Health, Seoul, Korea), and an injection of EB (SY Esrone, Samyang, Seoul, Korea) 36 h later, followed by TAI 36 h later (PG-EB group, n = 113). 2) An injection of 10 µg of a GnRH analog, buserelin acetate (Gestar, Over, San Vicente, Argentina), $PGF_{2\alpha}$ 7 days later, and GnRH 56 h later, followed by TAI 16 h later (Ovsynch group, n = 455) or 3) GnRH 6 days later, followed by Ovsynch (G6G group, n = 136). Conversely, the controls underwent AI at detected estrus (AIDE group, n = 546).

In the second part of the study, 617 sets of data (122 from heifer inseminations and 495 from cow inseminations) from 406 Hanwoo cattle that were inseminated artificially under conditions of heat stress (THI ≥72) were categorized into AIDE (n = 281), PG-EB (n = 194), or combined Ovsynch or G6G (combined n = 142) groups.

In these analyses, the probability of pregnancy per AI 45 days after AI and the probability of pregnancy loss between 30 and 45 days of gestation after AI in each treatment group were compared in both dairy cows and Hanwoo cattle under conditions of heat stress.

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). For statistical analyses, cow parity was categorized as either 1, 2, or ≥3 in dairy cows, whereas for Hanwoo cows and heifers. Dairy cows were also categorized according to whether their herds consisted of <60 or ≥60 lactating cows, while Hanwoo cattle were categorized according to whether their herds consisted of <40 or ≥40 head. The cattle were regarded as being under the heat stress when the daily maximum THI was ≥72 (8). The peri- or postpartum disorders included dystocia, retained placenta, ketosis, abomasal displacement, and clinical endometritis. Statistical analyses were performed using SAS (version 9.4; SAS Inst., Cary, NC, USA).

A comparison of the monthly mean maximum THI during 2015-2019 was carried out using an analysis of variance, followed by Duncan's multiple range tests.

The probability of pregnancy per AI 45 days after AI in dairy cows under heat stress was analyzed by logistic regression using the LOGISTIC procedure. The logistic regression model included treatment group (AIDE, PG-EB, Ovsynch, and G6G), herd size, parity, peri- or postpartum disorders (including dystocia, retained placenta, ketosis, abomasal displacement, or clinical endometritis), and AI year, and the interactions between these variables. The same analysis was applied to the probability of pregnancy per AI 45 days after AI in Hanwoo cattle under heat stress. The logistic regression model included treatment group (AIDE, PG-EB, and Ovsynch or G6G), herd size, parity, and AI year, and the interactions between these variables. Backward stepwise regression was used in all models, and elimination was performed according to the Wald statistic criterion when $P > 0.11$. Odds ratios (OR) and 95% confidence intervals (CIs) were determined by logistic regression, and the findings are displayed as percentages and ORs, with the respective 95% CIs.

The incidence of pregnancy loss between 30 and 45 days after AI was compared between the AIDE, PG-EB, Ovsynch, and G6G groups of dairy cows, and between the AIDE, PG-EB, and Ovsynch or G6G groups of Hanwoo cattle, using the Fisher's exact test. $P < 0.05$ was considered to represent statistical significance.

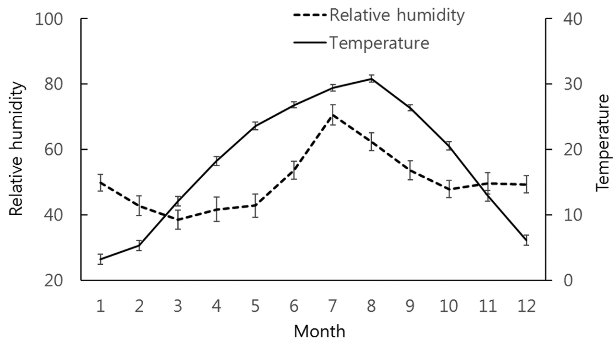

Fig 1. The monthly mean maximum temperature and minimum RH during January 2015 to December 2019 in the study area.

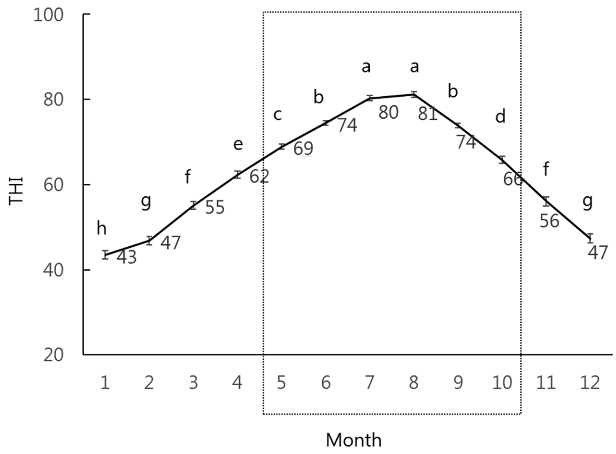

Fig 2. The monthly mean maximum THI during January 2015 to December 2019 in the study area. [a-h]Means with different superscripts differ ($p < 0.01$) between groups. The dotted square line represents the hot part of the year (THI ≥72), during which heat stress occurs.

Table 1. Adjusted odds ratios (ORs) for variables included in the logistic regression model regarding the probability of pregnancy per AI at detected estrus (AIDE[1]) and following TAI (using PG-EB[2], Ovsynch[3], or G6G[4]) in dairy cows during the period of heat stress (THI ≥72)

Variable	Level	Adjusted OR	95% CI[5]	*P*-value
Group	AIDE	Reference		
	PG-EB	1.68	1.080-2.625	< 0.05
	Ovsynch	1.48	1.111-1.969	< 0.01
	G6G	1.79	1.184-2.705	< 0.01
Parity	1	Reference		
	2	0.82	0.597-1.116	> 0.05
	3 ≤	0.66	0.489-0.891	< 0.01
Peri or postpartum disorders[6]	No	Reference		
	Yes	0.74	0.550-0.982	< 0.05

[1]Cows underwent AI at detected estrus.
[2]PG-EB (PGF_{2a}, and then estradiol benzoate 36 h later and AI 36 h after that).
[3]Ovsynch (GnRH-$PGF_{2\alpha}$ 7 days later, and then GnRH 56 h later and AI 16 h after that).
[4]G6G protocol (GnRH 6 days later, followed by Ovsynch).
[5]Confidence interval.
[6]Peri or postpartum disorders included dystocia, retained placenta, ketosis, abomasal displacement, and clinical endometritis.

Results

Figs 1 and 2 show the meteorological data during January 2015 to December 2019 in the study area. Fig 1 shows the monthly mean maximum temperature and minimum RH during the study period, while Fig 2 shows the monthly mean maximum THI, which demonstrates significant differences between the months ($p < 0.01$) and a peak in July and August.

The pregnancy rates of the dairy cows per AI 45 days after AI were 22.3% (122/546), 33.6% (38/113), 30.1% (137/455), and 33.1% (45/136) in the AIDE, PG-EB, Ovsynch, and G6G groups, respectively. Table 1 shows the factors that affected the probability of pregnancy per AI 45 days after AI following the AIDE and three TAI protocols, determined using a logistic regression model. The probability of pregnancy per AI 45 days after AI was higher ($P < 0.01$) in the PG-EB (OR: 1.68), Ovsynch (OR: 1.48), and G6G (OR: 1.79) groups than in the AIDE group. In addition, parity and peri- or postpartum disorders also affected the probability of pregnancy per AI: cows with parities ≥3 had a lower probability of pregnancy per AI (OR: 0.66, $P < 0.01$) than primiparous cows, and cows with peri- or postpartum disorders had a lower probability of pregnancy per AI (OR: 0.74, $P < 0.05$) than cows that did not develop such disorders. However, the incidence of pregnancy loss between 30 and 45 days after AI did not differ ($P > 0.05$) between the AIDE (13.5%, 19/141), PG-EB (2.6%, 1/39), Ovsynch (8.1%, 12/149), and G6G (11.8%, 6/51) groups.

The probability of pregnancy per AI 45 days after AI in Hanwoo cattle did not differ ($P > 0.05$) between the AIDE (65.5%, 184/281), PG-EB (63.4%, 123/194), and combined Ovsynch or G6G (64.8%, 92/142) groups. Furthermore, neither herd size, parity, nor AI year was not associated with the probability of pregnancy per AI 45 days after AI. Finally, the incidence of pregnancy loss between 30 and 45 days after AI also did not differ ($P > 0.05$) between the AIDE (2.8%, 4/142), PG-EB (4.1%, 5/121), and combined Ovsynch or G6G (1.5%, 1/66) groups.

Discussion

In this retrospective study, we compered the effects of TAI protocols, such as PG-EB, Ovsynch, or G6G, on the pregnancy rate per AI and incidence of pregnancy loss, with AIDE in dairy cows and Hanwoo cattle during periods of heat stress. The results show that all of the PG-EB, Ovsynch, or G6G protocols improved the probability of pregnancy per AI compared with AIDE in heat-stressed dairy cows, whereas there were no beneficial effects of the TAI protocols on the probability of pregnancy per AI in Hanwoo cattle. In addition, the TAI protocols did not affect the incidence of pregnancy loss between 30 and 45 days after AI in either dairy or Hanwoo cattle.

Based on the meteorological data collected for the period 2015-2019, heat stress (THI ≥72) began in the middle of June, lasted until the first week of October in the study area, and peaked in July and August. Therefore, measures to mitigate the adverse effects of heat stress on reproductive performance in cattle should be used between June and October.

In the present study, the probability of pregnancy per AI 45 days after AI was higher in the PG-EB, Ovsynch, and G6G groups than in the AIDE group among the dairy cows under heat stress, but there were no differences in the probabilities between the three TAI protocols. Our finding of a higher probability of pregnancy per AI 45 days after AI in the Ovsynch group than in the AIDE group is consistent with that of a previous study (10), in which a higher pregnancy rate per AI in heat-stressed dairy cows with Ovsynch (22.9%) was identified than in those who underwent AI following estrus detection (13.2%). However, other studies found no beneficial effects of Ovsynch protocols on pregnancy rate per AI *versus* estrus detection in dairy cows under heat stress (3,8,11,26). The reason for the discrepancies between these studies has not been clarified in the present study. Indeed, the mechanism by which heat stress reduces fertility is multifactorial and may vary according to the magnitude of the heat stress (23). In addition, we have not identified the mechanisms by which higher pregnancy rates per AI were achieved in the three TAI groups than in the EDAI groups of dairy cows under stress. However, we predict that oocytes might be ovulated from older dominant follicles in the EDAI group than in the three TAI groups when they are under heat stress (38). It has been suggested that heat stress can alter follicular dynamics by reducing follicular dominance, dominant follicles may develop earlier in the second follicular wave (49), and incomplete dominance may result in ovulation from aged follicles, which may explain the significantly lower pregnancy rates in the AIDE group (48). In addition, the PG-EB protocol might reduce the age of the preovulatory follicle because of $PGF_{2\alpha}$-induced luteolysis, resulting in a higher pregnancy rate than in the EDAI group. Conversely, the Ovsynch or G6G protocols might induce follicular turnover, resulting in the ovulation of dominant follicles in a new follicular wave, which might also be associated with a higher pregnancy rate than in the EDAI group. Consistent with this, a previous study also demonstrated that cows that underwent an Ovsynch protocol showed a higher pregnancy rate than those that were inseminated at detected estrus, which it was suggested might have been caused by the recruitment of fresh dominant follicles in the TAI group (10). Another possible explanation of the higher pregnancy rate per AI under heat stress in the three TAI groups than in the EDAI group among dairy cows might be the promotion of ovulation by GnRH just before TAI, which was part of all three TAI protocols.

We found no differences on the probabilities of pregnancy per AI between the three TAI protocols, which may suggest that an induction of follicular turnover and a new follicular wave using Ovsynch or G6G protocol did not have a greater effect on the viability of oocytes than the PG-EB protocol. Consistent with this, it has been reported that heat stress may affect bovine fertility during oocyte maturation and near ovulation (1,2).

In the present study, parity and peri- or postpartum disorders also affected the probability of pregnancy per AI in dairy cows. Our finding that cows with parities ≥3 had a lower probability of pregnancy per AI than primiparous cows is consistent with the findings of a previous study (30). In addition, the finding that cows with a peri- or postpartum disorder had a lower probability of pregnancy per AI than cows that did not have such a disorder has also been made in other studies (34,45). This might be because of the effect of such disorders on development, such as an impairment in the cleavage of potential zygotes or embryonic development in lactating dairy cows (35).

In contrast to the results obtained in dairy cows, the probability of pregnancy per AI 45 days after AI did not differ between the AIDE, PG-EB, and Ovsynch or G6G groups in Hanwoo cattle. It may be that Hanwoo cattle have certain advantages that limit the impact of heat stress on fertility, such as relatively low metabolic heat production (6), a genetic resistance to heat stress (20), and adaptation to the climate of Korea. These factors might be responsible for lower heat stress in Hanwoo cattle than in lactating dairy cows, which might explain the lack of superiority of any of the TAI protocols over AI following the detection of estrus regarding the probability of pregnancy per AI in Hanwoo cattle.

Late embryonic and early fetal death might represent an important source of reproductive loss in cattle, especially in high-producing dairy cattle that are under heat stress. It has been reported that the prevalences of pregnancy loss between days 34 or 45 and day 90 of gestation were 2% for cows in the cool season and 12% during the warm season (17). Moreover, pregnancy loss peaks just before day 50 of gestation, when the placenta is still not fully established (39). The uterine environment might be altered by higher uterine temperatures, in association with lower blood flow to the uterus (36). We found no differences in the incidence of pregnancy loss between 30 and 45 days after AI between the groups of both dairy cows and Hanwoo cattle. Consistent with these findings, a previous study showed that there were no differences in the prevalence of pregnancy loss (6.1% to 7.4%) between the TAI protocols in lactating dairy cows under stress (13). These findings may suggest that although TAI protocols increase the pregnancy rate per AI *versus* insemination at detected estrus in dairy cows under heat stress, the protocols used might not protect the embryo or fetus against high temperature-induced embryonic or fetal death (10). Therefore, pregnancy loss occurring between 30 and 45 days after AI might be associated with the heat stress at the time of embryonic and/or fetal growth period. Consistent with this, it has been reported that the peri-implantation period is critical in the life of the embryo, which implies that acute heat stress during this period would predispose pregnant cows to early fetal loss (17).

Conclusion

We have shown, using reproductive data collected between June 2015 and October 2019, that the implantation of a TAI protocol (PG-EB, Ovsynch, or G6G) improves the pregnancy rate per AI in dairy cows under conditions of heat stress, whereas there were no advantages of any of the TAI protocols for reproductive outcomes in Hanwoo cattle. Therefore, the use of a TAI protocol, together with the use of cooling systems, including shade, fens, or others, might help to mitigate an impairment in dairy herd fertility during the hot months (between late June and early October in Korea).

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