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RESEARCH ARTICLE

GnRH and hCG Improve the Function of Corpus Luteum and Uterine Receptivity at Timed Artificial Insemination in Postpartum Dairy Cows

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ABSTRACT

This study aimed to improve the uterine receptivity and luteal function following timed artificial insemination (TAI) by using gonadotropin releasing hormone (GnRH) and human chorionic gonadotropin (hCG), and to evaluate the effects of these treatments on pregnancy rates in dairy cows. In experiment I, dairy cows (n=141) were subjected to TAI protocol 50 days after calving. The cows were randomly divided into the control group (n=52), GnRH group (n=31, given 100 μg GnRH each) and hCG group (n=58, given 1500 IU hCG each) five days after TAI to study their effects on the blood progesterone levels and the pregnancy rates. In experiment II, bovine endometrial epithelial cells were cultured and treated with GnRH and hCG to study effects of these hormones on expression of specific genes including Toll-like receptor 4 (TLR-4), Nuclear factor-kappa B (NF-κB), Leukemia inhibitory factor (LIF) and Interleukin-1 (IL-1). The results showed that the injection of GnRH and hCG 5 days after AI significantly increased blood progesterone concentrations. However, differences in pregnancy rates between the cows of three groups (51.61, 53.45 & 36.54% in GnRH, hCG and control groups, respectively) were non-significant. PGF2a secreted by hCG-treated endometrial cells was significantly higher than that of the GnRH-treated cells and the control group, and the difference between the latter two groups was non-significant. GnRH and hCG treatment inhibited TLR-4 and NF-κB signaling pathways and promoted expression of LIF gene. In conclusion, the injection of GnRH and hCG 5 days after AI was beneficial to the function of the corpus luteum and uterine receptivity, although improvement in pregnancy rates was non-significant.

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INTRODUCTION

Continuous genetic progress in cows leads to increased milk yield, but it is associated with declining fertility of dairy cows (Pursley *et al.*, 1997). Pursley *et al.* (1995) introduced the simultaneous ovulation with timed insemination protocol in dairy cow production. In this protocol, gonadotropin releasing hormone (GnRH) was injected on any day of estrous cycle to promote the development and ovulation of the dominant follicles that may exist on the ovary and form a new corpus luteum. Prostaglandin F2 (PGF2α) was injected 7 days later to induce the dissolution of the functional corpus luteum on the ovary, so as to promote the development of dominant follicles. After 56 hours of PGF2α injection, GnRH was injected again to promote the impulse secretion of luteinizing hormone (LH) and cause ovulation of mature follicles. Artificial insemination (AI) was performed in all cows 16-18 hours after the second GnRH injection.

Later, many researchers used two times PGF2α pretreatment (Moreira *et al.*, 2001), double estrus synchronization (Souza *et al.*, 2008), treatment with CIDR for 5 days (Rabaglino *et al.*, 2010; Lima *et al.*, 2011), human chorionic gonadotrophin (hCG) instead of GnRH for the second injection (Garcia-Ispierto *et al.*, 2018), and two CIDR treatments (Melo *et al.*, 2018; Carvalho *et al.*, 2019) to improve the results of simultaneous ovulation after timed insemination. Ovulation rate and fertilization rate of oocytes are increased through improvements in the above protocols in dairy cows. However, the problems of low embryo implantation rate and high pregnancy losses remain unresolved (Nascimento *et al.*, 2013; Niles *et al.*, 2019).

Many researchers used hCG and GnRH after AI in order to improve the implantation rate of embryos (Colazo *et al.*, 2013; Nascimento *et al.*, 2013; Niles *et al.*, 2019; Zolini *et al.*, 2019). However, there are contradictory reports regarding the improvement in the fertility rate of cows through the use of hCG or GnRH after timed artificial insemination (TAI). Niles *et al.* (2019) found that hCG treatment after TAI had no effect on the pregnancy rate of cows. Zolini *et al.* (2019) used hCG treatment 5 days after insemination and found that the increase in pregnancy rate was related to the genotype of individual cows. Recently, López-Gatius and Garcia-Ispierto (2020) conducted a series of studies and confirmed that the treatment with GnRH after AI (early corpus luteum) could improve the fertility of dairy cows and reduce pregnancy losses.

During implantation, series of changes take place in the uterine environment of dairy cows. The physiological changes of endometrium are mainly realized by the coordination of steroid hormones, cytokines and adhesion molecule networks. Under the action of hormones, inflammatory cytokines (interleukin (IL)-1, IL-6, IL-10, etc.) produced by endometrial cells promoted endometrial inflammatory reaction and embryo implantation (Moraes *et al.*, 2018). Steroids also stimulate the secretion of epidermal growth factor (EGF), heparin binding epidermal growth factor, and leukemia inhibitory factor (LIF), which promote the differentiation and proliferation of embryonic cells. During the implantation process of the embryo and endometrial epithelial cells interaction, the endometrium produces immune and inflammation-related responses. The ability of the endometrium to support successful embryo implantation is termed as uterine receptivity (Garrett *et al.*, 1988; Rogers, 1992; Pillai *et al.*, 2019). Therefore, expression of Toll-like receptor 4 (TLR-4), Nuclear factor-kappa B (NF-κB), IL-6, and LIF genes can be used to assess the uterine receptivity. In the process of the interaction between endometrial cells and embryonic trophoblasts, the maternal endometrium is under a certain degree of immuno-suppression. TLR, as an important immune signaling pathway, is involved in the regulation of early embryo implantation (Wu *et al.*, 2018).

Based on the above facts, this study further investigated whether the injection of GnRH and hCG after TAI can increase pregnancy rate and reduce pregnancy loss and whether GnRH and hCG can directly act on endometrial epithelial cells to improve the uterine receptivity in dairy cows. Results of this experiment are expected to lay a theoretical foundation for further research on pregnancy losses in dairy cows.

MATERIALS AND METHODS

Experimental animals: In experiment I, 141 Holstein cows in their 2nd parity, kept at a dairy farm in Beian City (October 2018 to July 2019), Heilongjiang Province, China, were selected. The cows were acyclic and at 50 days postpartum stage, with body condition score of 2.5-3.5. They were kept in a cowshed with total mixed rations (TMR). TMR included silage corn, alfalfa, leymus, compressed corn and lactation feed of dairy cow. Feed was prepared according to National Research Council Nutrient Standard (Anonymous, 2001). Feed and water were provided ad libitum. Cows were milked 3 times a day, and the average milk yield was >30 kg/d. PGF2α (0.4 mg) was injected in all cows on the 14th day and the 28th day after parturition to promote uterine involution.

All trials were conducted as per guideines of the Animal Processing Ethics Committee of Northeast Agricultural University, China. Hormones (GnRH, PGF2α and hCG) used in the experiment were purchased from Ningbo Sansheng Pharmaceutical Company, China and were administered through intra-muscular route.

Experimental design: All experimental cows were subjected to TAI protocol consisting of GnRH (100 μg), PGF2α (0.4 mg) and GnRH (100 μg). The day of the first GnRH injection was herein called day 0, PGF2α was injected 7 days later, 2nd GnRH injection was given 56 h afterwards, and cows were artificially inseminated 16 h after the 2nd GnRH injection. For insemination, frozen-thawed semen, 0.25 ml containing at least 10 million motile sperm (Semex Alliance, Canada) was used. For pregnancy diagnosis, cows were examined ultrasonographically 35 days after AI, using a B-mode, real time ultrasound scanner fitted with a 6.0 MHz frequency trans-rectal probe (Beijing Eastern Bell Technology Group, Beijing, China).

After insemination, the experimental cows were randomly assigned to three groups, as given below:
(1): Control group (52): Animals of this group were subjected to TAI protocol described above.
(2): GnRH group (31): Animals of this group were subjected to TAI protocol as for control group plus additional GnRH (100 μg) injection on the 5th day after AI.
(3): hCG group (58): In addition to the TAI protocol given to control group, animals of this group were also injected with hCG (1500 IU) on the 5th day after AI.

Progesterone detection: Blood was collected from the tail root vein of each cow on 5, 7, 9, 16, and 23 days after AI. Blood samples were placed in the dark for 2 to 3 h, serum was separated using a centrifuge and stored at -80°C. A progesterone ELISA kit (Shanghai Xinle Biotechnology Co., Shanghai, China) was used to determine serum progesterone concentrations according to the manufacturer's instructions. Briefly, samples and strandards were processed as per instructions provided with the kit. Then absorbance of samples and standards was determined at 450 nm wavelength, using a microplate reader. The concentrations of progesterone in samples were determined from a standard curve generated between concentrations and absorbance values of the standards. The intra- and inter-assay coefficients of variation were 10.0 and 15.0%, respectively.

In vitro culture of endometrial cells: In experiment II, the laboratory-preserved bovine endometrial cells (Otwo Biotech Inc, Shenzhen, China) were thawed and cultured in a 60 mm culture dish at a density of 1×10^5/mL with DMEM/F_{12}+10% FBS+1% penicillin-streptomycin, 5% CO_2, 37.5°C, and saturated humidity for 24h. When the cell growth confluence was more than 80%, the culture medium was replaced. The cell samples were divided into three groups with 6 plates in each group. The endometrial

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cells were cultured in GnRH (1.0 µg/mL) or hCG (10.0 IU/mL) for 12h. Then, the culture medium in the culture dish was centrifuged at 1500 rpm for 5 minutes and the supernatant was drawn to determine PGF2α contents. The culture dish was washed three times with PBS, and the cells were dispersed with pipette. After centrifugation, the cells were collected for total RNA and proteins extraction. The independent experiment was repeated three times.

Analysis for PGF2α contents in culture medium: The cell-free culture medium was used to measure PGF2α contents, using an ELISA kit (Shanghai Xinle Biotechnology Co. Ltd, Shanghai, China) and following the instructions provided by manufacturer. The protocol of the assay was the same as described above for serum progesterone.

Real-time quantitative PCR analysis: Total RNA was extracted from the cultured bovine endometrial cells, using TRIzol reagent and following the instructions provided by the manufacturer (Invitrogen, China), as described previously (Wang *et al.*, 2019). After reverse transcription, the expression of IL-6, TLR-4, NF-Kb, and LIF was assayed by real-time quantitative PCR (RT-qPCR). The special primers of the detected genes are listed in Table 1 and were synthesized by BGI Company. Reference gene was bovine β-actin. The relative expression of the detected genes was calculated according to $2^{-\Delta\Delta Ct}$ method (Wang *et al.*, 2019).

Western blot analysis: The total protein was lysed using RIPA buffer (Invitrogen) for 10 min, using the method described by Wang *et al.* (2019). Antibody concentrations were as follows: Primary antibodies 1:300 (IL-6, TLR-4, NF-κB, and LIF); HRP labeled secondary antibody 1:3000. The immune response bands were detected by the ECL-Western blot assay system (Amersham Biosciences, USA). Image J software (NIH, Bethesda, MD) was used to quantify the band density.

Statistical analysis: SPSS software (version 19.0, IBM Corp., New York, USA) was used to perform statistical analysis. Comparison of percentages were made by chi-square test and other data were analysed by ANOVA. Multiple comparisons were performed using the Duncan's multiple range test. Data are presented as the means± standard deviation (SD).

RESULTS

Effects of GnRH and hCG on the pregnancy rates: The results of the present study showed that the pregnancy rates of cows in both GnRH and hCG treated groups were higher than that of the control group, but the difference was non-significant. Also, there was non-significant difference in the pregnancy rates of cows between GnRH and hCG treatment groups (Table 2).

Effects of GnRH and hCG on blood progesterone secretions: In the present study, blood progesterone concentrations were measured to monitor the effects of GnRH and hCG treatment on activity of corpus luteum in cows. The results showed that blood progesterone levels in cows of GnRH, hCG and control groups increased gradually from day 5 to day 23 after insemination. On the 5th day after insemination, there was non-significant difference in serum progesterone levels among cows of three groups (Table 3). However, on days 7, 9, 16, and 23 after insemination, serum progesterone levels in aninals of GnRH and hCG groups were significantly higher (P<0.05) than those of the control group, while the difference between the former two groups was non-significant (Table 3).

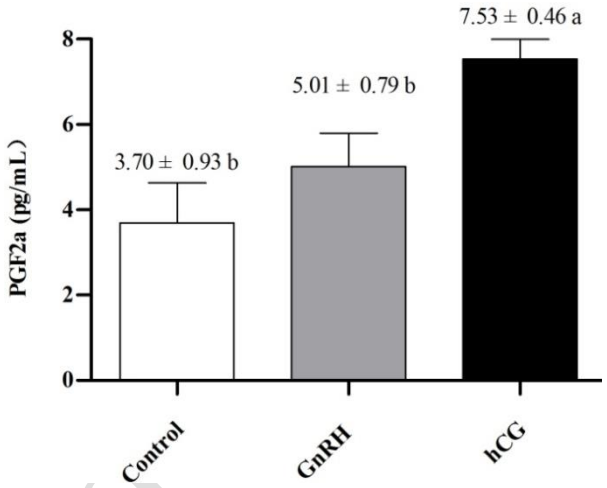

Fig. 1: Effects of GnRH and hCG on prostaglandin secretion by endometrial cells. Bars represent mean±SD (n=6). The groups with different alphabets are different significantly (P<0.05).

Table 1: Primers with their sequences used in the experiment

Genes	Genomic sequence	Primer sequence (5`–3`)	Product length (bps)
IL-6	AC_000161.1	TCC AGA ACG AGT ATG AGG CAT CCG AAT AGC TCT CAG	263
LIF	NM_173931	CATCCCTGTCCCAGCAACCTCATG ATGATGCGGTACAGCTCCACCAG	215
TLR-4	DQ839567.1	GGATGAAGACTGGGTGCGGAATG CTGGATGATATTGGCGGCGATGG	124
NF-κB	NM_001076409	CGCTTCCGCTACAAGTGTGAGG TCTTGATAGTGGGGTGGGTCTTGG	91
β-actin	NM_173979.3	GCGGCATTCACGAAACTACCTT TCCTGCTTGCTGATCCACATCT	268

Table 2: Effects of GnRH and hCG on the pregnancy rates in dairy cows

	Number of cows inseminated	Number of pregnant cows	Conception rate (%)	*P*-value*
GnRH group	31	16	51.61	0.179
hCG group	58	31	53.45	0.075
Control group	52	19	36.54	

* : *P* value compared with the control group.

Table 3: Effects of GnRH and hCG on progesterone secretion (ng/ml) by corpus luteum on different days after insemination in dairy cows

Groups	Number of days after artificial insemination				
	5	7	9	16	23
GnRH (n=31)	5.8±1.4a	7.9±1.2a	8.6±1.3a	10.2±1.1a	11.6±1.4a
hCG (n=58)	5.9±1.2a	8.2±1.1a	9.5±1.4a	11.9±1.2a	12.3±1.3a
Control (n=52)	5.6±0.9a	6.2±1.2b	6.9±1.1b	8.3±1.2b	9.2±1.3b

Groups are shown with number of cows evaluated. Values with different alphabets in the same column indicate significant differences (P<0.05).

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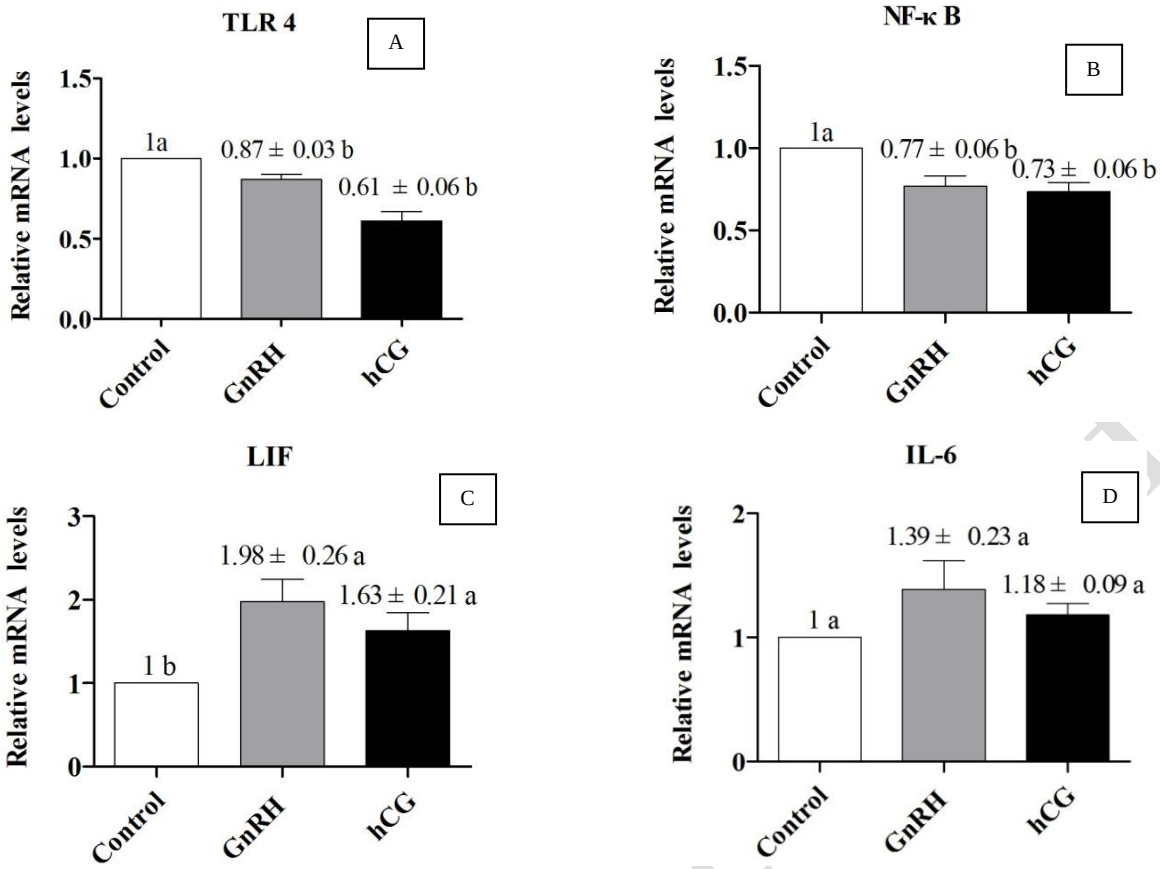

Fig. 2: Effects of GnRH and hCG on the mRNA expression of TLR-4, NF-κB, LIF, and IL-6 in endometrial cells. Bars represent mean±SD (n=6). The groups with different alphabets are different significantly (P<0.05).

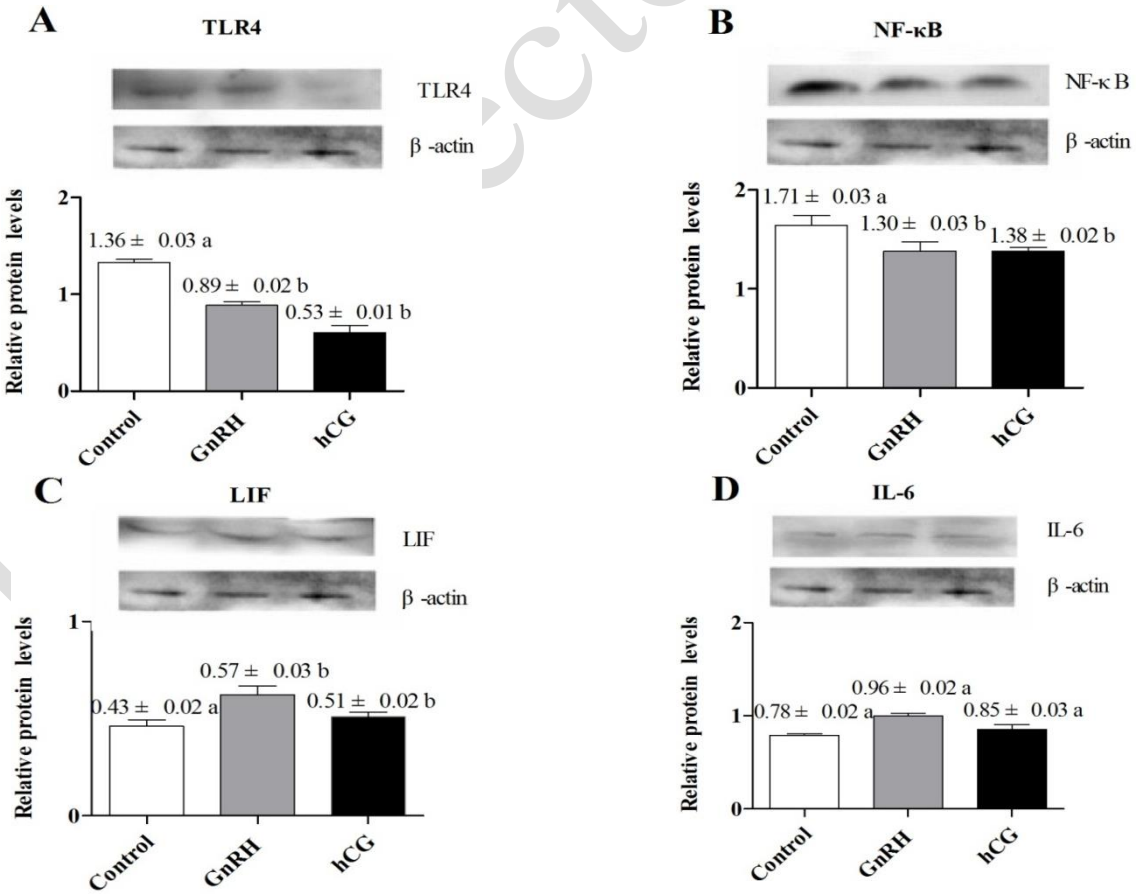

Fig. 3: Effects of GnRH and hCG on the protein expression of TLR-4, NF-κB, LIF, and IL-6 in endometrial cells. Bars represent mean±SD (n=6). The groups with different alphabets are different significantly (P<0.05).

Effects of GnRH and hCG on PGF2α secretion by endometrial cells: In the present study, the amount of PGF2α secreted by hCG-treated endometrial cells was significantly higher than that of GnRH-treated and control groups ($P<0.05$). However, the difference in level of prostaglandin secreted by endometrial cells of GnRH treated and control groups was non-significant (Fig. 1).

Effects of GnRH and hCG on the expression of receptivity genes in endometrial cells: The results regarding the effects of GnRH and hCG on the expression of uterine receptivity genes in endometrial cells have been displayed in Figs. 2 and 3. These figures show that GnRH and hCG treatment promoted the expression of TLR-4 and NF-κB genes, while it inhibited the expression of LIF gene. However, GnRH as well as hCG treatment had no effect on the expression of IL-6 gene in endometrial cells.

DISCUSSION

The effect of GnRH and hCG on the fertility: Estrus synchronization and TAI has been shown to improve the fecundity of cows through increasing the ovulation rates (Giordano *et al.*, 2015); however, the pregnancy rate was only 40-50% at 35 days. This suggests that the application of TAI technology did not reduce early embryo losses in cows. In our experiment, administration of GnRH and hCG 5 days after TAI increased pregnancy rates compared to the control group, however, the difference was statistically non-significant. It might have been due small sample size in this study. Further studies with larger sample size seem necessary for better understanding in this regard.

Serum progesterone levels in cows: The period of implantation has been considered as the key period of embryo loss in cows. The rate of embryo loss at this stage is 25 to 30 percent in cows. Colazo *et al.* (2013) confirmed that progesterone supplementation reduced pregnancy loss after TAI. According to Clemente *et al.* (2009), progesterone promoted embryo development and implantation through the endometrium pathway. In the present study, administration of both GnRH and hCG 5 days after TAI increased progesterone level of blood during the period of study (up to 23 days after TAI), though the increase in pregnancy rate was non-significant. It appears that both GnRH and hCG can promote the development of early corpus luteum, and then improve the level of progesterone, so as to promote the establishment of embryo development, implantation and pregnancy recognition (López-Gatius and Garcia-Ispierto, 2020).

It is well known that after bovine embryo enters the uterus, trophoblast cells of the embryo secrete interferon-τ, which inhibits the secretion of PGF2α by endometrial cells and prevents lysis of corpus luteum. So, it maintains the continuous secretion of progesterone and the establishment of uterine receptivity, which is conducive to embryo implantation and pregnancy establishment (Martal *et al.*, 1997). In addition, PGF2α can also be synthesized from bovine preimplantation embryos, which stimulates vascular endothelial growth factor, thereby stimulating angiogenesis and increasing vascular permeability to prepare for embryo implantation (Grycmacher *et al.*, 2019). Therefore, the basic secretion of prostaglandins is beneficial to embryo implantation, while the pulse release of PGF2α secreted by uterus causes luteal lysis and affects pregnancy.

Secretion of PGF2α by endometrial cells: In the present study, in-vitro treatment of bovine endometrial cells with 10.0 IU of hCG significantly increased the secretion of PGF2α compared to controls. However, no such effects were seen with GnRH. When hCG acts on the uterus, it binds to LH receptors of endometrium, promotes the secretion of PGF2α by endometrial cells, and affects embryo implantation (Zolini *et al.*, 2019). In the TAI program of dairy cows, different studies used different concentrations of hCG (1500-3300 IU) and obtained variable results, with the effects on pregnancy rate ranging from +14.0% to -7.7% (Nascimento *et al.*, 2013). This was attributed to the fact that excessive hCG promotes the secretion of PGF2α, affects the function of pregnancy recognition, stimulates the lysis of corpus luteum, and reduces the pregnancy rate. Therefore, use of low concentrations of hCG (1000-1500 IU) can be suggested after TAI in dairy cows. GnRH can be used to improve the implantation rate and pregnancy rate of dairy cows, as it had little effect on prostaglandins secreted by endometrial cells, which may be more conducive to maintain endocrine balance in dairy cows.

Expression of uterine receptivity related genes: As stated above, during maternal recognition of pregnancy in cows, interferon-τ secreted by embryonic trophoblasts inhibits the secretion of PGF2α by the endometrium. Resultantly, the corpus luteum persists and is transformed into gestational corpus luteum, which continues to secrete progesterone and maintains pregnancy (Brooks *et al.*, 2014). In addition, the immune response of trophoblasts and endometrial cells is also involved during pregnancy (Wu *et al.*, 2018). TLR-4, as an important immune factor, is involved in the local immune regulation of the endometrium. It activates NF-κB by triggering intracellular signaling pathways involved in the early stage of immune response and various stages of inflammatory response. It also plays an important role in the release of inflammatory factors, cell proliferation and apoptosis. During implantation, the maternal immune function is generally supressed to a certain extent. In the present experiment, in-vitro treatment of bovine endometrial cells with GnRH and hCG inhibited the signal pathways of TLR-4 and NF-κB, and promoted the expression of LIF, improving uterine receptivity. By inhibiting the immune response of uterus, the uterine environment was conducive to embryo implantation, which would improve the pregnancy rates.

Conclusions: Administration of GnRH and hCG at 5 days after TAI could improve the uterine receptivity in dairy cows through increasing functional activity of corpus luteum, which alters the expression of genes related to uterine receptivity.

Authors contribution: PZ and HH conceived the idea and designed the study. PZ, RF, FH, LH and MW

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executed the experiments and analyzed the sera and tissue samples. PZ also analyzed the data. All authors participated in interpretation of the data, critically reviewed the manuscript for important intellectual contents and approved the final version.

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