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RESEARCH ARTICLE

The relationship between progesterone and Th-related cytokines in plasma during early pregnancy in cows

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Abstract In cows, progesterone (P4) is essential for the maintenance of pregnancy and successful embryo development is dependent on the maternal immunomodulation of Th-related cytokines. However, *in vivo* investigation of the relationship between P4 and Th immunity in cattle remains incomplete. Therefore, we evaluated plasma P4 concentrations and expressions of three Th-related cytokines, interleukins IL-1β, IL-4 and IL-6, in 15 pregnant and 11 non-pregnant cows 0, 14, 18, 21, and 28 d post artificial insemination. Pregnant cows had significantly higher plasma P4 levels and pregnant cows with higher P4 on 14 d tended to have higher P4 in the subsequent period of pregnancy. There was no difference in IL-4 and IL-6 expression between pregnant cows and non-pregnant cows, whereas plasma IL-1β was temporally upregulated on 21 d. The cytokines measured were not affected in either the high-P4 group ($>$ 11.1 ng·mL^{-1}) or the low-P4 group ($<$ 11.1 ng·mL^{-1}) in pregnant cows. A weak negative correlation between IL-1β and IL-6 was observed, but none of the cytokines was associated with a change in plasma P4. In conclusion, there was no clear relationship between P4 and Th immunity in maternal plasma in the pregnant cows, which differs from what occurs in humans and mice during early pregnancy.

Keywords dairy cow, progesterone, pregnancy, cytokine

1 Introduction

In cows, the establishment of pregnancy requires multifactorial regulation, including hormones and cytokines[1,2]. In particular, high levels of plasma progesterone (P4) are essential for both the maternal recognition and the maintenance of pregnancy. Studies in humans and mice have indicated that successful pregnancy is linked to the predominance of Th2 immunity[3,4]. Although the importance of the Th1/Th2 paradigm in pregnancy has been questioned[5,6], new evidence indicates that a switch from a Th1 immune response to a higher Th2 immune response may be necessary in pregnant cows[7,8]. Circulating immune cells, together with cytokines are critical in regulating luteal functions and maternal recognition of pregnancy[9,10]. Elevation of P4, which is primarily secreted by the immunoregulated corpus luteum, can be detected in peripheral blood[11]. It is noteworthy that an *in vitro* study using peripheral blood mononuclear cells (PBMCs) has indicated that P4 is an important regulator of Th1/Th2/Th17 and Treg immunity during pregnancy in cows[7]. However, the *in vivo* relevance of changes in the Th-related cytokines to P4 has not been fully investigated.

In addition to P4, changes in a wide array of maternal genes (e.g., IFN-stimulated genes) and immunomodulators, which are caused by conceptus signaling during the period of early pregnancy, can be detected in peripheral blood leukocytes (PBL)[12,13]. To better understand whether P4 affects the expression of Th-related cytokines in plasma during early bovine pregnancy, we investigated levels of plasma P4 and typical Th cytokines in both pregnant cows and cows with pregnancy losses.

2 Materials and methods

2.1 Animals and artificial insemination

All experiments involving animals were approved by the Biological Studies Animal Care and Use Committee of Hubei Province, China. All procedures were conducted in

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Chinese Holstein cows ($n = 26$) from a commercial dairy farm (Wuhan Hui'erkang Yangtze Diary Co., Ltd., Wuhan, China) were used in this study. All cows were kept under the same feeding regime. Estrous synchronization was carried out following a fixed-time artificial insemination (AI) protocol (Sansheng Pharmaceutical Co. Ltd, Ningbo, China). Briefly, estrous cycles were synchronized by intramuscular administration of 100 µg gonadotropin-releasing hormone (GnRH) followed by injection of 0.4–0.6 mg cloprostenol sodium 7 d later (i.e., 8 d), then 100 µg of GnRH was administrated again after 48 h. Cows were inseminated 18–20 h after the second GnRH administration. Pregnancy was determined using rectal palpation 60 days after AI.

2.2 Sampling

Blood samples were collected from the caudal vein on 0, 14, 18, 21 and 28 d following the last round of AI. Samples were collected in 10 mL EDTA-K2 heparinized blood collection tubes (Aosaite Medical Devices Co. Ltd, Shandong, China). Samples were kept on ice during the transportation from facility to laboratory. Plasma was obtained by centrifugation at 3000 *g* for 15 min at 4°C, and was stored at −80°C until used.

2.3 P4 assay

P4 concentration in plasma was determined by chemiluminescence immunoassay as described previously[14]. The assay was carried out using an Access2 Immunoassay System (Beckman Coulter Inc., Brea, CA, USA). The range of the standard concentration was 0.08–40 $ng \cdot mL^{-1}$, and the sensitivity of the procedure was 0.08 $ng \cdot mL^{-1}$. Results were reported within 30 min. The required reagents, quality control and operations strictly followed the manufacturer's recommendations.

2.4 Cytokine assays

Cytokines were examined on the iMark™ Absorbance Microplate Reader with Microplate Manger® 6 software (Bio-Rad, Hercules, CA, USA). Concentrations of three Th-related cytokines, interleukins IL-1β, IL-6, and IL-4, in plasma were accessed using commercially available ELISA kits according to the manufacturer's instructions: Bovine IL-1β ELISA Reagent Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA); Bovine IL-4 ELISA Kit (Bio-Rad); Bovine IL-6 ELISA Kit (RapidBio, West Hills, CA, USA). Each sample was run in duplicate.

2.5 Statistical analysis

Data are presented as mean±SEM. For statistical analysis of P4 and cytokine data, the Sigma Stat Statistical Software Version 3.5 (Systat Software Inc., San Jose, CA, USA) was used. One-way ANOVA was used to analyze the significance of differences. When this analysis indicated a significant effect for a model, the LSD was use to test for significant differences. Correlations were calculated by Pearson Correlation Coefficients using 2-tailed test of significance. $P \leqslant 0.05$ was considered statistically significant.

3 Results and discussion

3.1 Changes of P4 in pregnant and non-pregnant cows during early pregnancy

Th-related cytokines in bovine plasma during early pregnancy up to implantation were monitored. Plasmatic concentrations of P4 were determined in parallel from the single-cell embryo (zygote) in oviduct on 0 d through blastocyst elongation on 14 d, maternal recognition of pregnancy on 18 d, appearance of caruncles/cotyledons on 21 d, and progressive implantation on 28 d. In pregnant cows, P4 increased steadily until implantation on 28 d, with a significant elevation detected on 14 d (Fig. 1a). Although the differences in the measurements between 14, 18 and 21 d were not significant, P4 concentration on 21 d was significantly higher than 14 d. These results agreed with the observations of Shirasuna et al.[11]. Non-pregnant cows displayed a similar trend, whereas P4 levels were significantly lower than those in pregnant cows and changes plateaued from 14 to 28 d. Higher P4 concentrations in bovine plasma prevent immunological rejection of the fetal allograft and benefit pregnancy probably by suppressing NK cell activity and specific components of the immune system[15]. Additionally, P4 induces the endometrium of the ewe to produce ovine uterine serpin, which is also present in cattle, and inhibits a wide variety of immune responses by inhibiting protein kinase C and interleukin-2 pathways[16]. The significant P4 elevation on 14 d and slight increase at the subsequent time points in non-pregnant cows indicate that early embryonic death might have occurred. In contrast to this study, our detections in another group of non-pregnant heifers showed that levels of P4 declined on 21 d post AI and increased on 28 d, suggesting that cows without successful pregnancy enter into the next estrous cycle (unpublished data).

3.2 Characterization of bovine plasma cytokine expression

Expression profiles of the three Th-related cytokines (IL-1β, IL-6, and IL-4) were characterized in plasma by ELISA. There were no significant changes during early pregnancy in either pregnant or non-pregnant cows (Fig. 1b, Fig.1c, Fig.1d). In humans and primates, IL-1β

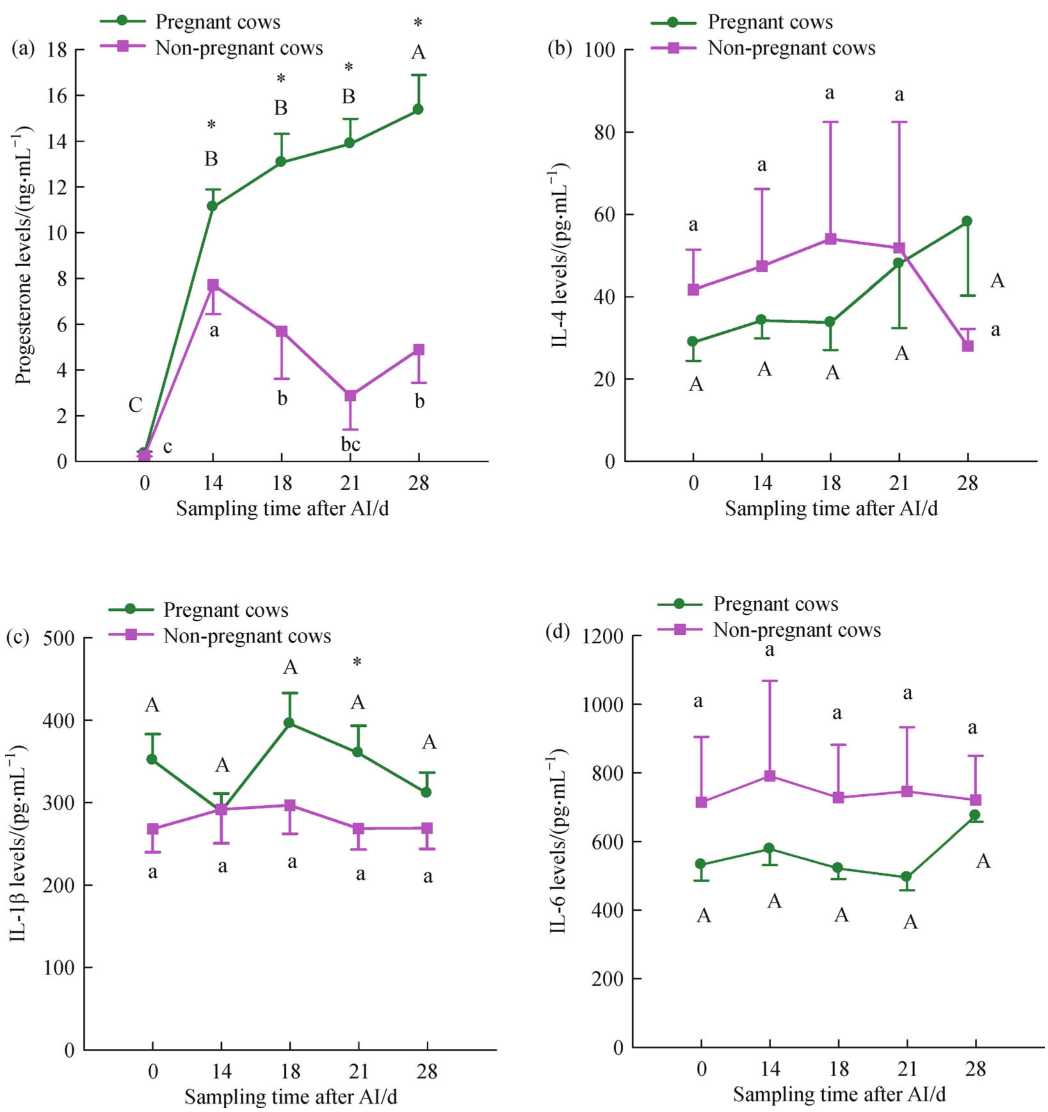

Fig. 1 Changes in P4 and cytokines in pregnant (n = 15) and non-pregnant (n = 11) cows after artificial insemination (AI). (a) Levels of P4; (b) levels of cytokine IL-4; (c) levels of cytokine IL-1β; (d) levels of cytokine IL-6. Data are shown as mean±SEM; Different letter case (uppercase = pregnant, lowercase = non-pregnant) indicate significant differences ($P \leqslant 0.05$) within the same group of cows; * means significance ($P \leqslant 0.05$) between pregnant and non-pregnant groups.

is involved in embryonic implantation and establishment of pregnancy through acting as a physiological mediator of acute phase response during conceptus invasion and placental formation[17]. In contrast to humans[18], our results show that plasma IL-1β concentration in pregnant cows was significantly higher ($P \leqslant 0.05$) than non-pregnant cows on 21 d (Fig. 1c). Consistent with our findings, it has been reported that IL-1β mRNA in bovine endometrium was temporally upregulated during early pregnancy[8,19].

Based on the mean P4 concentration (11.1 ng·mL^{-1}) on 14 d in pregnant cows, the pregnant cows were divided into two groups: high-P4 cows (> 11.1 ng·mL^{-1}, n = 6) and low-P4 cows (< 11.1 ng·mL^{-1}, n = 9). Significant difference in P4 were seen between the two groups of cows at each time point (Fig. 2a). However, as illustrated in Fig. 2, the expression of the three cytokines, IL-1β, IL-6, and IL-4, was not significantly upregulated in either group. Elevation of IL-4 transcription in PBMCs and IL-6 expression in plasma have been observed during normal pregnancy of women[20,21]. In humans, both cytokines can inhibit the generation of Th1 cells and secretion of Th1-derived cytokines[22,23]. Consistent with our results, Oliveira et al. reported that the accumulation of IL-6 mRNA was not upregulated in bovine endometrial tissue during early pregnancy[8]. In contrast, elevated mRNA for IL-6 in elongating conceptus and endometrium of cows has been observed[24]. As is well known, cows have no implantation per se, but a process of elongation and attachment, and their placenta is syndesmochorial with low

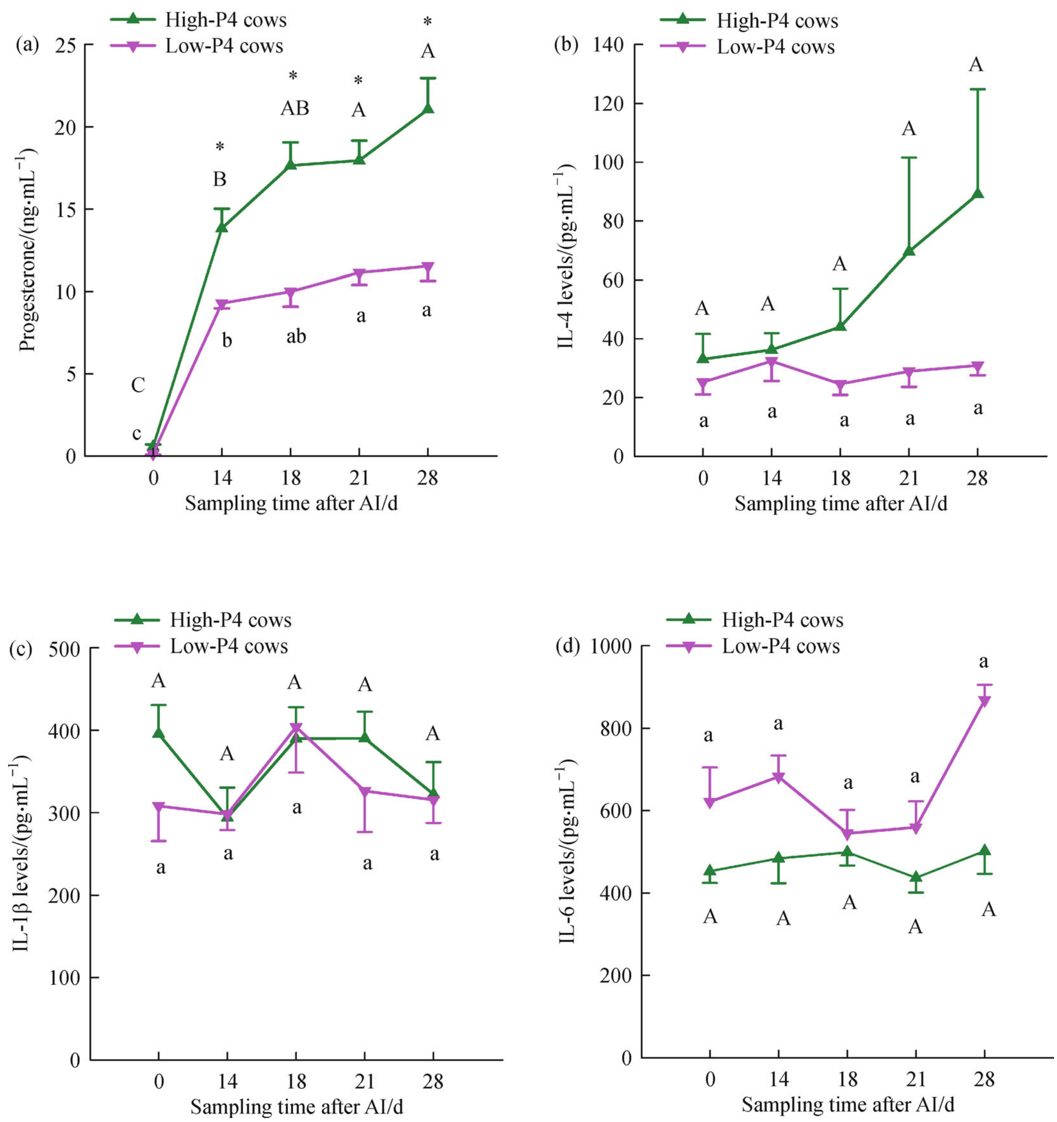

Fig. 2 Levels of P4 and cytokines in high-P4 and low-P4 pregnant cows. (a) Levels of P4; (b) levels of cytokine IL-4; (c) levels of cytokine IL-1β; (d) levels of cytokine IL-6. Pregnant cows were divided into two groups based on the progesterone plasma levels on 14 d: high-P4 cows ($>$ 11.1 ng·mL^{-1}, n = 6) and low-P4 cows ($<$ 11.1 ng·mL^{-1}, n = 9); Data are shown as mean±SEM; Different letter case (uppercase = high-P4 cows, lowercase = low-P4 cows) indicate significant ($P\leqslant 0.05$) differences within the same group of cows; * means significance ($P\leqslant 0.05$) between high-P4 and low-P4 groups.

invasiveness and restricted areas of attachment. In human and murine, their invasive implantation and erosive placentation (hemochorial) is regarded as diametrally different. Therefore, we speculate that the different regulation of cytokines between humans and cows is due to different mechanisms of maternal recognition of pregnancy or embryo tolerance.

3.3 Analysis of correlations between P4 and cytokines

Correlation analysis was performed to further investigate relationships between P4 and the three cytokines, as well as relationships among these cytokines. As shown in Table 1, there were no significant correlations between the plasma P4 concentrations and the cytokines during early pregnancy. Notably, plasma IL-1β was found to have a weak negative correlation with the IL-6 in both pregnant (r = –0.27458, P = 0.01713) and non-pregnant cows (r = –0.30617, P = 0.02435). Knowledge of IL-1β and IL-6 in pregnant cows is still limited, and further studies are needed. In contrast to our *in vivo* investigation, Maeda et al. reported that P4 strongly inhibit the differentiation of Th cells into Th1 and Th17 in the pregnant cows by decreasing the expression of T-bet and RORC (Th1 and Th17 transcription factors, respectively) and enhancing IL-4 expression in *in vitro* cultured PBMCs[7]. However, this only occurred with the highest dose of P4 (10 μg·mL^{-1}), which is much higher than detected in the serum during

Table 1 Pearson correlation analysis of P4 and three cytokines (IL-4, IL-1β, and IL-6) in pregnant and non-pregnant cows from 0 to 28 d post artificial insemination

Cytokine		Pregnant cows (n = 15)				Non-pregnant cows (n = 11)			
		IL-4	IL-1β	IL-6	P4	IL-4	IL-1β	IL-6	P4
IL-4	r	1	0.17801	−0.05879	0.20415	1	−0.04775	0.10087	0.01629
	P	–	0.12652	0.61637	0.07894	–	0.74455	0.49041	0.91154
IL-1β	r		1	−0.27458*	0.01779		1	−0.30617*	0.07586
	P		–	0.01713	0.87958		–	0.02435	0.58562
IL-6	r			1	0.09991			1	0.07364
	P			–	0.39373			–	0.59314
P4	r				1				1
	P				–				–

Note: *, significant at $P \leqslant 0.05$.

pregnancy[25]. It is probably that the peripheral P4 is too low to change cytokine secretion, thus no change in their plasma concentrations was detected in this study. Given that P4 concentrations in the uterine tissue, the uterine artery, and the ovarian artery are very high[26], it is possible that the switch to Th2 immunity mainly occurs in local tissues. For example, a significant downregulation of IL-1β was detected in uterine fluid (UF) on 8 d of pregnancy[27] and in bovine endometrial tissue, transcription of IL-1β is lowest on 13 and 16 d[8]. In fact, as has been suggested, cytokines are likely to be major participants, as autocrine factors, that direct the events of early pregnancy[28].

It is only recently that the Th1/Th2 cytokine pattern during pregnancy has been observed in ruminants[7,8]. Also, recent studies have found that monocytes/macrophages and dendritic cells in bovine maternal endometrium are important in regulating the cytokine network[29,30]. This confirms earlier speculation that the major source of cytokines in the reproductive tract might be the non-lymphoid cells of the endometrium and trophoblast[1].

4 Conclusions

It is concluded that the properties of some bovine Th-related cytokines in plasma are considerably different from those in humans or mice during early pregnancy, in that there is no clear relationship between maternal P4 and Th immunity in the pregnant cows.

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All applicable institutional and national guidelines for the care and use of animals were followed.

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