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...
ELISA kits according to the manufacturer
plasma were accessed using commercially available
Th-related cytokines, interleukins IL-1
(Bio-Rad, Hercules, CA, USA). Concentrations of three
Microplate Reader with Microplate Manger® 6 software
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 used to test for
significant differences. Correlations were calculated by
Pearson Correlation Coefficients using 2-tailed test of
significance. P≤0.05 was considered statistically signifi-
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 concentra-
tions 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

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

2.3 P4 assay
P4 concentration in plasma was determined by chemilu-
minescence 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·mL
–1, and the sensitivity of the procedure was 0.08 ng·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

accordance with the Hubei Provincial Regulation on
Administration of Laboratory Animals (10/1/2005).

Chinese Holstein cows (n = 26) from a commercial dairy
farm (Wuhan Hui’erkan 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 administra-

21 d, and successive implantation on 28 d. In pregnant
cows, P4 increased steadily until implantation on 28 d,
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the immune system[15]. Additionally, P4 induces the
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2.5 Statistical analysis

Data are presented as mean±SEM. For statistical analysis
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-1\(\beta\) concentration in pregnant cows was significantly higher \((P \leq 0.05)\) than non-pregnant cows on 21 d (Fig. 1c). Consistent with our findings, it has been reported that IL-1\(\beta\) mRNA in bovine endometrium was temporally upregulated during early pregnancy\[8,19\].

Based on the mean P4 concentration (11.1 ng\(\cdot\)mL\(^{-1}\)) on 14 d in pregnant cows, the pregnant cows were divided into two groups: high-P4 cows \((> 11.1 \text{ ng}\cdot\text{mL}\(^{-1}\), \(n = 6\)) and low-P4 cows \((< 11.1 \text{ ng}\cdot\text{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-1\(\beta\), 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\[19\]. 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

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**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-1\(\beta\); (d) levels of cytokine IL-6. Data are shown as mean\(\pm\)SEM; Different letter case (uppercase = pregnant, lowercase = non-pregnant) indicate significant differences \((P \leq 0.05)\) within the same group of cows; * means significance \((P \leq 0.05)\) between pregnant and non-pregnant groups.
invasiveness and restricted areas of attachment. In human and murine, their invasive implantation and erosive placentation (hemochorial) is regarded as diametrically 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 \((10 \, \mu g \cdot mL^{-1})\), which is much higher than detected in the serum during

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**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 \( (> 11.1 \, ng \cdot mL^{-1}, n = 6)\) and low-P4 cows \( (< 11.1 \, ng \cdot mL^{-1}, n = 9)\); Data are shown as mean±SEM; Different letter case (uppercase = high-P4 cows, lowercase = low-P4 cows) indicate significant \((P \leq 0.05)\) differences within the same group of cows; * means significance \((P \leq 0.05)\) between high-P4 and low-P4 groups.
during pregnancy. 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, 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 and in bovine endometrial tissue, transcription of IL-1β is lowest on 13 and 16 d of pregnancy. In fact, as has been suggested, cytokines are likely to be major participants, as autocrine factors, that direct the events of early pregnancy.

It is only recently that the Th1/Th2 cytokine pattern during pregnancy has been observed in ruminants. Also, recent studies have found that monocytes/macrophages and dendritic cells in bovine maternal endometrium are important in regulating the cytokine network. 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.

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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Compliance with ethics guidelines Lei Cheng, Youdong Xin, Xiaohua Liu, Xiuzhong Hu, Min Xiang, Dingfa Wang, and Shuhong Zhao declare that they have no conflict of interest or financial conflicts to disclose.

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

References

1. Hansen P J. Interactions between the immune system and the ruminant conceptus. Journal of Reproduction and Fertility, 1995, 49: 69–82
2. Bazer F W. Pregnancy recognition signaling mechanisms in ruminants and pigs. Journal of Animal Science and Biotechnology, 2013, 4(1): 23
3. Wegmann T G, Lin H, Guilbert L, Mosmann T R. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunology Today, 1993, 14(7): 353–356
4. Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. Immunology Today, 1997, 18(10): 478–482
5. Arck P, Hansen P J, Mulac Jericevic B, Piccinni M P, Szekeres-Bartho J. Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress. American Journal of Reproductive Immunology, 2007, 58(3): 268–279
6. Chaouat G. The Th1/Th2 paradigm: still important in pregnancy? Seminars in Immunopathology, 2007, 29(2): 95–113
7. Maeda Y, Ohtsuka H, Tomioka M, Oikawa M. Effect of progesterone on Th1/Th2/Th17 and regulatory T cell-related genes in peripheral blood mononuclear cells during pregnancy in cows. Veterinary Research Communications, 2013, 37(1): 43–49
8. Oliveira L J, Mansouri-Attia N, Fahey A G, Browne J, Forde N, Roche J F, Lonergan P, Fair T. Characterization of the Th profile of the bovine endometrium during the oestrous cycle and early pregnancy. PLoS ONE, 2013, 8(10): e75571
9. Shirasuna K, Nitta A, Sineenard J, Shimizu T, Bollwein H, Miyamoto A. Vascular and immune regulation of corpus luteum development, maintenance, and regression in the cow. Domestic Animal Endocrinology, 2012, 43(2): 198–211
10. Walusimbi S S, Pate J L. Physiology and endocrinology symposium: role of immune cells in the corpus luteum. Journal of Animal Science, 2013, 91(4): 1650–1659
11. Shirasuna K, Matsumoto H, Kobayashi E, Nitta A, Haneda S, Matsui M, Kawashina C, Kida K, Shimizu T, Miyamoto A. Upregulation of interferon-stimulated genes and interleukin-10 in peripheral blood immune cells during early pregnancy in dairy
12. Dixit V D, Parvizi N. Pregnancy stimulates secretion of adrenocorticotropic and nitric oxide from peripheral bovine lymphocytes. *Journal of Reproduction and Development*, 2012, **58**(1): 84–90

13. Gifford C A, Racicot K, Clark D S, Austin K J, Hansen T R, Lucy M C, Davies C J, Ott T L. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. *Journal of Dairy Science*, 2007, **90**(1): 274–280

14. Kohen F, Kim J B, Lindner H R, Collins W P. Development of a solid-phase chemiluminescence immunoassay for plasma progesterone. *Steroids*, 1981, **38**(1): 73–88

15. Scheibl P, Zerbe H. Effect of progesterone on the immune system in consideration of bovine placentation retention. *Deutsche Tierarztliche Wochenschrift*, 2000, **107**(6): 221–227

16. Peltier M R, Hansen P J. Immunoregulatory activity, biochemistry, and phylogeny of ovine uterine serpin. *American Journal of Reproductive Immunology*, 2001, **45**(5): 266–272

17. Geisert R, Fazleabas A, Lucy M, Mathew D. Interaction of the conceptus and endometrium to establish pregnancy in mammals: role of interleukin 1β. *Cell and Tissue Research*, 2012, **349**(3): 825–838

18. Daponte A, Pourmaras S, Deligeoroglou E, Skentou H, Messinis I E. Serum interleukin-1β, interleukin-8 and anti-heat shock 60 Chlamydia trachomatis antibodies as markers of ectopic pregnancy. *Journal of Reproductive Immunology*, 2012, **93**(2): 102–108

19. Groebner A E, Schulke K, Scheifeld J C, Fusch G, Sinowatz F, Reichenbach H D, Wolf E, Meyer H H, Ulbrich S E. Immunological mechanisms to establish embryo tolerance in early bovine pregnancy. *Reproduction, Fertility, and Development*, 2011, **23**(5): 619–632

20. Marzi M, Vigano A, Trabattoni D, Villa M L, Salvaggio A, Clerici E, Clerici M. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy.

21. Palm M, Axelsson O, Wernroth L, Larsson A, Basu S. Involvement of inflammation in normal pregnancy. *Acta Obstetricia et Gyneco-logic Scandanavica*, 2013, **92**(5): 601–605

22. O’Garra A, Arai N. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends in Cell Biology*, 2000, **10**(12): 542–550

23. Prins J R, Gomez-Lopez N, Robertson S A. Interleukin-6 in pregnancy and gestational disorders. *Journal of Reproductive Immunology*, 2012, **95**(1–2): 1–14

24. Schäfer-Somi S. Cytokines during early pregnancy of mammals: a review. *Animal Reproduction Science*, 2003, **75**(1–2): 73–94

25. Mann G E, Lamming G E. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction*, 2001, **121**(1): 175–180

26. Weems C W, Lee C N, Weems Y S, Vincent D L. Distribution of progesterone to the uterus and associated vasculature of cattle. *Endocrinologia Japonica*, 1988, **35**(4): 625–630

27. Muñoz M, Corrales F J, Caamaño J N, Diez C, Trigal B, Mora M I, Martín D, Carrocera S, Gómez E. Proteome of the early embryomaternatal dialogue in the cattle uterus. *Journal of Proteome Research*, 2012, **11**(2): 751–766

28. Mathialagan N, Roberts R M. A role for cytokines in early pregnancy. *Indian Journal of Physiology and Pharmacology*, 1994, **38**(3): 153–162

29. Mansoury-Attia N, Oliveira L J, Forde N, Fahey A G, Browne J A, Roche J F, Sandra O, Reinaud P, Lonergan P, Fair T. Pivotal role for monocytes/macrophages and dendritic cells in maternal immune response to the developing embryo in cattle. *Biología de Reproducción*, 2012, **37**(5): 123

30. Oliveira L J, Barreto R S, Perecin F, Mansoury-Attia N, Pereira F T, Meirelles F V. Modulation of maternal immune system during pregnancy in the cow. *Reproduction in Domestic Animals*, 2012, **47**(S4): 384–393