5-2024

The Potential Influence of Interferon-Gamma Induced Protein 10 on Early Gestation in Cattle

Peyton Spearry
pspearry@vols.utk.edu

Kyle McLean
University of Tennessee, Knoxville, kmclea10@utk.edu

Mary A. Leach
University of Tennessee, Knoxville, mleach11@vols.utk.edu

The Potential Influence of Interferon-Gamma Induced Protein 10 on Early Gestation in Cattle

Peyton E. Spearry, Mary A. Leach, & Kyle J. McLean

Department of Animal Science, Institute of Agriculture, University of Tennessee-Knoxville

ABSTRACT:

Reproductive efficiency is paramount for dairy producers in order to maintain a consistent and profitable calving window, especially with abnormally low milk prices and greater input costs. While advanced reproductive technologies (ART) are useful tools, further knowledge must be acquired about the intricacies of the physiological changes these techniques elicit during early gestation for optimization of the benefits of ART. A complex relationship exists between the maternal immune system and the growing conceptus, especially during the initial stages of pregnancy. Cytokines play a crucial role in maintaining a balance between immunity and viability, as they mediate inflammatory responses and cellular communication. Interferon gamma-induced protein 10 (IP-10) is a chemokine that is present in the uterus of pregnant animals. This study aims to characterize the role of IP-10 during early gestation by examining its concentration within vaginal fluid during the establishment of pregnancy in two separate experiments. In experiment one, the objective was to use vaginal fluid collected on d7 or d8 as a potential biomarker for pregnancy. The concentration of IP-10 was increased ($P < 0.001$) on d7 (1,244.2 ± 195 pg/mL) compared with d8 (337.7 ± 195 pg/mL). There were also positive correlations found between concentrations of IP-10, INF-γ, TNFα, and MIP-1β**.** However, there was no difference ($P = 0.67$) in IP-10 concentrations between open (2507.4 ± 365 pg/mL) and pregnant (2245.1 ± 483 pg/mL) cows. In experiment two, vaginal fluid collected on d0 and d7

was used to further elucidate the role of IP-10 during early gestation. The changes in IP-10 between d0 and d7 in open (3107 ± 1327 pg/mL) vs. pregnant (934.1 ± 1991 pg/mL) cows were not different ($P = 0.38$). Seasonality, however, did have an influence on concentrations and changes in IP-10 between days of gestation. There was a significant difference ($P = .02$) in the total concentration of IP-10 in the fall (5409 ± 1450 pg/mL), which was higher than in the spring (1422 ± 1146 pg/mL). The change in concentration of IP-10 between d0 and d7 in the Fall (5173 ± 660 pg/mL) was significant ($P < 0.001$). The difference in the concentration of IP-10 between d0 in the fall and d0 in the spring (4534 ± 522 pg/mL) was significant ($P < 0.001$). And the change in concentration of IP-10 between d0 in the fall and d7 in the spring (5043 ± 646) was also statistically significant ($P < 0.001$). These data provide valuable information on the role of cytokines, specifically IP-10, following the use of ART, during the establishment of pregnancy, and the potential impacts on reproductive efficiency in cattle.

INTRODUCTION:

As milk prices are at one of their lowest points in the past two decades, and milk-to-feed margins at a record low of $4/cwt, it is more crucial than ever for dairy producers to maximize productivity on their farms to improve profit margins.[1] In order to achieve this, producers should maintain a consistent 12-14 month calving interval to maximize lactation yield for cows in their herd. Competency and effective usage of advanced reproductive techniques (ART), such as artificial insemination (AI), estrus detection, and pregnancy detection methods, play paramount roles in attaining this ideal window. Technological advancements in ART have dramatically increased the efficiency and efficacy of dairy operations and bovine reproduction.[2] However, while these technologies have been critical for efficient external reproductive management,

understanding the physiological changes within the reproductive and immune systems is paramount.

An intricate relationship exists between the maternal immune and reproductive systems throughout the stages of the estrous cycle and into early pregnancy, with one of the first major transitions occurring following mating as the female and male reproductive systems first interact. Following the deposition of semen into the female tract and the interaction of spermatozoa with the uterine epithelial cells stimulates the migration of polymorphonuclear neutrophils (PMNs) into the uterus.[3] These PMNs bind to and phagocytize dead and excess spermatozoa, therefore removing them from the female reproductive tract. Seminal plasma plays an important role in these preliminary interactions, as it contains a variety of biomolecules important for the nourishment and transport of spermatozoa. Interaction of seminal plasma with cervical and uterine epithelial cells triggers the secretion of cytokines from the female reproductive tract.[4] This preliminary release of cytokines begins to prepare both the endometrial tissue and maternal immune system for gestation, by initiating the first steps in the creation of an immunotolerant environment for the fetus: an effect that is intensified as the events of early pregnancy continue to transpire.[5, 6]

The foundational immunotolerant environment is supported by cytokine activity. Macrophage Inflammatory Protein-1β (MIP-1β) maintains fetal tolerance while enhancing antagonistic response towards foreign microbes in humans and rodents.[7] Tumor Necrosis Factor-alpha (TNF-α) has destructive effects on oocyte maturation and increased blastomere apoptosis in cattle.[8] Interferon-gamma (IFN-γ) is known to assist with initiation of vascular remodeling in the endometrium, angiogenesis, and maintenance of maternal placenta in humans and rodents.[9] Another cytokine of interest is Interferon gamma-induced protein 10 (IP-10) which

IFN-γ stimulates.[10] The predominant function of IP-10 is to assist with the migration of the embryo from the site of fertilization to the uterus in ruminants. It is postulated that this is achieved through endometrial IP-10 establishing a concentration gradient, which the trophoblast cells of the embryo sense and migrate towards.[11] Furthermore, migration into the uterus and the close proximity of the conceptus to the caruncular areas of the endometrium is a prerequisite for successful placental attachment, followed by a high concentration of Interferon-τ during the height of maternal recognition signaling.[12] Interferon gamma-induced protein 10 is known to influence other events of early pregnancy and implantation in human and rodent models.[2] Due to the discrepancies in placenta classifications between species, the known cascading effects of IP-10 in hemochorial placenta species are not directly transitive to the events of embryo migration and attachment in ruminants, which have epitheliochorial placentas.[13,14] Therefore, the complete influence of IP-10 on the events of early pregnancy in cattle is yet to be uncovered. The focus of this study is to further investigate the behavior of IP-10, a cytokine known to play an important role in the establishment of pregnancy, in cows.

MATERIALS & METHODS:

Animal Management and Sample Collection:

All experimental procedures described below were conducted in accordance with approved procedures by the University of Tennessee Institutional Animal Care and Use Committee.

Experiment One: A mixture of primiparous and multiparous Holstein and Jersey cows (n=88) from two dairy farms: Little River Dairy Unit (LRD) and Sweetwater Valley Farm (SVF) were utilized to complete the objectives of this study. After a voluntary waiting period of 60 d

post-calving, all cows underwent estrus synchronization and were AI as per standard operating procedures of the location. All cows were granted *ad libitum* access to a total mixed ration for lactation and water. Cows were vaginally flushed with 20 mL of sterile saline on either d 7 (SVF) or d 8 (LRD) following insemination.

Experiment Two: Holstein cows (n = 77) from LRD were used to complete the objectives of this study. All cows were synchronized and underwent AI as per the standard operating procedure of the farm. On d 0, animals were vaginally flushed with 20 mL of sterile saline, had a blood sample collected, and bred by a single AI technician. On d 7, all cows were vaginally flushed and a blood sample was taken. On d 21, a blood sample was collected for progesterone analysis. On d 35, a blood sample was collected for pregnancy-associated glycoproteins (PAG) analysis and an ultrasound was performed to determine pregnancy status. On d 65, cows underwent a final pregnancy confirmation via ultrasonography.

Vaginal Flush Collection:

Vaginal flush samples were collected by inserting a 14-gauge Foley catheter into the fornix vagina while the cervical os was manually obstructed via rectal palpation. Sterile saline, 20 mL, was inserted into the vaginal environment and mixed with vaginal fluid via manual manipulation. The flush fluid sample was then removed back through the Foley catheter. All samples were stored on ice for no more than 2 hours before being frozen at -80°C until cytokine quantification could be completed.

Cytokine Quantification:

Cytokine concentrations of TNFα, IFN-γ, MIP-1β/CCL-4, and IP-10 were quantified within vaginal fluid using the MILLIPLEX MAP Bovine Cytokine/Chemokine Magnetic Bead Panel (MilliporeSigma; Burlington, MA) according to the manufacturer protocol. The plates

were analyzed on a Luminex 200 instrument (Luminex; Austin, TX) at the University of Tennessee Institute of Agriculture Genomics Hub.

Statistical Analyses:

Experiment One: A complete randomized design was utilized to elucidate differences in cytokine concentrations between pregnancy status, day of gestation, and the interaction of pregnancy status × day of gestation via separate mixed model analysis of variances and mean separations in SAS 9.4 (SAS Institute; Cary, NC). The individual cow was the experimental unit. Means were reported as different when $P < 0.05$ and tendencies are reported at a $P < 0.10$. The correlation procedure in SAS 9.4 was utilized to evaluate correlations between IP-10, INF-γ, TNFα, and MIP-1β.

Experiment Two: A complete randomized design was utilized to elucidate differences in cytokine concentrations and the change in concentration from d0 to d7 between pregnancy status, season, and the interaction of pregnancy status × season via separate mixed model analysis of variances and mean separations in R Studio. The individual cow was the experimental unit. Means were reported as different when $P < 0.05$ and tendencies were reported at a $P < 0.10$.

RESULTS:

Experiment One

These data show a decrease ($P < 0.001$) in the concentration of IP-10 between d7 (1,244.2 ± 195 pg/mL) and d8 (337.7 ± 195 pg/mL) of gestation (Fig. 1). When examining the relationship between cytokines (Table 1), IP-10 and IFNγ were positively correlated (R = 0.87). A similar relationship between MIP1β and TNFα with IP-10 was observed, where MIP1β and IP-10 (R = 0.63), as well as TNFα and IP-10 (R = 0.52) were positively correlated (Table 1).

Experiment Two:

There was no significant difference ($P = 0.67$) in the mean concentration of IP-10 when comparing open (2507.4 ± 365.2 pg/mL) and pregnant (2245.1 ±483.1 pg/mL) animals (Fig.2). Additionally when comparing the prevalence of detectable IP-10 between open and pregnant animals, it was most common that IP-10 was within the standard curve for both classifications (42.98% open; 45.90% pregnant; Table 2). Both classifications had the lowest percentage of cows who exhibited detectable levels of IP-10 below the standard curve (25.44% open; 14.75% pregnant; Table 2). The change in the concentration of IP-10 across the first 7 days of gestation was not significant ($P = 0.38$) when comparing open (3106.96 ± 1327.08 pg/mL) and pregnant (934.08 ± 1990.62 pg/mL) cows (Fig. 3). However, there was an effect of season on the change in IP-10 concentrations from d0 to d7. Regardless of pregnancy status, the change in the IP-10 across the first 7 days of gestation was greater ($P = 0.02$) in the fall (5409.2 ± 1450.5 pg/mL) when compared to the spring (1422.9 ± 1146.7 pg/mL; Fig. 4). Finally, there was also a significant ($P < 0.001$) decrease in the concentration of IP-10 on d0 of gestation in the Fall when compared to d0 in the spring, d7 in the fall, and d7 in the spring (4534 ± 522 pg/mL, 5173 ± 660 pg/mL, 5043 ± 646 pg/mL, respectively; Fig. 5).

DISCUSSION:

The innate immune system plays an integral role in regulating the activity of the luteal phase, as macrophages, dendritic cells, and PMNs all exist within the corpus luteum (CL). These cells support the normal development of the CL by maintaining luteal vasculature and progesterone (P4) secretory patterns.[15] In non-pregnant animals, the CL undergoes luteolysis following the Prostaglandin F2-alpha (PGF2α) and oxytocin feedback loop. This causes an

increase in the number of macrophages and T lymphocytes recruited to the ovary.[16] These T lymphocytes in turn stimulate cytokines such as TNFα, IFN-γ, and Interleukin-1β (IL-1β) that inhibit steroidogenesis and initiate apoptosis, ultimately causing the CL to regress completely.[17] A decrease in P4 produced by the CL reduces inhibitory effects on Gonadotropin-releasing hormone (GnRH) and therefore causes follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to increase as the cow transitions into the follicular phase. The increase in FSH, along with the activity of the cytokine interleukin-7 (IL-7) stimulates the maturation of the dominant, preovulatory follicle.[18] This preovulatory follicle produces enough estradiol to cause the surge in LH. During the LH surge and subsequent ovulation, the number of leukocytes in the ovary dramatically increases, along with cytokine activity, specifically interleukin-6 (IL-6) and interleukin-8 (IL-8) to promote follicular pressure, activate proteolytic pathways, reorganization of follicular stroma, loss of follicular epithelium structure, and other physiological processes.[17,19] Cytokines play many complex roles throughout cyclicity and gestation, and effective communication between cytokines is essential for reproductive success. It is known that the actions of IFN-γ directly influence IP-10, and the results of this experiment support this understanding, as IP-10 and IFN-γ have the strongest correlation of the cytokine relationships studied in experiment 1.[10] The correlation between IP-10 and both TNFα and MIP1β was unexpected and should be a focus of further research into understanding this relationship's effect on the success of dairy cow reproduction.

On d7 of gestation, the cow embryo is categorized as a blastocyst, containing differentiated trophoblast cells.[20] As stated previously, IP-10 is known to assist with the migration of the embryo into the uterus, due to communication with the trophoblast cells in the blastocyst.[11] The results from experiment 1 are unexpected given this information. The high

concentration of IP-10 on d7 aligns with the current understanding of IP-10's relationship with the trophoblast cells, however, it is interesting that the concentration of IP-10 decreases on d8 (Fig. 1). One may theoretically postulate that the activity of IP-10 would increase following d7, as the blastocyst gets closer in proximity to the uterine tissue, and approaches attachment.

Although cows are considered polyestrous animals, there is evidence to suggest that seasonality affects bovine cyclicity, and it is well-documented that heat stress negatively impacts bovine reproductive efficiency.[21,22] More specifically, heat stress causes an increase in the cortisol level produced, influencing the production of many cytokines, one of which is IFN-γ; therefore, potentially IP-10 as well. So, although the overall mean concentration of IP-10 is not different in open vs. pregnant cows (Fig.2), the changes in IP-10 concentrations as exhibited in the seasonality data may provide insight into potential fertility. The change in concentration of IP-10 was significantly higher in the fall than in the spring, as well as a higher concentration of IP-10 on d0 of gestation in the fall when compared to the spring (Fig. 4 & Fig.5). Additionally, in the current dataset, pregnancy rates of experiment 2 were 43.1% (31 out of 72 inseminations) in the spring compared with 16.7% (5 out of 30 inseminations) in the fall (data not shown). With successful pregnancy rates in the fall being over double the number of successful pregnancies in the spring, this supports the possibility that IP-10 concentration on d0 of pregnancy is a potential biomarker for the indication of a successful pregnancy.

In summary, experiment one confirmed the correlation between the activity of IP-10 and other cytokines associated with the reproductive system, as well as heightened IP-10 activity on the d7 of gestation. In experiment two, it was concluded that the average concentration of IP-10, and changes in IP-10 concentration between d 0 and d 7 is not different between open and pregnant cows. However, experiment two also highlighted the fact that seasonality plays a role in

reproductive efficiency in dairy cows. When comparing the significant changes in concentrations of IP-10 and pregnancy rates between seasons, it may be possible that the concentration of IP-10 on d 0 of pregnancy is a biomarker for fertility. The cellular communication network is extremely complex, with many cytokines playing different roles depending on the stage of gestation. Further research is needed to better understand the changes in concentrations of cytokines throughout the entirety of bovine gestation, and how that may be applied to enhance reproductive management practices for dairy cattle.

LITERATURE CITED:

1. OSU: Hartschuh, J. (2024). *Current 2024 U.S. Dairy Outlook.* Buckeye Dairy News. https://shorturl.at/hzA13
2. Jiang, Y., Huang, F., Chai, X., Yuan, W., Ding, H., & Wu, X. (2021). The role of IP-10 and its receptor CXCR3 in early pregnancy. *Molecular Immunology, 140*, 59-69. https://link.springer.com/chapter/10.1007/978-1-4614-8887-3_6
3. Katila, T. (2012). Post-mating inflammatory Responses of the Uterus. *Reproduction in Domestic Animals, 47*(s5), 31-41. https://onlinelibrary.wiley.com/doi/10.1111/j.1439-0531.2012.02120.x
4. https://my.clevelandclinic.org/health/body/24585-cytokines cytokine definition
5. Fair, T. (2015) The Contribution of the Maternal Immune System to the Establishment of Pregnancy in Cattle. *Frontiers in Immunology, 6*(7). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4309202/

6. Velázquez, M. M. L., Peralta, M. B., Angeli, E., Stassi, A. F., Gareis, N. C., Durante L., Cainelli, S., Salvetti, N. R., Rey, F., & Ortega, H. H. (2019). Immune status during postpartum, peri-implantation and early pregnancy in cattle: An updated review. *Animal Reproduction Science, 206*, 1-10. https://www.sciencedirect.com/science/article/pii/S0378432019301940#:~:text=The%20uterine%20immune%20system%20of,et%20al.%2C%202008
7. True, H., Blanton, M., Sureshchandra, S., & Messaoudi, I. (2023). Monocytes and Macrophages in Pregnancy: The Good, the Bad, and the Ugly. *Immunological Reviews*, *308*(1), 77-92. https://doi.org/10.1111/imr.13080
8. Soto, P., Natzke, R. P., & Hansen, P. J. (2003) Actions of Tumor Necrosis Factor - α on Oocyte Maturation and Embryonic Development in Cattle. *American Journal of Reproductive Immunology, 50*(5), 380-388. https://doi.org/10.1034/j.1600-0897.2003.00101.x
9. Sharma, S., Godbole, G., & Modi, D. (2016). Decidual Control of Trophoblast Invasion. *American Journal of Reproductive Immunology, 75*(3), 341-350. https://doi.org/10.1111/aji.12466
10. Dufour, J. H., Dziejman, M., Liu, M. T., Leung, J., Lane, T. E., & Luster, A. D. (2002) IFN-γ-Inducible Protein 10 (IP-10; CXCL10)- Deficient Mice Reveal a Role for IP-10 in Effector T Cell Generation and Trafficking. *The Journal of Immunology, 168*(7), 3195-3206. https://journals.aai.org/jimmunol/article/168/7/3195/34941/IFN-Inducible-Protein-10-IP-10-CXCL10-Deficient
11. Nagaoka, K., Nojima, H., Watanabe, F., Chang, K., Christenson, R. K., Sakai, S., & Imawaka, K. (2003). Regulation of blastocyst migration, apposition, and initial adhesion

by a chemokine, interferon gamma-inducible protein 10 kDa (IP-10), during early gestation. *Journal of Biological Chemistry, 278*(31), 29048-29056. https://pubmed.ncbi.nlm.nih.gov/12756249/

12. Roberts, M. (2007). Interferon-tau, a type 1 interferon involved in maternal recognition of pregnancy. *Chemokine & Growth Factor Reviews, 18*(5-6), 403-408.https://pubmed.ncbi.nlm.nih.gov/17662642/#:~:text=Interferon%2Dtau%20is%20a%20major,begins%20to%20form%20a%20placenta.
13. Furukawa, S., Tsuji, N., & Sugiyama, A. (2019). Morphology and physiology of rat placenta for toxicological evaluation. *Journal of Toxicologic Pathology, 31*(1), 1-17. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6361663/#:~:text=The%20placentas%20of% 20humans%20and,and%20is%20categorized%20as%20hemomonochorial.
14. *Green, J.A., Geisert, R.D., Johnson, G.A., Spencer, T.E. (2021). Implantation and Placentation in Ruminants. In: Geisert, R.D., Spencer, T. (eds) Placentation in Mammals. Advances in Anatomy, Embryology and Cell Biology, vol 234. Springer, Cham. https://doi.org/10.1007/978-3-030-77360-1_7*
15. Talukder, A. K., Marey, M. A., Shirasuna, K., Kusama, K., Shimada, M., Imakawa, K., & Miyamoto, A. (2020). Roadmap to pregnancy in the first 7 days post-insemination in the cow: Immune crosstalk in the corpus luteum, oviduct and uterus. *Theriogenology, 150*(1), 313-320. https://www.sciencedirect.com/science/article/pii/S0093691X20300844
16. Shirasuna, K., Jiemtaweeboon, S., Raddatz, S., Nitta, A., Schuberth, H., Bollwein, H., Shizimu, T., & Miyamoto, A. (2012). Rapid Accumulation of Polymorphonuclear Neutrophils in the *Corpus luteum* during Prostaglandin F2α-Induced Luteolysis in the

Cow. *PLOS ONE, 7*(1). https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0029054#:~:text=Prostaglandin%20F2%CE%B1%20

17. Pate, J. L. *(2018). Luteolysis. In: Skinner, M. (ed) Encyclopedia of Reproduction, (*vol. 2, 106-113) *American press https://doi.org/10.1007/978-3-030-77360-1_7*
18. Field, S. L., Dasgupta, T., Cummings, M., & Orsi, N. M. (2013). Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation. *Molecular Reproduction and Development*, *81*(4), 284-314. https://onlinelibrary.wiley.com/doi/full/10.1002/mrd.22285
19. Avila, L. M., Madero, J. I., Escobar, M., Lopez, C., Leon, M. F., Ardila, J., Abdalla, L., Guerra, O., Aparicio, A., & Ruiz, J. (2004). Proinflammatory cytokines IL-1β , IL-8 and IL-6 in the follicular fluid of healthy women. *International Congress Series, 1271*, 291-295. https://www.sciencedirect.com/science/article/pii/S0531513104012981#:~:text=Conclusion,reactions%20(IL%2D1)
20. Guillomot, M. (1995). Cellular interactions during implantation in domestic ruminants. *Journal of reproduction and fertility, 49*, 39-51.https://pubmed.ncbi.nlm.nih.gov/7623329
21. White, F. J., Wettemann, R. P., Looper, M. L., Prado, T. M., & Morgan, G. L. (2002). Seasonal effects on estrous behavior and time of ovulation in nonlactating beef cows. *Journal of Animal Science, 80*(12), 3053-9. https://pubmed.ncbi.nlm.nih.gov/12542143/
22. Khan, I., Mesalam, A., Heo, Y., Lee, S., Nabi, G., & Kong, I. (2023). Heat Stress as a Barrier to Successful Reproduction and Potential Alleviation Strategies in Cattle. *Animals, 13*(14), 2359. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10376617/#:~:text=The%20Impact%20of%20Heat%20Stress%20on%20the%20Estrous%20Cycle&text=A%20significant%20reduction%20in%20the,to%20declining%20estrus%20%5B38%5D.

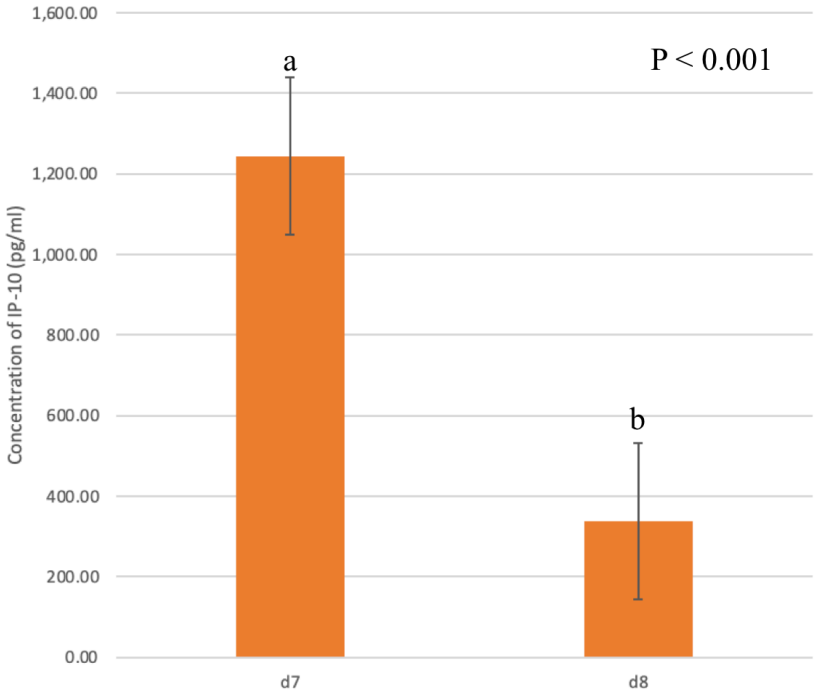

Figure 1: Comparison between the concentrations of IP-10 in vaginal fluid on d7 and d8 of gestation regardless of pregnancy status. Means without a common superscript differ by ($P <$ 0.05). The pooled standard error of the means (SEM) was 194.

Table 1: Correlations between IP-10 and MIP1β, TNFα, or IFNγ in vaginal fluid of dairy cattle following artificial insemination.

Cytokine 1	Cytokine 2	R value	P value
IP-10[1]	MIP1β[2]	0.63	< 0.001
IP-10	TNFα[3]	0.52	0.015
IFNγ[4]	IP-10	0.87	< 0.001

[1] IP-10: Interferon gamma induced protein-10
[2] MIP1β: Macrophage inflammatory protein 1 beta
[3] TNFα: Tumor Necrosis Factor alpha
[4] IFNγ: Interferon gamma

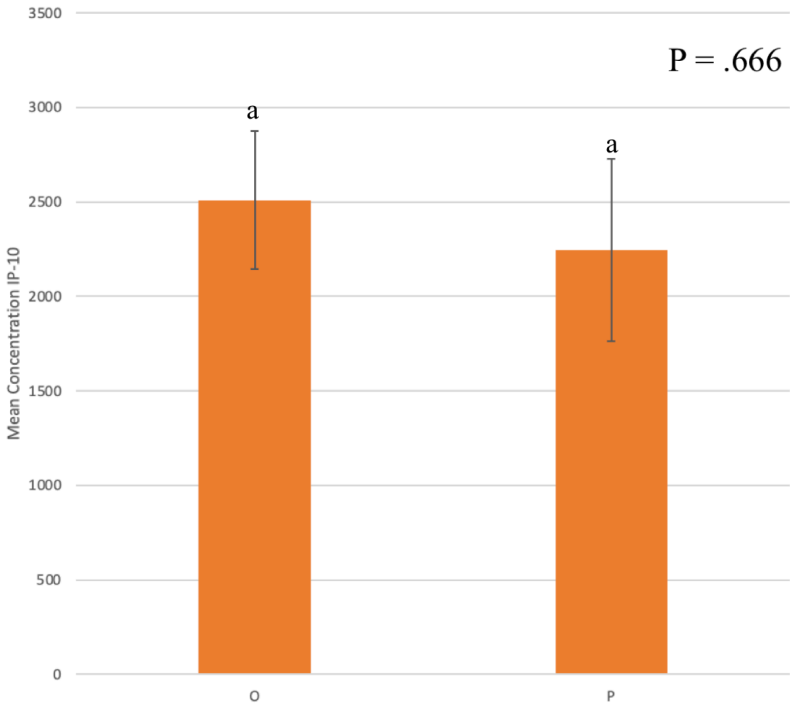

Figure 2: Mean concentration of IP-10 for open (O) vs. pregnant (P) cows. Means without a common superscript differ by ($P < 0.05$). The pooled standard error of the means (SEM) is 605.

Table 2: Prevalence of detectable IP-10[1] within vaginal fluid of lactating dairy cows following artificial insemination.

Pregnancy Classification	Above[2]	Below[3]	Within[4]
Open[5]	36 (31.58%)	29 (25.44%)	49 (42.98%)
Pregnant[6]	24 (39.34%)	9 (14.75%)	28 (45.90%)

[1] IP-10: Interferon gamma induced protein-10
[2] Above: The incidence and percentage of values that were found to be above the standard curve
[3] Below: The incidence and percentage of values that were found to be below the standard curve
[4] Within: the incidence and percentage of values that were found to be within the standard curve
[5] Percentage of total number of open cows.
[6] Percentage of total number of pregnant cows.

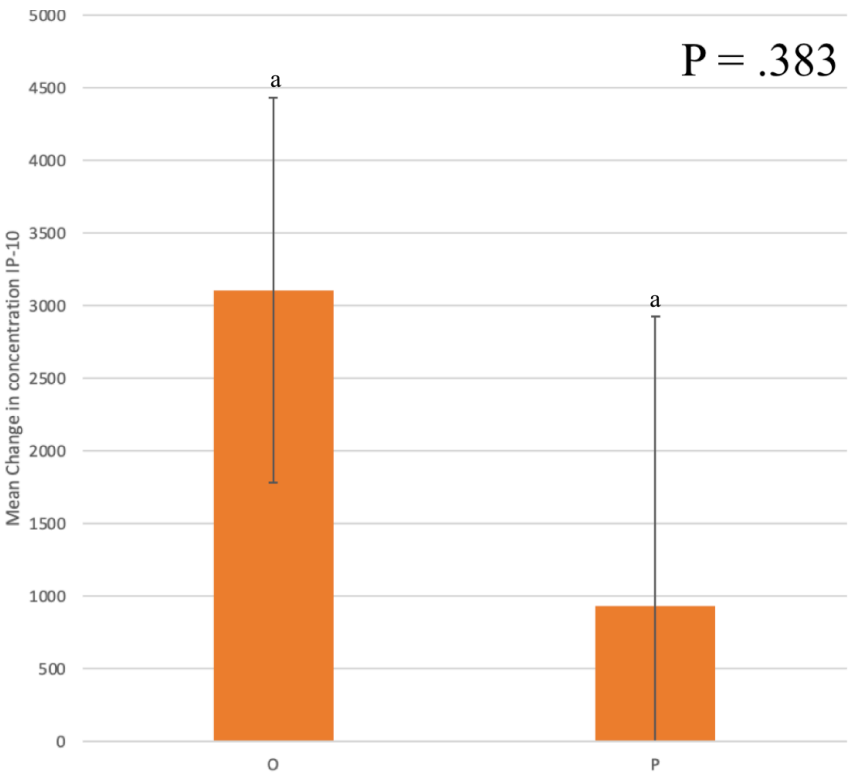

Figure 3: Mean changes in concentration of IP-10 (pg/ml) from d0 to d7 in open (O) vs. pregnant (P) cows. Means without a common superscript differ by ($P < 0.05$). The pooled standard error of the means (SEM) is 2392.

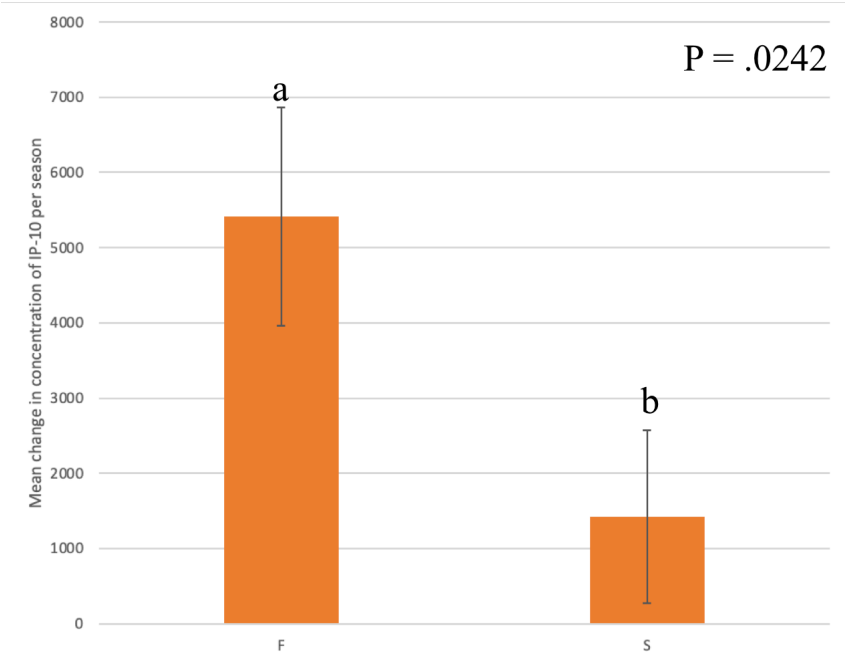

Figure 4: Changes in the concentration of IP-10 from d0 to d7 in all cows between Fall (F) and Spring (S). Means without a common superscript differ by ($P < 0.05$). The pooled standard error of the means (SEM) is 1299.

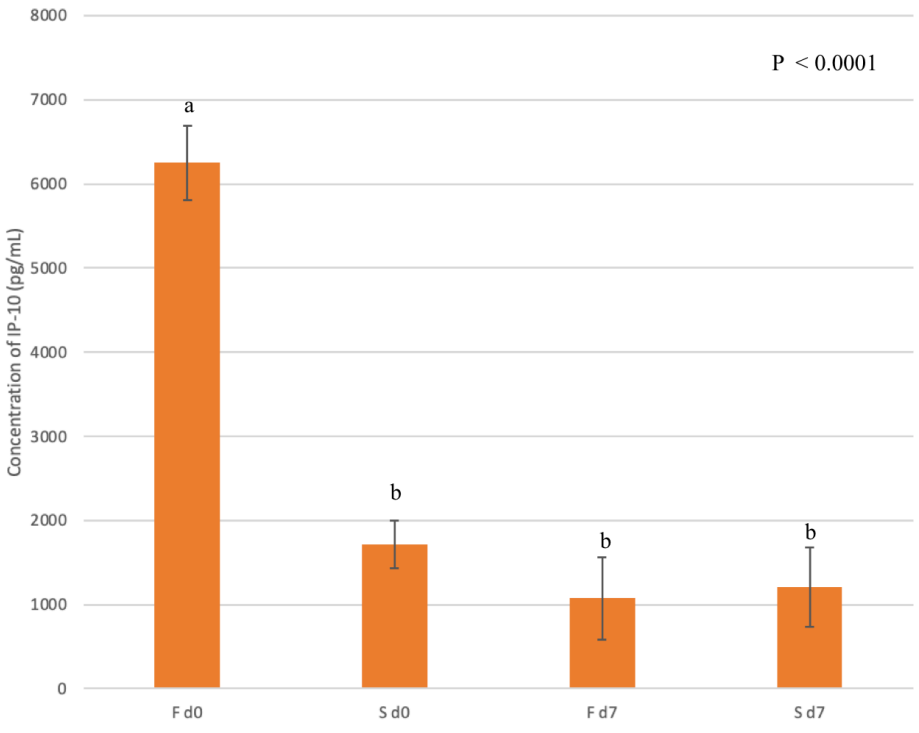

Figure 5: Concentrations of IP-10 Based on day of gestation and season. Means without a common superscript differ by ($P < 0.05$). The pooled standard error of the means (SEM) is approximately 199.