

Effect of flunixin meglumine on prostaglandin metabolites and progesterone in lactating dairy cows

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Abstract

The objectives of this study were to determine the effects of flunixin meglumine (FM), a non-steroidal anti-inflammatory drug on prostaglandin F₂α (PGF) secretion, by characterizing plasma prostaglandin metabolites (PGFM), and luteal function by characterizing plasma progesterone (P₄) concentrations in lactating Holstein dairy cows during the luteal phase of the estrous cycle. On day -35, estrous cycles were synchronized using a Presynch-Ovsynch protocol. Transrectal ultrasonography was performed on days -9, 0, 3, 7, and 15 to confirm ovulation and formation of a corpus luteum (CL). On day 15, cows were stratified by parity and randomly assigned to either treatment or control groups. The treatment group (n = 9) received a total of 2.0 mg/kg body weight of FM (iv), whereas the control group (n = 8) received saline. Jugular blood samples were collected at 30 and 0 minutes before treatment for PGFM concentrations. Following treatment, blood samples were collected at 30 minutes and every hour for additional 7 hours. Blood samples were also collected daily from days 15 to 22 to characterize P₄ concentrations. Plasma PGFM concentrations did not differ between groups before treatment. Following treatment, plasma PGFM remained unchanged in the control group, whereas the mean PGFM concentrations decreased in FM-treated cows (P < 0.05). Mean PGFM concentration decreased within 60 minutes following treatment and remained low through the experimental period. Mean P₄ concentrations before treatment (day 15) did not differ between the control and treatment groups. Mean P₄ concentrations decreased between days 16 to 22. However, the rate of P₄ decline over time tended to be smaller for FM-treated cows compared with controls (P = 0.09). These results suggest that FM treatment decreases plasma PGFM and therefore, may inhibit PGF secretion in lactating dairy cows during the luteal phase of the estrous cycle. Moreover, FM may help to extend luteal function by reducing the rate of P₄ decline.

Keywords: Flunixin meglumine, prostaglandin, luteal phase, dairy cows

Introduction

Reproductive efficiency is an important contributor to dairy farm profitability. Many factors can cause reproductive inefficiencies and decrease pregnancy rates (PR). Embryonic loss is one factor that contributes to dairy cattle reproductive efficiency and negatively impacts PR.^{1,2} In dairy cattle, fertilization rates are similar between lactating and nonlactating cows, and it has been reported to be as high as 88% to 98%. Others have reported that fertilization rates in dairy cows are approximately 90%.³⁻⁵ Conception rates (CR) at day 27 to 31 after artificial insemination (AI) are approximately 35 to 45%, indicating that the majority of cows that conceive do not maintain pregnancy following fertilization.³ The majority of embryonic mortality (70 to 80% of the total loss) occurs between days 8 and 16 following fertilization.⁵

A specific sequence of events must occur in order for embryos to survive and for pregnancy to be maintained. In cattle, these events are in part dependent upon sufficient P₄ secretion from the CL and timely secretion of interferon-tau (IFNτ) from the embryo. Interferon-tau prevents synthesis and secretion of PGF and stops the luteolytic mechanism.⁶⁻⁸ Any disruption in the previously mentioned events may lead to early embryonic death. Therefore, it is critical to investigate methods to regulate luteolysis and assist in the maintenance of early pregnancy in dairy cattle.

High concentrations of P₄ prior to fertilization are necessary to prime the uterus for embryo development and inhibit secretion of PGF⁹ However, if there are insufficient concentrations of P₄, embryonic mortality increases because of an unsuitable uterine environment for embryonic development.¹⁰ Another possible scenario that may lead to early embryonic loss is if the embryo is

incapable of producing sufficient IFN τ , or if secretion of IFN τ is delayed. This may result in untimely maternal recognition, leading to secretion of PGF, luteolysis and embryonic loss.¹¹

Several *in vivo* studies have shown the negative effects of premature secretion of PGF on embryonic survival in beef cows.¹²⁻¹⁴ Given that the majority of embryonic loss occurs between days 8 and 16, and maternal recognition of IFN τ by the embryo occurs between days 14 and 16, embryonic loss may occur due to the inability of the embryo to prevent PGF secretion. Any strategy to inhibit or reduce PGF secretion near the time of maternal recognition may help to reduce embryonic loss and improve reproductive performance in lactating dairy cows.

Non-steroidal anti-inflammatory drugs (NSAID), such as FM, are known inhibitors of prostaglandin synthesis and are used therapeutically for alleviation of pain, fever, and inflammation.^{15,16} This specific NSAID has been studied to improve PR under various circumstances in beef and dairy cattle.

In dairy heifers, the use of FM before luteolysis improved the overall PR.¹⁷ A study by Schrick and colleagues¹⁸ showed that when FM was administered during embryo transfer (ET) in beef cows, the overall PR was significantly improved. Another study by Pfeifer et al¹⁹ also demonstrated an improvement of PR on day 30 (37% FM vs. 17% control) following AI in lactating dairy cows administered FM 12 hours apart on day 15 and 16 of the estrous cycle. These authors suggested that the observed results were due to the attenuation of PGF_{2 α} secretion, therefore delaying luteolysis.^{17,19} Nevertheless, PGF nor its metabolites (PGFM) were measured by these investigators.

Although some studies have demonstrated a decrease in PGFM and/or an increase in PR/AI, other studies in lactating beef and dairy cows, as well as beef and dairy heifers have shown no difference or a decline in PR/AI when FM was administered following AI. For example, von Kruger and Heuwieser²⁰ and Rabaglino et al,²¹ observed no differences in PR when FM was administered twice 12 or 24 hour apart on day 15 and 16 after AI in dairy heifers. In lactating beef cows and beef heifers, administration of FM 13 days following AI had either negative effects or showed no improvements on PR/AI.²² Once again, PGF nor its metabolites were measured in any of these studies. To our knowledge there are only four studies in which PGFM was measured following FM administration. One study examined FM effects in cyclic dairy heifers and oophorectomized cows, and another used only cyclic dairy heifers. In these studies, a limited number of animals was used (n = 2 to 6), and FM was administered orally 2-4 times daily or intravenously (iv) four times daily for either 7 to 9 days beginning on day 15 or 16 of the estrous cycle.^{23,24} In both of these studies it was concluded that administration of FM 4 times daily orally or iv decreased PGFM concentrations in cows and heifers and prolonged the CL lifespan in cyclic dairy heifers. An additional study that measured blood PGFM after an intense treatment of FM for 10 days (4 x daily) following ET, also demonstrated a decrease in PGFM concentrations following FM treatment.²⁵ Lastly, a study in nonlactating beef cows demonstrated that when FM was administered 13 days after AI, PGFM concentrations decreased; however no improvements on PR were observed.²⁶ An intense regiment of FM administration was used in most of these studies, which makes it impractical to implement in a production operation.

The results on the effects of FM to improve PR have been inconsistent. Moreover, the effects of FM on PGF secretion have not been studied in lactating dairy cows during the luteal phase of the estrous cycle. To our knowledge, there is a lack of evidence on FM effects on PGF secretion and P₄ secretion during the maternal recognition period in lactating Holstein dairy cows. Our hypotheses were that FM would decrease PGFM concentrations and affect P₄ secretions following treatment. The objectives of this investigation were to examine the effects of FM administration on PGF secretion by characterizing plasma PGFM, as well as peripheral P₄ secretion during the luteal phase of the estrous cycle, around time of maternal recognition.

Materials and methods

This study was conducted at the University of Idaho Dairy Research and Education Center located in Moscow, Idaho. The University of Idaho Animal Care and Use Committee approved all procedures used in this experiment. Milk from FM-treated cows was discarded for a minimum of 36

hours following treatment, based on milk withdrawal guidelines established by the US Department of Agriculture, Food and Drug Administration (USDA-FDA).

Experimental design

On day -35, seventeen lactating Holstein dairy cows (7 primiparous and 10 multiparous), were presynchronized using two intramuscular (im) injections of PGF (25 mg; Lutalyse®; Zoetis, Florham, NJ) 14 days apart (Fig. 1). On day -9 (12 days after second PGF) after detection of a CL by transrectal ultrasonography (Sonovet 600®, 5 MHz probe; Universal Ultrasound, Bedford Hills, NY) all cows were enrolled into an Ovsynch protocol and administered gonadotropin-releasing hormone (GnRH; 100 µg, im; Cystorelin®; Merial, Athens, GA; Fig. 1). Seven days later (day -2), all cows received a PGF injection (25 mg; im) to regress the existing CL previously confirmed on day -9 (Fig.1). Approximately 48 hours later (day 0) all cows received the final GnRH (100 µg; im; Fig. 1). All cows were visually observed (3 to 4 times daily) for estrus behavior from days 15 to 22 of experimental protocol.

Ovarian examination

All cows were subjected to transrectal ultrasonography (Sonovet 600®, 5 MHz probe) on days -9, 0, 3, 7, and 15, in order to assess follicular dynamics and to determine ovulation of the dominant follicle and the formation of a CL in the same location (Fig.1). Ovulation was defined as the disappearance of a follicle ≥ 10 mm diameter and the formation of a CL in the same location. The presence of CL was also confirmed with blood P₄ concentrations > 1 ng/mL.²⁷

Treatment

On day 15, six hours prior to serial blood collection, cows were weighed, assigned a body condition score (BCS), and the ovaries were examined by transrectal ultrasonography. Following detection of a CL, cows were stratified by parity and randomly assigned to either treatment or control groups. The treatment group (n = 9; 5 multiparous and 4 primiparous) received 2.0 mg/kg body weight (BW) of FM (Banamine®, Schering-Plough Animal Health, Union, NJ) via the jugular vein (iv) administration, and the control group (n = 8; 5 multiparous and 3 primiparous) received the same volume of saline also via the jugular vein (Fig. 1). The average days in milk (DIM) for all cow at the time of treatment was 59 ± 4 days.

Blood collection and processing

On the day of experiment (day 15), 6 hours before treatment, cows were fitted with jugular catheters. Jugular blood samples were collected at 30 and 0 minutes before treatment, and at 30 minutes and every hour after for 7 hours following treatment to measure plasma PGFM concentrations (Fig. 1). Additional blood samples were obtained on day 16 to quantify PGFM concentrations 24 hours after treatment. Plasma PGFM concentrations are the stable metabolites of PGF in circulation and are known to be correlated to uterine-ovarian PGF in both ovine and bovine species.²⁸⁻³² Daily coccygeal blood samples were also collected from days 15 to 22 for quantification of P₄ concentrations (Fig. 1). Blood samples were collected using chilled collection tubes containing 30 IU heparin and immediately placed on ice. Blood tubes were then centrifuged at 4°C for 30 minutes at $2750 \times g$. Plasma was harvested and stored at -20°C until assayed for PGFM and P₄ concentrations.

Hormone assays

Serial blood samples from day 15 were assessed for PGFM concentrations using an ELISA assay (Cayman Chemical kit 13,14-dihydro 15-keto PGF_{2 α}) as previously described.³³ Samples were diluted 1:1 with an assay buffer, and the standard curve ranged from 2.3 to 5,000 pg/mL. All samples and standard curves were run in duplicates, and the intra- and inter-assay coefficient of variance (CV) were 8.8 and 6.8%, respectively. The sensitivity of this assay was 15 pg/mL. Daily plasma blood samples from days 15 to 22 were analyzed for P₄ concentrations using a solid-phase radioactive-immunoassay (RIA; Siemens, Los Angeles, CA). All samples were assayed in duplicates and the standard curve ranged from

0.1 to 40 ng/mL. This assay was conducted under equilibrium conditions and the intra-assay CV was 7.8%, with an assay sensitivity of 0.1 ng/mL.

Statistical analysis

Differences between treatment groups for BW, BCS, DIM, and milk yield were analyzed using the General Linear Model (GLM) in SAS 9.2 (SAS Inst. Inc., Cary, NC). The model included treatment, parity, and treatment by parity effects.

Analysis of repeated measures using the mixed model procedure, Autoregressive Moving Average (ARMA 1,1) in SAS 9.2 was utilized to analyze plasma PGFM and P₄ concentrations between groups.³⁴ The model included treatment effects, the repeated measure of time, and the interactions between treatment and time. The random effect was cow within treatment, and pre-treatment hormone concentrations were used as a covariate in the model. Statistical significance was declared at a $P < 0.05$ and a tendency at $P \leq 0.1$.

A linear regression analysis, using GLM procedures of SAS, were also carried out to analyze the effect of treatments on the P₄ concentration over time (day 15 to 22). The fitted model for each treatment took the form of

$$Y = \beta_0 + \beta_1 x + \varepsilon_1$$

where Y was the P₄ concentrations, x represented time, β_0 was the intercept, β_1 was the rate of decline for P₄ concentration over time, and ε_1 represented the random error under classical regression assumptions.

Results

Mean BW, DIM, BCS, and milk yield were not different between treatment groups (Table). There was no effect of parity or parity by treatment interaction on DIM and BCS. As expected, milk yield tended to be different between primiparous and multiparous cows (28.4 ± 3.2 vs. 35.9 ± 2.7 kg, $P = 0.08$), but there was no effect of parity by treatment interaction on milk yield. In addition, BW differed between primiparous and multiparous cows (552.5 ± 20.0 vs 650.4 ± 16.6 kg; $P < 0.05$), but there was no effect of parity by treatment interaction on BW.

Ovarian structures were mapped and recorded using transrectal ultrasonography on days -9, 0, 3, 7, and 15 of experimental protocol (Fig. 1). All cows included in this data did not express estrus prior to day 0, the last GnRH injection in the Ovsynch protocol. The presence of a dominant follicle, and subsequent formation of a CL in the same location of the dominant follicle were observed in all cows by day 7 and 15 of experiment. All cows had a CL before treatment and on the day of treatment (day 15). Also, all cows had plasma P₄ concentrations > 1.0 ng/mL on day 15. Mean P₄ concentrations before treatments were not different between the groups (6.3 vs. 7.2 ± 0.5 ng/mL, for FM and control, respectively).

There was an effect of treatment ($P < 0.05$) and treatment by time interaction ($P < 0.01$) on mean PGFM concentrations. The mean plasma PGFM concentrations 30 and 0 minutes before treatment did not differ between FM-treated and control cows (129 ± 39 pg/mL for control vs 152 ± 41 pg/mL for FM; Fig. 2). Flunixin meglumine decreased PGFM secretion ($P < 0.05$) following treatment and the mean PGFM concentrations were lesser ($P < 0.05$) in FM-treated cows by 60 minutes after treatment administration and remained lower ($P < 0.05$) for the FM-treated cows throughout the remainder of the sampling period compared with the control group (57.5 ± 30 pg/mL; Fig. 2). Plasma PGFM remained unchanged in the control group throughout the sampling period (176 ± 40 pg/mL; Fig. 2). Also, mean PGFM concentrations on day 16 (22-24 hours following treatment) tended ($P = 0.09$) to be lower for FM than for control cows (51.4 ± 35 vs. 125.6 ± 33 pg/mL).

Progesterone concentrations decreased ($P < 0.01$) over time for both control and treatment groups. There was an effect of treatment by day interaction on plasma P₄ concentrations ($P < 0.08$), which indicated that the rate of P₄ decline over time was not similar between the groups. In fact, the slope or the rate of P₄ decline over time tended ($P = 0.09$) to be smaller for FM-treated cows compared with control cows (Fig. 3). Based on serum P₄ concentrations during day 15 to 22, two cows did not exhibit luteolysis ($P_4 < 0.5$ ng/mL) by day 22 in the control group. In contrast, four cows did not exhibit

luteolysis in FM-treated group. During the seven days after treatment, more cows were detected in estrus in the control group compared with the FM group. In the control group 6 cows exhibited estrus, whereas in the FM group only 4 cows were detected in estrus.

Discussion

In this experiment, FM treatment on day 15 of the estrous cycle decreased plasma PGFM concentrations within 60 minutes following treatment (Fig. 2). This indicates that FM has an inhibitory effect on PGF secretion, as plasma PGFM concentrations are known to be correlated to uterine-ovarian PGF concentrations in ovine and bovine species.²⁸⁻³² Therefore, it can be speculated that FM may have inhibited PGF secretion of the uterine-ovarian origin. Progesterone concentrations were not different between treatment and control groups on day 15 before FM administration. After treatments, the pattern of blood P₄ decline was inconsistent between the FM-treated and the control group. Flunixin meglumine treatment tended to delay the rate of decline in P₄ concentrations following treatment between days 16 and 22 (Fig. 3), indicating that perhaps FM delayed the process of luteolysis. These results suggest that FM may inhibit PGF secretion in lactating dairy cows during the luteal phase of the estrous cycle and affect P₄ secretion during the time of maternal recognition.

Our results are similar to other studies in cyclic dairy heifers, oophorectomized dairy cows and non-lactating beef cows in which FM treatment was able to decrease PGFM concentrations.^{23,24,26} Pfeifer et al¹⁹ demonstrated that administration of two doses of FM, 12 hours apart, on day 15 and 16 of the estrous cycle significantly improved PR on day 30 following AI in lactating dairy cows (37% FM vs. 17% control). Although the exact mechanism for the improved PR cannot be determined, the authors suggested that the improvement in PR may be due to the attenuation of PGF secretion during the late luteal phase. The findings of the current study support this hypothesis.

In the current study, although we did not measure PR/AI, our results indicate that FM treatment may improve PR/AI on day 15, the time in which maternal recognition occurs in cattle by decreasing PGF secretion. Flunixin meglumine may be a beneficial reproductive tool in preventing secretion of PGF during the time of maternal recognition, which may cause early embryonic loss. Since some studies have indicated an increase in PR/AI in beef cows¹⁸ and lactating dairy cows,¹⁹ treatment of FM may prove more beneficial when early embryonic loss is more prevalent such as heat stress situations. Future research is needed in order to determine the effects of FM during heat stress situations, as well as potential effects on reducing the occurrence of early embryonic loss.

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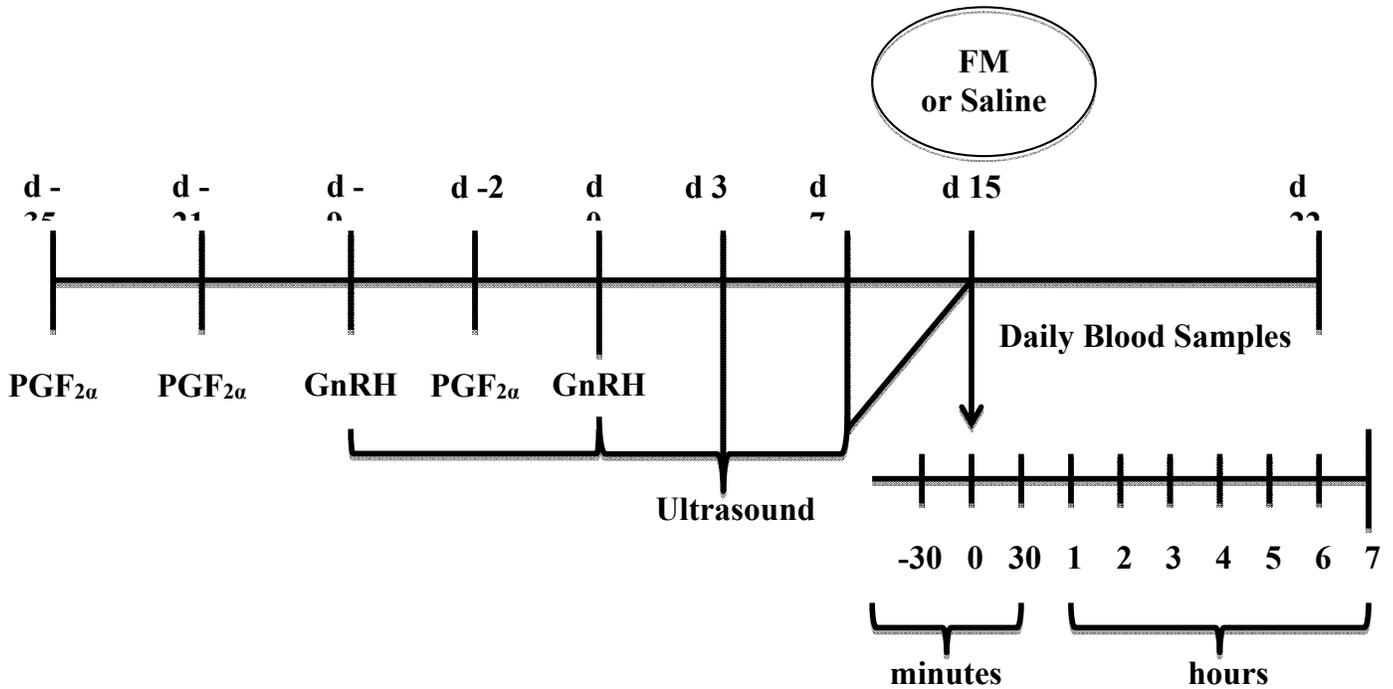


Figure 1. Experimental protocol. Holstein dairy cows were synchronized using a Pre-synch-Ovsynch protocol. Ultrasonography was conducted on days -9, 0, 3, 7, and 15 in order to determine ovulation of a dominant follicle and formation of a corpus luteum in the same location. On day 15, cows received 2.0 mg/kg body weight of flunixin meglumine or the same volume of saline. Jugular blood samples were collected at 30 and 0 minutes before treatment, and at 30 minutes and every hour after for 7 hours following treatment to measure plasma PGFM concentrations. From days 16 to 22, daily coccygeal blood sampling was obtained to examine progesterone concentrations.

Table. Descriptive statistics. Mean \pm standard error (SE) for body weight (BW), days in milk (DIM), body condition score (BCS), and milk yield in Holstein cows treated with flunixin meglumine (FM) or control.

Treatment	BW (kg)	DIM ¹	BCS ²	Milk yield (kg) ³
Flunixin (n = 9) ⁴	612.9 \pm 23.8	61 \pm 4	2.8 \pm 0.1	33.3 \pm 2.9
Control (n = 8) ⁴	606.7 \pm 25.3	58 \pm 4	2.7 \pm 0.1	32.6 \pm 3.1

¹ DIM on day -8 of experimental protocol.

² BCS on scale of 1 to 5 in 0.25 increments (1 = emaciated; 5 = over conditioned).

³ Milk yield based on closest test date to day of treatments.

⁴ Flunixin = 5 multiparous and 4 primiparous; Control = 5 multiparous and 3 primiparous.

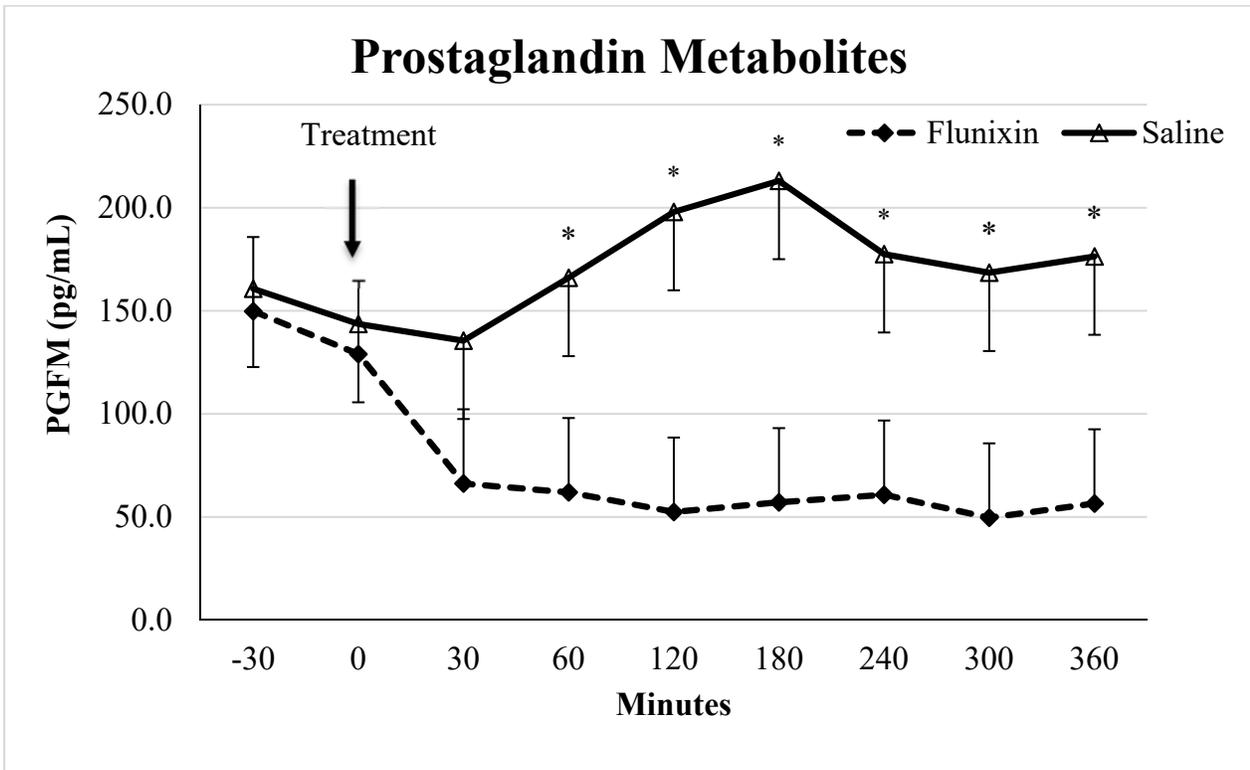


Figure 2. Prostaglandin metabolite (PGFM) concentrations, between flunixin meglumine (n = 9) and control (saline; n = 8) groups of lactating Holstein dairy cows during the luteal phase of the estrous cycle. Time -30, and 0 minutes are considered to be jugular blood samples collected prior to treatment. Following treatment, blood samples were obtained at 30 minutes, 60 minutes, and every hour for 6 consecutive hours following treatment. * Significant difference between treatment groups (P < 0.05).

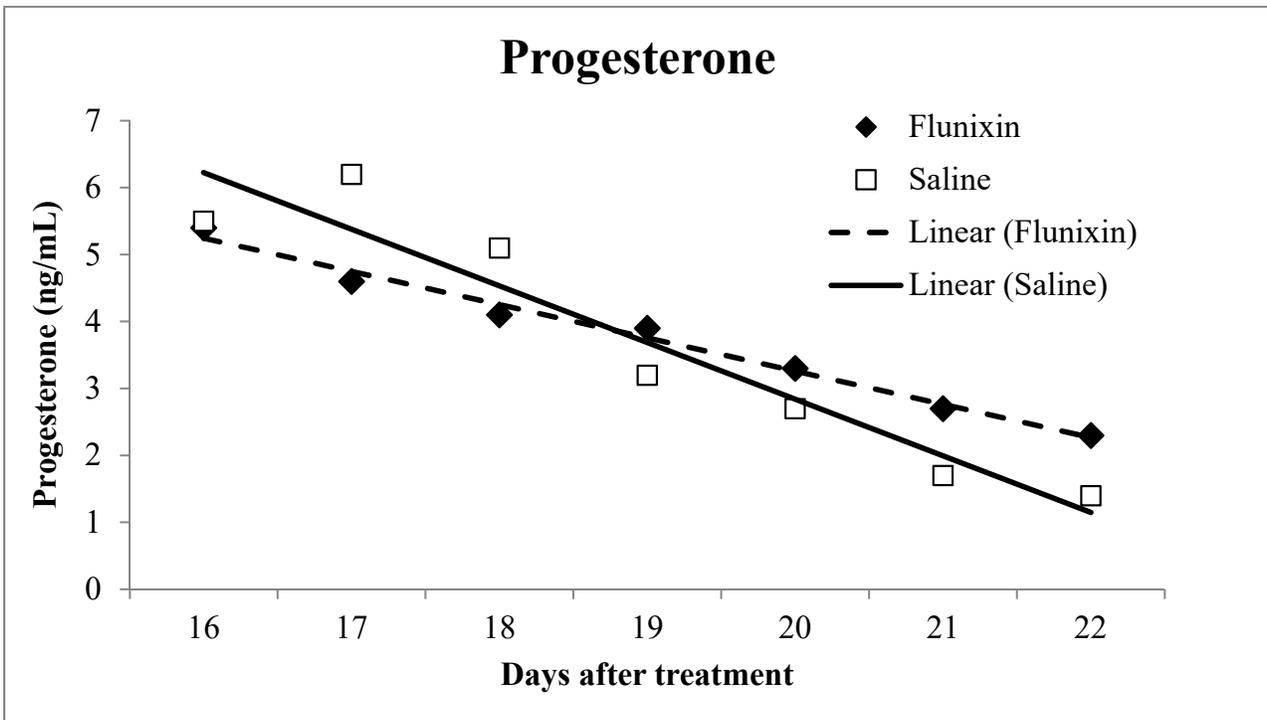


Figure 3. Linear regression analysis of progesterone between days 16 and 22 for flunixin meglumine and saline (control). The rate of decline (or slope) tended (P = 0.09) to be smaller for the FM-treated cows when compared to the control group.