Dynamics of Progesterone, TNF-α, and a Metabolite of PGF2α in Blood Plasma of Beef Cows following Embryo Transfer

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1. Introduction

Progesterone (P4) is abundantly reported in the literature as the primary and most intrinsic hormone associated with embryonic survival during early pregnancy [1, 2]. The emphasis in studying P4 is due to the ability exhibited by P4 in regulating uterine receptivity during implantation [3]. Moreover, low concentrations of P4 are associated with retention of pregnancy in beef cows [4]. Therefore, several studies have been recently designed to examine the effects of supplementing exogenous P4 on embryonic retention after the transfer of embryos. At present, this is commonly done by inserting a controlled internal drug releasing device (CIDR) at the time of breeding.

Our laboratory previously reported [5] that inserting a CIDR immediately after the transfer of embryos enhanced retention rates in recipient lactating and nonlactating beef cows. Similar results have been observed in cattle by other investigators [6, 7]. Conversely, Purcell et al. [5] did not detect beneficial effects on pregnancy rates by placing CIDRs immediately subsequent to embryo transfer in dairy cows.

Several factors might be responsible for the inconsistency in the research outcomes observed by supplementing exogenous P4 aimed to enhance embryonic retention of transferred embryos. In some cases, this may be attributed to the fact that a single CIDR may not deliver enough P4 to support pregnancy on recipients experiencing low circulating P4 [8] or perhaps failing to supplement adequate levels of P4 [6] at the time when majority of embryonic rejections have been suggested to occur after the transfer [7, 9, 10]. Furthermore, several trials have clearly demonstrated that exogenous supplementation of P4 impairs endogenous luteal production of P4 [10, 11] and caused marked regression of the corpus luteum (CL) during early pregnancy in cattle [12].
A more recent study conducted by our laboratory showed cows in the control group having increased pregnancy rates in parallel with increased concentrations of P4 during the first week after embryo transfer compared to a treated group with exogenous P4 via CIDR’s [10]. Therefore, strategies to enhance endogenous production of P4 may be an alternative method to examine its role on key factors associated with embryonic retention of transferred embryos.

Some of the potential strategies to achieve this goal may be by either inducing the formation of an accessory CL or by boosting the synthesis of luteal tissue in the existing CL with the use of hormones. Hence, several hormonal treatments have been reported to manipulate secretion of endogenous P4 in cattle. Gonadotropin releasing hormone (GnRH) is reported to alter the synthesis of P4 [13] by manipulating growth of the follicle [14] and number of CL [15]. Consequently, administration of GnRH at the time of insemination results in increased conception rates in cattle [16–18]. In addition, human chorionic gonadotropin (hCG) has also commonly been used in cattle to boost endogenous concentrations of P4 in blood [19–21]. The increase in P4 may be a result of the formation of an accessory CL [22,23] combined with promoting growth of the existing CL [22,24]. Nevertheless, hCG inconsistently improves pregnancy rates [23–25]. Consequently, these hormones (GnRH and hCG) were used to boost endogenous P4 in the present study.

Prostaglandin F2α (PGF2α) and tumor necrosis factor-alpha (TNF-α) have been linked to retention of pregnancy by several investigators. Thus, it is well known that PGF2α is responsible for inducing regression of the CL [26], which is synthetized by the uterus and regulated by P4 [27]. In cyclic sheep, loss of P4 receptors allows for the uterine release of luteolytic pulses of PGF2α suggesting an inverse relationship. Tumor necrosis factor-alpha has both luteotropic and luteolytic functions [28]. Progesterone is considered to be a potent inhibitor of TNF-α messenger RNA (mRNA) and TNF-α protein production [29]. A decrease in TNF-α concentration on d 7 after the transfer of embryos may be associated with the decreased concentrations of P4 observed in the nonpregnant animals in a previous trial [10]. Therefore, the objective of this study was to assess the effectiveness of four treatments in increasing blood P4 and its effects on TNF-α and PGF2α. Our working hypothesis was that high concentrations of circulating P4 creates a window of time that facilitates synchrony between the embryo and the uterine environment by regulating concentrations of PGF2α and TNF-α in the uterine environment of the recipient.

2. Materials and Methods

2.1. Experimental Design and Hormonal Protocol. The study was approved by the Institutional Animal Care and Use Committee of Mississippi State University (II-023) and implemented at The Coastal Plain Branch Experiment Station of Mississippi State University in Newton, MS in the Spring of 2011. Lactating Angus crossbred cows were synchronized for estrous by receiving a CIDR (Eazi-Breed CIDR; Zoetis, Madison NJ) for 7 d. One d after removal, all cows (n = 62) received an injection of PGF2α (25 mg IM; Lutalyse; Zoetis). Cows were observed for estrus (d 0) four times per d (1 h at each time) during the 80 h post-PGF2α. Following manual evaluation of the CL via palpation per rectum, all cows exhibiting estrus with a CL received an embryo in the uterine horn ipsilateral to the CL on 7 d after estrus. At the time of transfer, cows were assigned to 1 of 4 treatments: no further treatment (Control, n = 16), a CIDR insert (CIDR, n = 16), an injection of hCG (1000 IU, IM; Sioux Biochemical, Inc, Sioux Center, IA; hCG, n = 15) or an injection of GnRH (100 μg, IM; Cystorelin; Merial, Duluth, GA; GnRH, n = 15).

2.2. Animals and Embryos. Animals were body condition scored (scale of 1 = emaciated; 9 = obese) by visual appraisal at the beginning of the project according to Whitman [30]. Embryos used in the study were donated by Mississippi State University. Flushing and freezing of the embryos were performed on d 7 after insemination. Embryos were a quality grade 1 [31] and developmental stages 4 and 5; the embryos were frozen in ethylene glycol and stored in liquid nitrogen until their use. The transfer of embryos was performed by an embryo transfer practitioner (Mid-South Reproductive Services, Baton Rouge, LA). Pregnancy diagnosis via palpation per rectum was determined at 60 d after transfer of the embryos.

2.3. Collection and Laboratory Analysis of Blood Samples. All samples were collected in 6.0 mL plastic vacutainers with no additives (Fisher Scientific, Pittsburg, PA) from the tail vein. Immediately after collection the samples were stored on ice until they could be centrifuged for 15 min at 1800g, which was followed by long term storage at −20°C until later analysis. Blood samples for determination of 13, 14-dihydro-15-keto PGF2α metabolite (PGFM) were collected from half the animals within each treatment group on d 14 and the remaining half on d 21. On each of these two days, animals selected for collection of blood were additionally divided in two groups and collected every 15 min for 2 h in two individual restraining systems. Synthesis of PGF2α in each blood sample was inhibited as previously described by [32]. Blood samples were collected from all cows on d 7 (day of transfer), d 14, and d 21 for analysis of P4 and TNF-α.

The concentration of P4 in peripheral blood plasma was determined via radioimmunoassay that has been validated for use in bovine (Coat-a-Count Progesterone, Los Angeles, CA) and used according to the manufacturer’s procedure. Plasma samples were assayed for concentrations of TNF-α via a double antibody radioimmunoassay as described by Kenison et al. [33], with the following changes. Antibody (rabbit anti-bovine TNF-α R7-93) generated against recombinant bovine TNF-α (kindly donated by Ciba-Geigy, Basel, Switzerland) was used as the primary antibody at a final tube dilution of 1:120,000 and recombinant bovine TNF-α (Kingfisher Biotech, St. Paul, MN) was radiiodinated and used as the assay tracer. Concentrations of PGFM were measured using an enzyme-linked immunosorbent assay (ELISA; Oxford Biomedical Research, Oxford, MI) and used according to the manufacturer’s instructions. The intrasay
and interassay coefficients of variation were 6.25 and 9.38%, respectively.

2.4. Statistical Analysis. Body condition scores of experimental animals were analyzed using the GLM procedure (SAS, Inst. Inc., Cary, NC). Data on conception rates (%) was also analyzed using the GLM procedure with a significance level of 5%; treatment means were compared using the Duncan multiple range test. Concentrations of P4, TNF-α, and PGFM in blood were analyzed using the MIXED procedure SAS (SAS Inst., Inc.) with repeated measures. The repeated measures model for the response plasma hormone concentrations on d 0, d 7, and d 14 contained the fixed effect of the treatments and the repeated factors of day and their corresponding interactions. Least squares means by the Bonferroni adjustment were analyzed and separated when the their corresponding interaction with cows failing to maintain pregnancy in the hCG group had significantly greater concentrations of P4 (P ≤ 0.05) on d 14 (5.40 ± 0.58 ng/mL) and d 21 (2.91 ± 0.61 ng/mL) compared to nonpregnant cows in any other treatment groups on d 14 (2.27 ± 0.63, 2.32 ± 0.68, and 2.57 ± 0.44 ng/mL) and on d 21 (0.91 ± 0.63, 0.46 ± 0.44, and 1.24 ± 0.47 ng/mL) for the control, CIDR, and GnRH groups, respectively. Although nonpregnant cows in the control and CIDR groups had similar concentrations on d 7 and d 14, a decrease (P ≤ 0.05) in the concentration of P4 occurred from d 14 (2.28 ± 0.56, 2.31 ± 0.68 ng/mL) to d 21 (0.90 ± 0.56, 0.46 ± 0.68 ng/mL; Table 1); Animals in these same two experimental groups are the only groups in the study experiencing a decrease in P4 from d 7 to d 21. Non-pregnant animals in the GnRH group also had a decline (P ≤ 0.05) in P4 from d 14 (3.34 ± 0.44 ng/mL) to d 21 (1.24 ± 0.47 ng/mL); whereas, animals in the hCG group had an increase (P ≤ 0.05) in concentration of P4 from d 7 (2.67 ± 0.59 ng/mL) to d 14 (5.4 ± 0.59 ng/mL); nevertheless, they similarly had a decrease (P ≤ 0.05) in concentration of P4 from d 14 (5.4 ± 0.59 ng/mL) to d 21 (2.90 ± 0.62 ng/mL). Conversely, animals that maintained pregnancy in the control, CIDR and hCG group had an increase (P ≤ 0.05; Table 1) in P4 from d 7 (2.27 ± 0.49, 1.54 ± 0.33, 2.17 ± 0.49 ng/mL) to d 14 (3.44 ± 0.49, 2.98 ± 0.36, 4.53 ± 0.78 ng/mL) along with a significant decline from d 14 to d 21.

It is believed that hCG may have increased overall secretions of P4 from the primary CL as well as from an induced secondary luteal structure during the first week of the study [39, 40]. Moreover, Mason et al. [10] also observed a significant increase in P4 7 d after the transfer in control and CIDR-treated cows retaining the embryos to completion of pregnancy.

### 3. Results and Discussion

3.1. Progesterone. It is well documented in the literature that body condition of animals influences systemic P4 concentrations of cows [34, 35]. No significant differences were observed in body condition scores among cows in the hCG (5.76 ± 0.21), control (5.47 ± 0.18), GnRH (5.68 ± 0.11), and CIDR (5.67 ± 0.49) groups of this study. Pregnancy diagnosis via palpation per rectum at 60 d after transfer of the embryos revealed retention rates of 56.2% (9/16) for the control group, 62.5% (10/16) for the CIDR group, 13.3% (2/15) for the GnRH group, and 46.6% (7/15) for the hCG group. Pregnancy rates were not different between cows in the control, CIDR, and hCG groups (P > 0.05); however, percent pregnancy rate was lower (P < 0.05) in the GnRH group when compared to the control and CIDR groups. Other investigators have also observed a negative effect on conception rates in lactating dairy cows receiving treatment with GnRH right after artificial insemination [36]. Nevertheless, it has been shown to improve conception rate in repeat-breeder dairy cows when injected at the time of the fourth insemination [37].

An overall comparison between pregnant and nonpregnant animals (Figure 1) revealed that pregnant cows had increased (P ≤ 0.05) concentrations of P4 on d 21 compared to nonpregnant cows in this study (Figure 1). These results are supported by previous reports revealing that majority of embryo losses occur between d 14 and d 21 of the gestation [7, 9, 10]. However, both nonpregnant and pregnant cows had an increase (P ≤ 0.05) in concentration of P4 from d 7 to d 14, but a decrease (P ≤ 0.05) from d 14 to d 21. However, regardless of the treatment only nonpregnant cows experienced a significant decrease in P4 (P ≤ 0.05) from d 7 to d 21 of this study; this is attributed to the regression of the CL [38] due to factors impairing luteal activity taking place perhaps during the first days after the transfer [7]. Additionally, a previous study revealed that nonpregnant animals bearing a CIDR experienced an increase on P4 from d 7 to d 14 due to a P4 output by the regressing CL combined with the P4 released by the CIDR [10].

There was a significant treatment by pregnancy status interaction with cows failing to maintain pregnancy in the hCG group having significantly greater concentrations of P4 (P ≤ 0.05) on d 14 (5.40 ± 0.58 ng/mL) and d 21 (2.91 ± 0.61 ng/mL) compared to nonpregnant cows in any other treatment groups on d 14 (2.27 ± 0.63, 2.32 ± 0.68, and 2.57 ± 0.44 ng/mL) and on d 21 (0.91 ± 0.63, 0.46 ± 0.44, and 1.24 ± 0.47 ng/mL) for the control, CIDR, and GnRH groups, respectively. Although nonpregnant cows in the control and CIDR groups had similar concentrations on d 7 and d 14, a decrease (P ≤ 0.05) in the concentration of P4 occurred from d 14 (2.28 ± 0.56, 2.31 ± 0.68 ng/mL) to d 21 (0.90 ± 0.56, 0.46 ± 0.68 ng/mL; Table 1); Animals in these same two experimental groups are the only groups in the study experiencing a decrease in P4 from d 7 to d 21. Non-pregnant animals in the GnRH group also had a decline (P ≤ 0.05) in P4 from d 14 (3.34 ± 0.44 ng/mL) to d 21 (1.24 ± 0.47 ng/mL); whereas, animals in the hCG group had an increase (P ≤ 0.05) in concentration of P4 from d 14 (2.67 ± 0.59 ng/mL) to d 14 (5.4 ± 0.59 ng/mL); nevertheless, they similarly had a decrease (P ≤ 0.05) in concentration of P4 from d 14 (5.4 ± 0.59 ng/mL) to d 21 (2.90 ± 0.62 ng/mL). Conversely, animals that maintained pregnancy in the control, CIDR and hCG group had an increase (P ≤ 0.05; Table 1) in P4 from d 7 (2.27 ± 0.49, 1.54 ± 0.33, 2.17 ± 0.49 ng/mL) to d 14 (3.44 ± 0.49, 2.98 ± 0.36, 4.53 ± 0.78 ng/mL) and a significant decrease from d 14 to d 21.

It is believed that hCG may have increased overall secretions of P4 from the primary CL as well as from an induced secondary luteal structure during the first week of the study [39, 40]. Moreover, Mason et al. [10] also observed a significant increase in P4 7 d after the transfer in control and CIDR-treated cows retaining the embryos to completion of pregnancy.
Table 1: LSMeans and standard errors for concentrations of progesterone (ng/mL) in nonpregnant and pregnant cows within treatments.

| Day | Treatment | Nonpregnant | Pregnant |
|-----|-----------|-------------|----------|
|     | Control   | CIDR¹       | GnRH²    | hCG³     |
|     |           | Nonpregnant | Pregnant | Nonpregnant | Pregnant | Nonpregnant | Pregnant |
| 7   | 2.22 ± 0.56ᵃ | 2.27 ± 0.49ᵇ | 1.54 ± 0.33ᵃ | 2.57 ± 0.44ᵇ | 1.07 ± 1.66ᵃ | 2.67 ± 0.46ᵃ | 2.17 ± 0.49ᵇ |
| 14  | 2.28 ± 0.56ᵃ | 3.44 ± 0.49ᵇ | 2.31 ± 0.47ᵇ | 2.98 ± 0.36ᵇ | 3.35 ± 0.44ᵇ | 1.16 ± 1.66ᵃ | 5.40 ± 0.73ᵇ | 4.53 ± 0.78ᵇ |
| 21  | 0.90 ± 0.56ᵇₓ | 2.54 ± 0.49ᵇᵧ | 0.46 ± 0.33ᵇₓ | 1.73 ± 0.25ᵇₓ | 1.25 ± 0.47ᵇₓ | 0.85 ± 1.66ᵇₓ | 2.94 ± 0.94ᵇₓ | 2.11 ± 0.95ᵇₓ |

ᵃᵇᶜMeans within the same column lacking a common superscript are significantly different (P ≤ 0.05).

Table 2: LSMeans and standard errors for concentrations of TNF-α (pg/mL) in nonpregnant and pregnant cows within treatments.

| Day | Treatment | Nonpregnant | Pregnant |
|-----|-----------|-------------|----------|
|     | Control   | CIDR¹       | GnRH²    |
|     |           | Nonpregnant | Pregnant | Nonpregnant |
| 7   | 0.11 ± 0.02ᵃ | 0.12 ± 0.01ᵇ | 0.12 ± 0.03ᵃ |
| 14  | 0.01 ± 0.02ᵇ | 0.11 ± 0.01ᵇ | 0.12 ± 0.01ᵇ |
| 21  | 0.11 ± 0.01ᵇ | 0.12 ± 0.01ᵇ | 0.12 ± 0.01ᵇ |

ᵃᵇᶜMeans within the same column lacking a common superscript are significantly different (P ≤ 0.05).

Concentrations of P4 between treatment groups were not different at the time of transfer of the embryos (Figure 2) as a result of the previously synchronized estrus and the examination of the viability and presence of a well-developed CL in all animals on that day. Concentrations of P4 decreased (P ≤ 0.05) from d 14 to d 21 in cows from all treatment groups; however, only cows within the GnRH group experienced decline in P4 concentrations (P ≤ 0.05) from d 7 to d 21. This is in line with previous reports indicating that GnRH directly downregulates P4 release [41, 42]. On d 14, cows in the hCG group had increased concentrations of P4 compared to animals in all other treatment groups. On d 21, concentrations of P4 in cows in the hCG group were only greater (P ≤ 0.05) than those in the GnRH group on that same day. Also, cows in the hCG group were the only ones with an increase (P ≤ 0.05) in P4 from d 7 to d 14.

3.2. Tumor Necrosis Factor-α. Concentrations of TNF-α declined (P ≤ 0.05) in animals in the hCG group from d 7 to d 21 (Figure 3). This same figure also shows a greater (P ≤ 0.05) concentration of TNF-α in the hCG group compared to the GnRH group on d 7. The decrease (P ≤ 0.05) in TNF-α between d 14 and d 21 also follows the decrease (P ≤ 0.05) in concentrations of P4 within the hCG group.

The similar pattern of concentration between P4 and TNF-α suggests some type of link that allows this hormone and protein to act congruently [43]. When the treatment groups were looked at individually between the pregnant and nonpregnant cows (Table 2), decreased (P ≤ 0.05) concentrations of TNF-α from d 7 to d 21 were observed in the nonpregnant cows of the hCG group; additionally, an increased (P ≤ 0.05) concentration of TNF-α in the pregnant...
cows was observed when compared to the nonpregnant cows on d 21 on that same group. Similar results were observed in a previous study where concentrations of TNF-α increased after hCG administration, suggesting a relationship between hCG and TNF-α via the Interleukin-6 receptor system [44, 45].

An overall comparison between pregnant and nonpregnant animals (Figure 4) showed a decrease \( P \leq 0.05 \) in concentration of TNF-α from d 7 to d 21 in nonpregnant cows, which occurred similarly in P4 in this same experimental group. Previously, it has been reported by our laboratory that low concentrations of TNF-α are linked to low concentrations of P4 in nonpregnant cows [10]. The nonpregnant group additionally showed a greater \( P \leq 0.05 \) concentration of TNF-α on d 7 compared to the pregnant group for reasons unable to be determined with these results. However, it is noteworthy that contrary to nonpregnant animals, pregnant cows maintained steadier concentrations of TNF-α throughout the entire experimental period. Interestingly, TNF-α has been reported having luteolytic properties. Some investigators [46–48] have suggested that TNF-α is deleterious to young embryos and promotes the process of luteolysis, thereby stimulating the release of PGF2α. On the other hand, other investigators [28] suggest that TNF-α may provide both luteolytic and luteotropic tendencies. Thus, the increased concentrations of P4 in pregnant animals may have played a role in inhibiting the luteolytic properties of TNF-α [49, 50]; nevertheless, the decreasing concentrations of TNF-α in the nonpregnant cows, seems to be associated with the luteolytic properties and consequently low concentrations of P4 as it has been reported in some other species and in cattle [28, 51].

3.3. Prostaglandin F2α. There were no significant differences in concentrations of PGF2α (\( P \geq 0.05 \)) between the treatment groups on d 14 (0.30 ± 0.48, 0.37 ± 0.84, 0.33 ± 0.46, 0.43 ± 0.99 pg/mL in control, CIDR, GnRH, and hCG groups, resp.) or d 21 (0.51 ± 0.11, 0.64 ± 0.69, 0.55 ± 0.14, 0.62 ± 0.85 pg/mL in control, CIDR, GnRH, and hCG groups, resp.) or between the pregnant and nonpregnant animals within treatment groups. Many studies have associated increased concentrations of PGF2α with the termination of pregnancy [26], as PGF2α is released from the uterus to essentially cause spontaneous luteolysis in cattle. However, in these current data, animals in the pregnant group actually had more steady concentrations of PGF2α on d 21, inferred from the measurement of PGFM, compared to the nonpregnant group on that same day (Table 3).

Prostaglandin F2α is released in pulses from the endometrium of the uterus and 80% of it is metabolized during one passage of the lungs, which helps create a short half-life for PGF2α as well as fluctuations in concentrations [52]. As expected, variation existed among the six samples collected over the 2h period for each cow. These data are supported by fellow investigators [32, 53] who also reported variations within concentration of PGF2α between cyclic and noncyclic ewes. With the exception of pregnant cows on d 21, on both d 14 and d 21 there were consistently one or two samples within both the pregnant and nonpregnant animals that were different \( P \leq 0.05 \) than the other samples collected on one of those days (Table 3). Furthermore, concentrations of PGFM in samples 5 and 6 within d 21 were significantly increased \( P \leq 0.05 \) in the pregnant (0.69 ± 0.69; 0.57 ± 0.69) cows compared to the nonpregnant
cows (0.45 ± 0.49; 0.40 ± 0.49), respectively. Nevertheless, concentrations of PGFM were not correlated on either d 14 or d 21 of the study with concentrations of P4. These findings are supported by other investigators [27, 54] who found that PGF2α actually increases during pregnancy. This suggests that the pattern of uterine secretion is altered during pregnancy and that this increased concentration of PGF2α now becomes luteo-protective rather than a luteolytic pattern of secretion [55]. One possible luteo-protective mechanism for the pregnant animal is to lower their sensitivity to the luteolytic effects of PGF2α [56]. Sensitivity may be lowered by the steady release of PGF2α in pregnant animals, where nonpregnant animals have more peaks and variations in their PGF2α release [57]. This steady release would allow the CL to become desensitized and have less PGF2α receptors, which would induce a more rapid metabolism of PGF2α to the inactive PGFM. Alternatively, along with the steady secretion of PGF2α, the uterus may receive signals by the conceptus via interferon tau to induce the release of PGF2α, which would consequently reduce the luteolytic effects of PGF2α [58].

4. Conclusion

These results indicate that the strategy of boosting endogenous P4 in cattle by injecting GnRH immediately at the transfer of embryos results in low pregnancy rates. Although treatment with hCG resulted in being the best treatment to boost systemic P4, this did not translate into a higher percent pregnancy compared to the other treatments in this study. Instead, similar concentrations of P4 between d 7 and d 21 are more suggestive of the survival of transferred embryos. Furthermore, with the exception of GnRH, pregnant animals in the other experimental groups had a significant increase in concentrations of P4 from d 0 to d 7. In addition, increased concentrations of P4 seem to be linked with TNF-α, perhaps by inhibiting the luteolytic effects of TNF-α as more of these cows maintained pregnancy. Concentrations of PGFM were steadier in pregnant animals.

| Serum samples | Day 14 | Day 21 | Day 14 | Day 21 | All pregnant | All nonpregnant |
|---------------|--------|--------|--------|--------|--------------|-----------------|
|               | Pregnant | Nonpregnant | Pregnant | Nonpregnant |              |                 |
| 1             | 0.38 ± 0.60<sup>a</sup> | 0.68 ± 0.69<sup>a</sup> | 0.37 ± 0.32<sup>b</sup> | 0.67 ± 0.49<sup>a</sup> | 0.49 ± 0.15 | 0.52 ± 0.15 |
| 2             | 0.36 ± 0.54<sup>b</sup> | 0.68 ± 0.69<sup>a</sup> | 0.35 ± 0.32<sup>a</sup> | 0.66 ± 0.49<sup>a</sup> | 0.52 ± 0.16 | 0.51 ± 0.16 |
| 3             | 0.33 ± 0.60<sup>a</sup> | 0.71 ± 0.69<sup>a</sup> | 0.33 ± 0.32<sup>a</sup> | 0.67 ± 0.49<sup>a</sup> | 0.52 ± 0.19 | 0.50 ± 0.17 |
| 4             | 0.31 ± 0.54<sup>a</sup> | 0.77 ± 0.74<sup>a</sup> | 0.30 ± 0.32<sup>a</sup> | 0.68 ± 0.52<sup>a</sup> | 0.54 ± 0.23 | 0.49 ± 0.19 |
| 5             | 0.31 ± 0.54<sup>a</sup> | 0.69 ± 0.69<sup>a</sup> | 0.29 ± 0.32<sup>a</sup> | 0.45 ± 0.49<sup>b</sup> | 0.50 ± 0.19 | 0.37 ± 0.08 |
| 6             | 0.51 ± 0.54<sup>b</sup> | 0.57 ± 0.69<sup>a</sup> | 0.46 ± 0.32<sup>a</sup> | 0.40 ± 0.49<sup>b</sup> | 0.54 ± 0.03 | 0.43 ± 0.03 |

Mean 0.36 ± 0.03<sup>a</sup> 0.68 ± 0.03<sup>a</sup> 0.35 ± 0.03<sup>a</sup> 0.59 ± 0.05<sup>a</sup> 0.52 ± 0.16<sup>a</sup> 0.47 ± 0.12<sup>a</sup>

<sup>a,b,c</sup>Means within the same column lacking a common superscript are significantly different.
<sup>x,y</sup>Means within row and within treatment group lacking a common superscript are significantly different (P ≤ 0.05).

<sup>(P ≤ 0.05).</sup>

1Serum samples taken 15 minute apart on each day.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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