The Effects of Prostaglandin E_2 Treatment on the Secretory Function of Mare Corpus Luteum Depends on the Site of Application: An *in vivo* Study

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We examined the effect of prostaglandin (PG) E_2 on the secretory function of equine corpus luteum (CL), according to the application site: intra-CL injection vs. an intrauterine (intra-U) administration. Moreover, the effect of intra-CL injection vs. intra-U administration of both luteotropic factors: PGE_2 and human chorionic gonadotropin (hCG) as a positive control, on CL function was additionally compared. Mares were assigned to the groups ($n = 6$ per group): (1) an intra-CL saline injection (control); (2) an intra-CL injection of PGE_2 (5 mg/ml); (3) an intra-CL injection of hCG (1,500 IU/ml); (4) an intra-U saline administration (control); (5) an intra-U administration of PGE_2 (5 mg/5 ml); (6) an intra-U administration of hCG (1,500 IU/5 ml). Progesterone (P_4) and PGE_2 concentrations were measured in blood plasma samples collected at −2, −1, and 0 (pre-treatment), and at 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after treatments. Moreover, effects of different doses of PGE_2 application on the concentration of total $PGF_{2\alpha}$ ($PGF_{2\alpha}$ and its main metabolite 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$– PGFM) was determined. The time point of PGE_2, hCG, or saline administration was defined as hour "0" of the experiment. An intra-CL injection of PGE_2 increased P_4 and PGE_2 concentrations between 3 and 4 h or at 3 and 12 h, respectively ($p < 0.05$). While intra-U administration of PGE_2 elevated P_4 concentrations between 8 and 24 h, PGE_2 was upregulated at 1 h and between 3 and 4 h ($p < 0.05$). An intra-CL injection of hCG increased P_4 concentrations at 1, 6, and 12 h ($p < 0.05$), while its intra-U administration enhanced P_4 and PGE_2 concentrations between 1 and 12 h or at 3 h and between 6 and 10 h, respectively ($p < 0.05$). An application of PGE_2, dependently on the dose, supports equine CL function, regardless of the application site, consequently leading to differences in both P_4 and PGE_2 concentrations in blood plasma.

Keywords: prostaglandin E2, human chorion gonadotropin, corpus luteum, progesterone, mare

INTRODUCTION

Corpus luteum (CL) is critical for reproductive cyclicity and pregnancy maintenance, which depends on the supportive action of progesterone (P$_4$) secreted by this transient endocrine gland (1–3). The lifespan of CL is controlled by numerous regulatory factors with luteotropic and luteolytic effects (4) such as cytokines, growth factors, P$_4$, 17β-estradiol (E$_2$), luteinizing hormone (LH), prostaglandin (PG) E$_2$, and PGF$_{2\alpha}$, respectively (5–7). Some of these factors are widely applied in veterinary practice for estrus synchronization. Mostly, PGF$_{2\alpha}$ is used for the regulation of the estrous cycles in the mare. However, application of PGE$_2$ or LH analogs (human chorionic gonadotropin; hCG and equine chorionic gonadotropin; eCG) are also key areas of veterinarian interests in the control of equine reproduction. In addition, the interesting issues in the veterinary practice are different models of drug administration have been investigated in farm animals (5, 8, 9).

Human chorionic gonadotropin is a glycoprotein purified from the urine of pregnant women (10). This glycoprotein acts as LH, sharing the same receptor (1). The evidence for the presence of the LH/CGR receptor in the reproductive tract of humans and other domestic animals has been previously described (11, 12). Moreover, in mares, the LH receptor is expressed in the endometrium and myometrium during the estrous cycle and anestrus (13). Intramuscular (i.m.) (14), subcutaneous (s.c.) (10), or intravenous (i.v.) (15–17) hCG administration has shown a good efficacy in the induction of ovulation to improve the time of mating in mares. Moreover, in mares at early diestrus, i.m. (18) or i.v. (19) hCG application results in an increase in circulating progestin concentrations. Other studies using hCG found promising results in breeding mares. Intravenous hCG administration has been advocated for use to increase fertility and early equine pregnancy rates (20). In addition, the positive effect of i.v. or i.m. hCG administration on an additional CL formation and an increase in pregnancy rates have been reported in cattle (21–24). Therefore, in our study hCG was used as a control–reference luteotropic factor.

Prostaglandins are key factors in many reproductive processes in mammals, such as luteolysis, fertilization, maternal recognition of pregnancy, and implantation (5). It has been previously demonstrated that PG are produced by the CL in numerous species (25–30). Prostaglandin E$_2$ is known as a luteotropic factor (1, 31, 32). Our preliminary *in vitro* study confirmed that PGE$_2$ plays a luteotropic role as an auto-paracrine factor stimulating P$_4$ production by equine luteal steroidogenic cells and CL tissues (33, 34). The effects of PGE$_2$ are mediated by four receptor subtypes, which are encoded by different genes: EP1, EP2, EP3, and EP4 (35). The expression of the EP2 and EP4 receptors in the uterus during the estrous cycle and pregnancy has been reported in mares (36). In contrast to PGE$_2$, PGF$_{2\alpha}$ is the main luteolytic agent secreted in pulses from the uterine endometrium of numerous mammals during luteolysis including mares (37–40). Ginther et al. (41) demonstrated that pulses of PGF$_{2\alpha}$ detected before the onset of luteolysis were less frequent per session and less prominent than during and after luteolysis.

According to our *in vitro* studies, in mare, many factors are involved in the secretion of PG from equine CL such as cytokine (42, 43) and from the endometrium such as P$_4$, E$_2$, oxytocin, LH, or cortisol (44–46) regulating modulating enzymatic cascade of AA metabolism. In the PG production cascade, prostaglandin–endoperoxide synthases (PTGS2) convert arachidonic acid (AA) into PGH$_2$. The conversion of PGH$_2$ into PGF$_{2\alpha}$ and PGE$_2$ is catalyzed by PGF$_{2\alpha}$ synthases (PTGFS) and PGE$_2$ synthases (PTGES), respectively. Prostaglandin H$_2$ is converted to PGI$_2$ by the action of PGI$_2$ synthases (PTGIS) (47). In addition, PGE$_2$ can be converted into PGF$_{2\alpha}$ through PGE2-9-ketoreductase (PGE2-9-K) activation, an enzyme which works also as 20-α-hydroxysteroid dehydrogenase (20α-HSD), converting P$_4$ into inactive 20-α-hydroxyprogesterone (20α-OHP) (4, 48). In mares, the aldo-keto reductase (AKR1C23), which has 20α-HSD activity, converting P$_4$ to its inactive metabolite, was expressed in the CL (30, 48) and placenta during placentitis (49). Moreover, 15-hydroxyprostaglandin dehydrogenase (PGDH), which is involved in the first step of PG inactivation, leading into the generation of 15-keto-metabolites, was expressed in mares in the CL (50), gravity uterus (51), and presented from 150 days of gestation onwards (52). Similar mechanisms that involved the activity of PGE2-9-K were confirmed in the rabbit ovary (53, 54) and bovine placenta (55, 56). Therefore, due to the analysis of the action of PGE$_2$, its conversion into PGF$_{2\alpha}$ should also be considered. The above effect may depend on different interactions between luteotropic PGE$_2$ and luteolytic PGF$_{2\alpha}$.
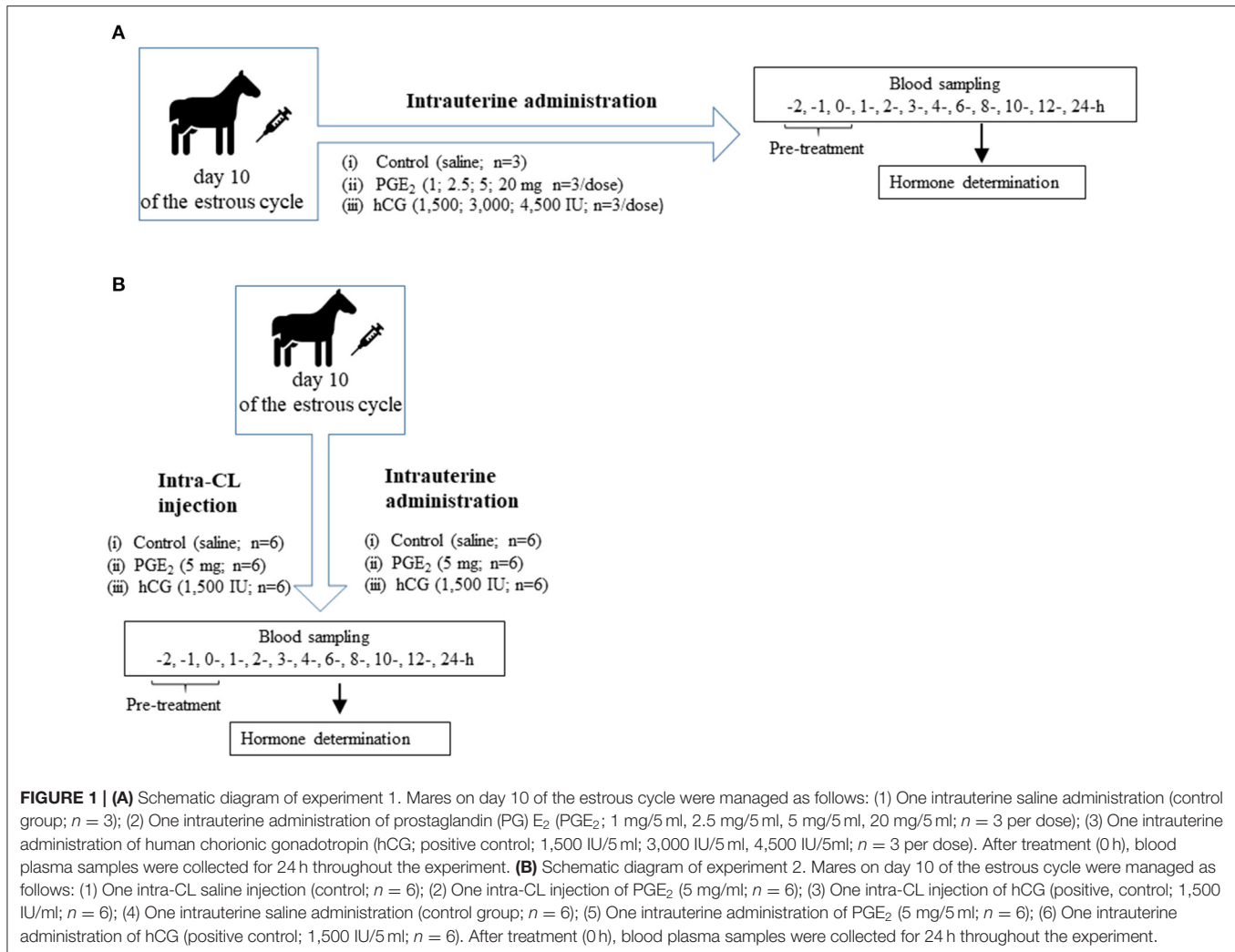
Many studies have discussed the benefits and disadvantages of different routes of PGE$_2$ administration and its proper dosages in mares (32, 57, 58). While some studies reported intrafollicular PGE$_2$ administration induced ovulation (58), in other studies intrauterine (intra-U) administration of PGE$_2$ resulted in prolonged CL (32). Moreover, the positive influence of intracervical administration of PGE$_2$ on the preparation of the uterine cervix to parturition in mares has been observed (59).

The area of research seeking the most effective routes and site for administration of luteotropic agents, used for manipulation of the reproductive processes in breeding mares, is still valuable for veterinary practitioners. To the best of our knowledge, no reports have demonstrated so far the action of PGE$_2$ on equine mid-luteal CL (day 10 of the estrous cycle) secretory function according to its application site. Therefore, the objective of this study was to determine the effects of PGE$_2$ on the secretory function of CL, according to the application site: ultrasound-guided intra-CL injection vs. intra-U administration. Moreover, the effect of intra-CL injection vs. intra-U administration of both luteotropic factors, PGE$_2$ and hCG (as a positive control), on CL function was additionally compared. Possibility of the conversion of luteotropic PGE$_2$ into luteolytic PGF$_{2\alpha}$ dependently on the dose was also examined.

MATERIALS AND METHODS

Animals and Surgical Procedures

Fifty-one clinically healthy, non-pregnant, and normally cycling mixed-breed mares (aged 3–13 years, weighing 400 ± 150 kg)

FIGURE 1 | **(A)** Schematic diagram of experiment 1. Mares on day 10 of the estrous cycle were managed as follows: (1) One intrauterine saline administration (control group; $n = 3$); (2) One intrauterine administration of prostaglandin (PG) E_2 (PGE_2; 1 mg/5 ml, 2.5 mg/5 ml, 5 mg/5 ml, 20 mg/5 ml; $n = 3$ per dose); (3) One intrauterine administration of human chorionic gonadotropin (hCG; positive control; 1,500 IU/5 ml; 3,000 IU/5 ml, 4,500 IU/5 ml; $n = 3$ per dose). After treatment (0 h), blood plasma samples were collected for 24 h throughout the experiment. **(B)** Schematic diagram of experiment 2. Mares on day 10 of the estrous cycle were managed as follows: (1) One intra-CL saline injection (control; $n = 6$); (2) One intra-CL injection of PGE_2 (5 mg/ml; $n = 6$); (3) One intra-CL injection of hCG (positive, control; 1,500 IU/ml; $n = 6$); (4) One intrauterine saline administration (control group; $n = 6$); (5) One intrauterine administration of PGE_2 (5 mg/5 ml; $n = 6$); (6) One intrauterine administration of hCG (positive control; 1,500 IU/5 ml; $n = 6$). After treatment (0 h), blood plasma samples were collected for 24 h throughout the experiment.

were used. The study was conducted between April and September 2016 in Poland. Mares were housed in private stables and were provided *ad libitum* access to water and fed hay and cereal grain. Horses deemed otherwise healthy based on a veterinary physical examination. Animal procedures were conducted in accordance with the EU Directive of the European Parliament and the Council on the protection of animals used for scientific purposes (22 September 2010; no 2010/63/EU), the Polish Parliament Act on Animal Protection (21 August 1997, Journal of Laws 1997 No 111 item 724) with further updates— the Polish Parliament Act on the protection of animals used for scientific or educational purposes (15 January 2015, Journal of Laws 2015 item 266). All animal procedures were designed to avoid or minimize discomfort, distress, and pain to the animals. Procedures were reviewed and accepted following the guidelines of the Local Ethics Committee for Experiments on Animals in Olsztyn, Poland (Approval No. 51/2011). Animals had no abnormalities of the reproductive tract detected by ultrasonic imaging. Prior to the experiment, mares received two doses of a $PGF_{2\alpha}$ analog (5 mg dinoprost, Dinolytic; Zoetis, Poland), 12

days apart, for synchronization of estrus. Follicular development was monitored in mares using transrectal palpation and USG at 12-h intervals during the periovulatory period until ovulation and every 2 days until day 10 (day 0 = day of ovulation). Moreover, structural changes of the CL during the entire estrous cycle were evaluated by ultrasonography with a 7.5-MHz linear probe (MyLabOne Vet Ultrasound System; ESOATE Pie Medica, Genoa, Italy), and visible signs of estrus (i.e., vaginal mucus and standing behavior) were assessed. In addition, the stage of the estrous cycle was confirmed by measurement of peripheral concentrations of P_4 in blood plasma samples collected from mares. **Figure 1** shows the *in vivo* study design where mares ($n = 51$) at day 10 of the estrous cycle were enrolled to the following experiments.

Intravenous Catheterization
Each mare was sedated with detomidine hydrochloride (Domosedan 0.01 mg/kg i.v.; Orion Pharma Poland Sp, Poland), followed by insertion of a temporary catheter (Intraflon IV cannulae 2.1 × 80 mm 14G, KRUUSE, 121805; KRUSSE Poland)

into the jugular vein of mares. Intravenous catheters were flushed with heparinized saline and used for frequent blood sample collections.

An Intra-CL Injection

Caudal epidural anesthesia was achieved with 4 ml procaine hydrochloride (2% Polocainum Hydrochloricum; Biowet Drwalew, Poland). All intra-CL injections were administered under ultrasound guidance (7.5 MHz linear array transducer, MyLab 30 VET Gold Color Doppler Diagnostic Ultrasound System; ESOATE Pie Medica) through a sterile 1.25 × 50 mm (2-in. 18-gauge) ovum pick-up disposable veterinary injection needle (Bovivet, Poznan, Poland). The transducer and needle guide were coated with a sterile lubricant (Medicum, Lodz, Poland), and positioned within the vagina. The convex transducer was placed in the vagina against the vaginal fornix ipsilateral to the target ovary. The needle was then passed through the vaginal wall, and intraluteal treatments, PGE$_2$ (PGE$_2$, P0409; Sigma-Aldrich, Saint Louis, Missouri, USA) or hCG (Chorulon; Intervet International B.V., The Netherlands) dissolved in sterile saline solution (1 ml), were injected directly into the CL.

Intrauterine (Intra-U) Administration

The luteotropic factors were administrated directly into the uterine lumen of mares. The catheter was protected by a sanitary sheath that was broken immediately before the catheter passed through the opening of the cervix. Prostaglandin E$_2$ or hCG dissolved in sterile saline solution was infused into the uterine horn using a 5-ml sterile syringe.

Experimental Design

Experiment 1. Dose-Dependent Effect of Prostaglandin E$_2$ on CL Function, Compared With Human Chorionic Gonadotropin Action

Experiment 1 design is shown in **Figure 1A**. The dose-dependent effect of PGE$_2$ on blood plasma P$_4$ concentrations in mares on day 10 of the estrous cycle was determined as follows: (1) one intra-U saline administration (control group; $n = 3$); (2) one intra-U administration of PGE$_2$ (1 mg/5 ml, 2.5 mg/5 ml, 5 mg/5 ml, 20 mg/5 ml; $n = 3$/per dose); (3) one intra-U administration of hCG (positive control; 1,500 IU/5 ml, 3,000 IU/5 ml, 4,500 IU/5 ml; $n = 3$/per dose).

Moreover, the possibility of PGE$_2$ conversion into PGF$_{2\alpha}$, dependently on the dose, was also examined. The concentration of total PGF$_{2\alpha}$ (PGF$_{2\alpha}$ plus its main metabolite 13,14-dihydro-15-keto-prostaglandin F$_{2\alpha}$– PGFM) in blood plasma of mares on day 10 of the estrous cycle was determined after different doses of PGE$_2$ application (**Table 2**). In mares, PGF$_{2\alpha}$ in the uterine vein reaches systemic circulation and is metabolized in the lungs much via PGDH, resulting in lower concentrations of PGF$_{2\alpha}$ (38, 60). The half-life of PGF$_{2\alpha}$ in mares is short (94 s); therefore, plasma concentrations of PGFM are used to represent changes in PGF$_{2\alpha}$ output (38, 60). The blood sampling was described in Blood Sampling section.

Experiment 2. The Comparison of Intra-CL Versus Intra-U Application Site of Prostaglandin E$_2$ on CL Function, Compared With Human Chorionic Gonadotropin Action

Experiment 2 design is shown in **Figure 1B**. To investigate the effect of PGE$_2$ according to the application site on the function of equine CL, mares on day 10 of the estrous cycle were managed as follows: (1) an intra-CL saline injection (control; $n = 6$); (2) one intra-CL injection of PGE$_2$ (5 mg/ml; $n = 6$); (3) an intra-CL injection of hCG (1,500 IU/ml; $n = 6$); (4) one intra-U saline administration (control; $n = 6$); (5) one intra-U administration of PGE$_2$ (5 mg/5 ml; $n = 6$); (6) one intra-U administration of hCG (1,500 IU/5 ml; $n = 6$). Mares (from experiment 1) with intra-U administrations of saline ($n = 3$), PGE$_2$ (5 mg/5 ml $n = 3$), and hCG (1,500 IU/5 ml; $n = 3$) were used in experiment 2, respectively. The blood sampling is described in Blood Sampling section.

Blood Sampling

In mares, blood was aspirated frequently from the jugular vein according to the schedule: at −2, −1, and 0 (pre-treatment), and at 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after injection/administration as shown in **Figures 1A,B**. The time point of intra-CL injection or intra-U administration of PGE$_2$, hCG or saline was defined as hour "0" of the experiment. Blood was aspirated into sterile 10-ml tubes containing 100 µl of 0.3 M EDTA and 1% acetylsalicylic acid, pH 7.4. After centrifugation (2,000 × g for 10 min at 4°C), plasma was stored at −20°C for determination of P$_4$, PGE$_2$, PGF$_{2\alpha}$, and PGFM concentrations.

Hormone Determination

Progesterone concentration in blood plasma was measured in duplicates via RIA (P4125 104 I" RIA kit, Immunotech, Czech Republic, IM1188), according to the manufacturer's instructions. The standard curve for P$_4$ ranged from 0.1 to 100 ng/ml. The intra- and inter-assay coefficients of variation (CV) were 6.5 and 8.6%, respectively.

Prostaglandin E$_2$ was determined in blood samples using commercial ELISA kit (Enzyme Immunoassay kit; Enzo Life Science, Farmingdale, New York, USA, #ADI-901-001), according to the manufacturer's instructions. The standard curve for PGE$_2$ ranged from 39.1 to 2,500 pg/ml. The sensitivity of the PGE$_2$ assay was 13.4 pg/ml. The cross-reactivity for various prostaglandins and their metabolites was as follows: PGE$_2$ 100%, PGE$_1$ 70%, PGE$_3$ 16.3%, PGF$_{1\alpha}$ 1.4%, PGF$_{2\alpha}$ 0.7%, and 6-keto-PGF$_{1\alpha}$ 0.6%. The intra- and inter-assay CV were 13.1% and 9.7%, respectively. The intra- and inter-assay CV were 5.8 and 5.1%, respectively.

13,14-Dihydro-15-keto-PGF$_{2\alpha}$ (PGFM) was determined in blood samples using a commercial ELISA kit (PGFM Enzyme Competitive ELISA Kit, Invitrogen, Thermo Fisher Scientific, #EIAPGFM, UK), according to the manufacturer's instructions. The standard curve for PGFM ranged from 50 to 3,200 pg/ml. The sensitivity of the PGFM assay was 20.8 pg/ml. The cross-reactivity for various prostaglandins and their metabolites was as follows: PGFM 100%, PGEM 1.5%, PGF$_{2\alpha}$ 0%, and PGE$_2$ 0%. The intra- and inter-assay CV were 7.5 and 9.6%, respectively.

TABLE 1 | The effect of one intrauterine administration of prostaglandin (PG) E_2 or human chorionic gonadotropin (hCG; positive control) on progesterone (P_4) concentrations in mares' blood plasma samples ($n = 3$ per dose) at day 10 of the estrous cycle.

Group	Dose	Progesterone	
		Baseline (ng/ml)	Total amount (mean ± SEM)
Control	Saline	10.78	80.44 ± 5.12
PGE_2	1 mg	10.31	79.31 ± 22.10
	2.5 mg	10.21	68.6 ± 37.52
	5 mg	11.59	115.00 ± 8.99*
	20 mg	12.29	38.36 ± 50.91
hCG (positive control)	1,500 IU	11.67	115.2 ± 10.17*
	3,000 IU	10.91	81.81 ± 52.74
	4,500 IU	12.91	31.76 ± 16.84

All values are expressed as total amount of P_4 secretion (area under curve). Baseline indicates average concentration of P_4 (ng/ml) in the period before treatment (pre-treatment time: −2 to 0 h). Asterisks indicate significant differences in P_4 concentrations in PGE_2- or hCG-treated group versus control group. The results were considered significantly different at $p < 0.05$.

Prostaglandin $F_{2\alpha}$ was determined in blood samples using a commercial $PGF_{2\alpha}$ ELISA kit (ENZO Life Sciences Inc., Farmingdale, NY, USA; ADI-901-069) according to the manufacturer's instructions. The standard curve for $PGF_{2\alpha}$ ranged from 3.05 to 50,000 pg/ml. The sensitivity of the $PGF_{2\alpha}$ assay was 6.71 pg/ml. The cross-reactivity for various prostaglandins and their metabolites was as follows: $PGF_{2\alpha}$ 100%, $PGF1_\alpha$ 11.82%, PGD_2 3.62%, 6-keto-$PGF_{1\alpha}$ 1.38%, PGI_2 1.25%, and PGE_2 0.77%. The intra- and inter-assay CV were 6.8 and 9.7%, respectively.

Statistical Analysis

For each statistical analysis, a Gaussian distribution was tested using D'Agostino and Pearson normality test (GraphPad Software version 8.3.0; GraphPad, San Diego, CA, USA). Parametric analyses were performed because normal distribution was assumed. Two-way ANOVA (GraphPad) test was used in experiment 1 (**Supplementary Tables 1–3**) and in experiment 2 (**Supplementary Tables 4, 5**). The results were considered significantly different at $p < 0.05$.

In experiment 1, the differences in P_4 concentrations in blood plasma samples between groups treated with different doses of PGE_2 or hCG and control group were measured as the area under the curve (AUC), using the total amount of P_4 concentrations (mean ± SEM) secreted during the experiments (**Table 1**). The differences in concentrations of P_4 (**Figure 2**), and total $PGF_{2\alpha}$ concentrations (**Table 2**) in blood plasma samples in response to treatment with different doses of PGE_2 or hCG were analyzed using a repeated measures design approach in which treatments and time of sample collection (h) were fixed effects and all interactions were included (two-way ANOVA test followed by Dunnett's multiple comparison test).

In experiment 2, data were analyzed by two-way ANOVA (treatments vs. the differences in P_4 and PGE_2 concentrations)

(**Figures 3, 4**) and blood plasma samples collected after application of PGE_2 or hCG were analyzed using a repeated measures design approach in which treatments and time of sample collection (h) were fixed effects and all interactions were included (two-way ANOVA test followed by Dunnett's multiple comparison test). All values are presented as percentage of the control. The differences in P_4 (**Table 3**) and PGE_2 (**Table 4**) concentrations in blood plasma samples between PGE_2 or hCG groups were measured as AUC, using the total amount of P_4 or PGE_2 concentrations secreted during the experiments and were calculated using two-way ANOVA, followed by Dunnett's multiple comparisons test.

RESULTS

Experiment 1. Dose-Dependent Effect of Prostaglandin E_2 on CL, Compared With Human Chorionic Gonadotropin Action

In mares, only one intra-U administration of PGE_2 at the dose of 5 mg/5 ml increased the total amount of P_4 concentrations in blood plasma, compared with the control group ($p < 0.05$; **Table 1**). An increase in P_4 concentrations was observed at 4 h and between 8 and 24 h after intra-U administration of PGE_2 at the dose of 5 mg/5 ml, compared with the control group and to its concentrations in the period before treatment (pre-treatment time) ($p < 0.05$; **Figure 2A**).

An increase in P_4 concentrations in blood plasma was observed at 2 h and between 4 h and 6 h after intra-U administration of PGE_2 at the dose of 1 mg/5 ml, compared with the control group ($p < 0.05$; **Figure 2A**), while intra-U administration of PGE_2 at the dose of 20 mg/5 ml decreased its concentrations at 6 h compared with the pre-treatment time, and at 8 h, compared with the control group ($p < 0.05$; **Figure 2A**).

The total amount of P_4 concentrations increased in blood plasma only after one intra-U administration of hCG at the dose of 1,500 IU/5 ml, compared with the control group ($p < 0.05$; **Table 1**). An increase in P_4 concentrations was observed at 1 h after one intra-U administration of hCG at the dose of 1,500 IU/5 ml compared with the control group ($p < 0.05$; **Figure 2B**). Moreover, an increase in P_4 concentrations was noticed at 1 h and between 10 and 12 h after intra-U hCG administration at the dose of 1,500 IU/5 ml, compared with its concentrations in the pre-treatment time ($p < 0.05$; **Figure 2B**).

An intra-U administration of hCG at the dose of 4,500 IU/5 ml decreased P_4 concentrations at 24 h, compared with the control group ($p < 0.001$; **Figure 2B**).

Concentrations of $PGF_{2\alpha}$ and its metabolite PGFM (total $PGF_{2\alpha}$) in blood plasma samples increased at 1 h after an intra-U administration of PGE_2 at the dose of 2.5 mg/5 ml, compared with its concentrations in the pre-treatment time ($p < 0.05$; **Table 2**). Moreover, an increase in total $PGF_{2\alpha}$ concentrations was observed between 1 and 3 h after an intra-U administration of PGE_2 at the dose of 20 mg/5 ml, compared with its concentrations in the pre-treatment time ($p < 0.05$; **Table 2**).
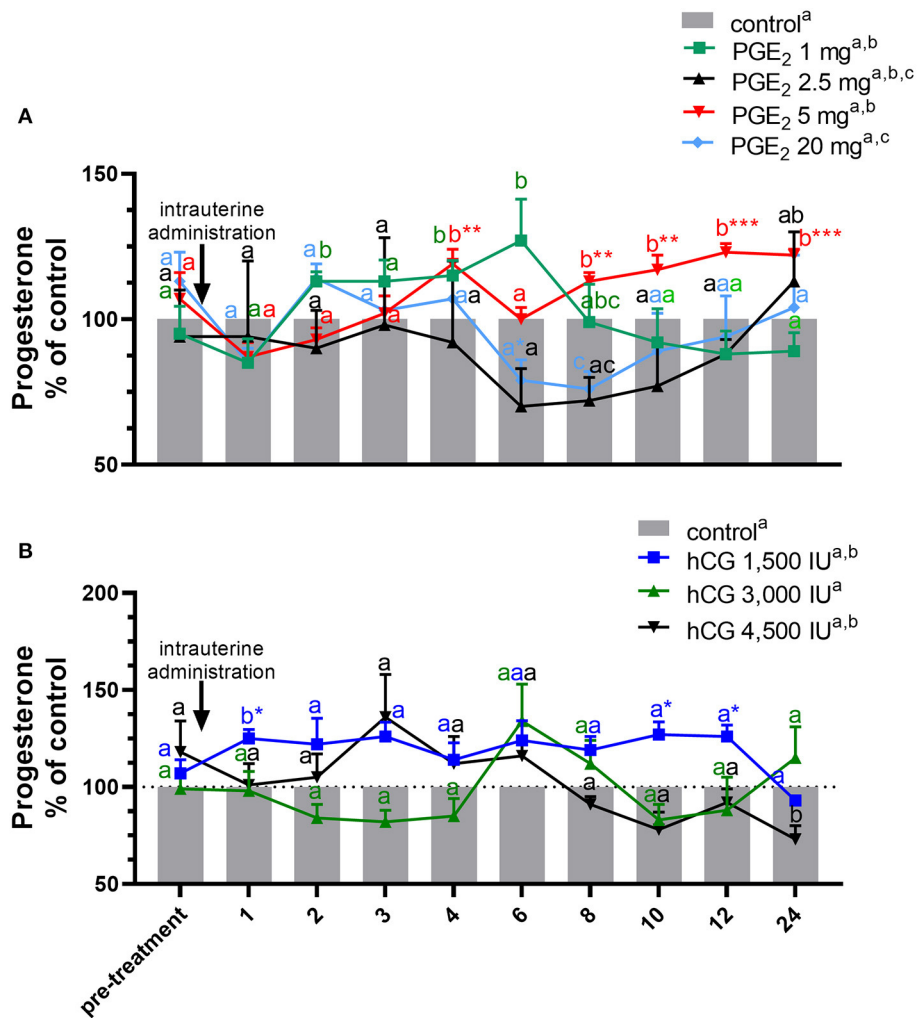
FIGURE 2 | Concentration of progesterone (P_4) in the jugular vein blood plasma in mares with one intrauterine administration of **(A)** four different doses of prostaglandin (PG) E_2 (PGE₂; 1, 2.5, 5, 20 mg/5 ml, $n = 3$/dose) and **(B)** three different doses of human chorionic gonadotropin (hCG; positive control; 1,500, 3,000, 4,500 IU/5 ml, $n = 3$/dose) on day 10 of the estrous cycle, compared with control groups. All values are presented as % of the control. Different superscript letters [a,b,c] indicate significant differences in P_4 concentrations between PGE₂- or hCG-treated group vs. control group at specific time points of blood sample collection. Asterisks indicate significant differences between P_4 levels in PGE₂- or hCG-treated group vs. average concentration of P_4 in blood plasma in the period before treatment (pre-treatment time: −2 to 0 h). The results were considered significantly different at $p < 0.05$.

Experiment 2. Comparison of Intra-CL vs. Intra-U Application of Prostaglandin E₂ on Corpus Luteum Function, Compared With Human Chorionic Gonadotropin Action

An increase in P_4 concentrations in blood plasma samples was noticed in mares, between 3 and 4 h after receiving one intra-CL injection of PGE₂, compared with its concentrations in the pre-treatment time within PGE₂-treated group ($p < 0.01$), and with respect to the control mares ($p < 0.01$; **Figure 3A**). At the same time, P_4 concentrations increased between 8 and 24 h after intra-U administration of PGE₂, compared with P_4 concentrations in the pre-treatment time within PGE₂-treated group ($p < 0.001$), as well as compared with the control mares ($p < 0.05$; **Figure 3B**).

In mares, an intra-CL injection of hCG elevated P_4 levels at 6 h, compared with P_4 levels in the pre-treatment time within PGE₂-treated group ($p < 0.05$; **Figure 3A**). Moreover, one intra-CL injection of hCG (positive control) increased P_4 concentrations in blood plasma at 1, 6, and 12 h after its application, compared with the control group ($p < 0.001$; **Figure 3A**), while its intra-U administration elevated P_4 concentrations between 1 and 12 h, compared with the control group, and to P_4 concentrations in the pre-treatment time within this group of mares ($p < 0.05$; **Figure 3B**). Total amount of P_4 found in mares with intra-U administration of hCG was greater compared with total amount of P_4 in mares with its intra-CL injection ($p < 0.05$; **Table 3**).

TABLE 2 | The effect of one intrauterine administration of prostaglandin (PG) E$_2$ on total prostaglandin F$_{2\alpha}$ (the sum of PGF$_{2\alpha}$ and PGF$_{2\alpha}$ metabolite 13,14-dihydro-15-keto PGF$_{2\alpha}$–PGFM) concentrations in mares' blood plasma samples ($n = 3$ per dose) at day 10 of the estrous cycle.

| Time (h) | Total prostaglandin F$_{2\alpha}$ (pg/ml) | | | | |
| | Intra-U administration | | | | |
	Saline (control)	1 mg PGE$_2$	2.5 mg PGE$_2$	5 mg PGE$_2$	20 mg PGE$_2$
−2	67.37 ± 10.57[a]	73.90 ± 13.79[a]	56.10 ± 1.56[a]	70.43 ± 13.03[a]	65.58 ± 2.57[a]
−1	82.19 ± 11.06[a]	89.00 ± 11.48[a]	70.48 ± 1.28[a]	89.48 ± 16.55[a]	79.96 ± 3.11[a]
0	92.00 ± 10.95[a]	96.10 ± 12.75[a]	68.48 ± 1.47[a]	92.53 ± 11.21[a]	80.01 ± 4.49[a]
1	88.64 ± 4.10[a]	102.59 ± 20.81[a]	138.47 ± 4.77[b]	86.91 ± 7.11[a]	161.11 ± 9.80[b]
2	76.62 ± 9.17[a]	74.10 ± 16.01[a]	51.75 ± 4.05[a]	74.66 ± 9.13[a]	109.39 ± 2.54[b]
3	101.21 ± 12.30[a]	79.16 ± 15.88[a]	68.21 ± 0.03[a]	73.25 ± 3.49[a]	133.30 ± 2.37[b]
4	84.06 ± 10.82[a]	59.50 ± 6.79[a]	57.88 ± 1.88[a]	62.08 ± 3.51[a]	73.45 ± 15.62[a]
6	61.84 ± 1.72[a]	84.92 ± 21.59[a]	73.89 ± 7.98[a]	57.93 ± 5.57[a]	65.57 ± 11.33[a]
8	127.73 ± 18.13[a]	67.26 ± 10.59[a]	86.80 ± 4.19[a]	85.94 ± 10.57[a]	76.49 ± 5.73[a]
10	96.13 ± 9.48[a]	65.37 ± 22.81[a]	57.63 ± 7.52[a]	58.96 ± 2.61[a]	89.50 ± 1.14[a]
12	64.18 ± 5.39[a]	93.44 ± 14.36[a]	79.25 ± 15.41[a]	77.94 ± 16.17[a]	64.58 ± 12.66[a]
24	58.29 ± 5.94[a]	71.36 ± 18.76[a]	80.52 ± 26.61[a]	70.45 ± 4.88[a]	63.70 ± 6.16[a]

All values are expressed as the mean ± SEM. Different superscript letters [a,b] within the column indicate significant differences in total PGF$_{2\alpha}$ concentrations in PGE$_2$-treated group versus the period before treatment (pre-treatment time: −2 to 0 h). The results were considered significantly different at $p < 0.05$.

In mares, an intra-CL injection of PGE$_2$ increased PGE$_2$ concentrations in blood plasma at 3 and 12 h after its administration, compared with the control group ($p < 0.05$; **Figure 4A**), while an intra-CL injection of hCG (positive control) increased its concentrations at 10 h after injection, compared with the control group ($p < 0.05$; **Figure 4A**). Prostaglandin E$_2$ concentrations were elevated after intra-U administration of PGE$_2$ at 1 h and between 3 and 4 h, relative to the control mares ($p < 0.001$), and to PGE$_2$ levels in the pre-treatment time ($p < 0.05$; **Figure 4B**). Moreover, intra-U administration of hCG (positive control) increased PGE$_2$ concentrations at 3 h and between 6 and 10 h after its administration, compared with the control group ($p < 0.01$; **Figure 4B**), and at 3 and 8 h after hCG administration, compared with PGE$_2$ levels in the pre-treatment time ($p < 0.01$; **Figure 4B**). No differences in the total amount of PGE$_2$ were observed between mares with intra-U administration and intra-CL injection ($p > 0.05$; **Table 4**).

DISCUSSION

Until now, many studies have been focusing on the different application route or sites of luteolytic/luteotropic factors that may be used in veterinary practices to regulate the estrous cycle in mares. In the literature, different ways of PGE$_2$ or hCG administrations have been demonstrated, for example, i.m., i.v., s.c., intrafollicular, or intracervical (10, 14, 17, 18, 58, 59). The ultrasound-guided intra-CL injection as a method for studying the direct effect of PGF$_{2\alpha}$ on reproductive function in mares was evaluated by Weber et al. (61). However, this technique is not widely known by practitioners. While intra-U administration of luteotropic PGE$_2$ on the CL function was described by Vanderwall et al. (32), in our study we demonstrated the effect of luteotropic factor PGE$_2$ on P$_4$ secretion, depending on the application site: intra-CL vs. intra-U in mares at day

10 of the estrous cycle. To the best of our knowledge, for the first time, we have showed that application of PGE$_2$ supports equine CL secretory function, regardless of the application site, consequently leading to differences in both P$_4$ and PGE$_2$ concentrations in blood plasma.

The role of PGE$_2$ on equine CL function is not fully understood. A previous *in vitro* study in cows confirmed that PGE$_2$ participates in luteoprotective mechanisms required for CL formation and maintenance (62), and stimulates the P$_4$ production by luteal steroidogenic cells (63). Moreover, in cows and ewes, there have been evidences that PGE$_1$ or PGE$_2$ prevented P$_4$-induced premature luteolysis by suppressing the loss of luteal LH receptors (64, 65).

Interestingly, our study shows that the action of PGE$_2$ on CL secretory function is determined by the application site and dose. An intra-CL injection of PGE$_2$ increased P$_4$ concentrations in blood plasma of mares at day 10 of the estrous cycle compared with the control group, suggesting its direct action. The aforementioned data are in agreement with a preliminary study conducted by our group (33, 34), showing that in mares PGE$_2$ plays a luteotropic role as an auto-paracrine factor stimulating P$_4$ production by luteal steroidogenic cells and CL tissues *in vitro*. Some decades ago, Vanderwall et al. (32) reported that a single intra-U administration of PGE$_2$ was capable to maintain prolonged luteal function in the mare *in vivo*. In the experiment of Vanderwall et al. (32), non-pregnant mares were continuously infused with 0.24 mg of PGE$_2$, from day 10 to 16 postestrus, using an osmotic minipump surgically placed into the uterine lumen. In our study, intra-U administration of PGE$_2$ increased P$_4$ concentrations in blood plasma on day 10 of the estrous cycle in mares, compared with the control group. Simple comparison between data obtained in our study and in the study of Vanderwall et al. (32) cannot be made because of differences in methodology of PGE$_2$ application.
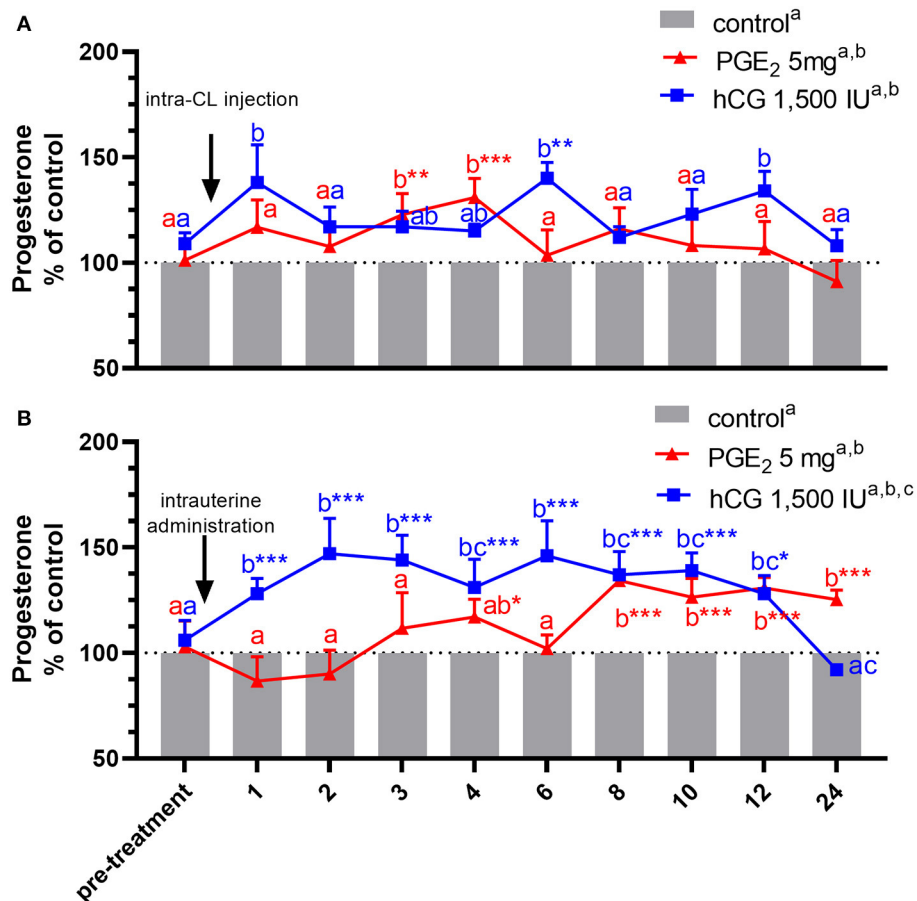
FIGURE 3 | Concentrations of progesterone (P$_4$) in the jugular vein blood plasma in mares with one **(A)** intra-CL injection of saline (control; gray bar), prostaglandin (PG) E$_2$ (PGE$_2$; 5 mg/ml; red line), or human chorionic gonadotropin (hCG, positive control; 1,500 IU/ml, blue line) or **(B)** one intrauterine administration of saline (control; gray bar), PGE$_2$ (5 mg/5 ml; red line), or hCG (positive control; 1,500 IU/5 ml, blue line) on day 10 of the estrous cycle. All values were presented as % of the control. Different superscript letters [a,b,c] indicate significant differences between blood P$_4$ level in PGE$_2$- or hCG-treated groups of mares vs. control group at specific time points of blood sample collection. Asterisks indicate significant differences in blood P$_4$ level within PGE$_2$- or hCG-treated group of mares vs. average concentration of P$_4$ in blood plasma in the period before treatment (pre-treatment time: −2 to 0 h). Average concentrations of P$_4$ in the blood plasma samples of control mares during the period before treatment (pre-treatment time) were **(A)** 10.92 ng/ml or **(B)** 11.15 ng/ml, respectively. The results were considered significantly different at $p < 0.05$.

We should take into account that in our study, whereas P$_4$ concentrations increased at 3–4 h after direct intra-CL injection of PGE$_2$, the positive effect of intra-U administration of PGE$_2$ on P$_4$ concentrations was observed between 8 and 24 h after treatment. We suppose that the aforementioned effect is a result of indirect action of PGE$_2$ on PGE$_2$ receptors in the uterus, involving the regulation of vasculature events and induction of other luteotropic factors engaged in luteal support, in the equine endometrium (e.g., growth factors, nitric oxide, and cytokines). Galvão et al. (42, 66) showed that cytokines interact with nitic oxide synthases and influence luteal angiogenesis in mares as angiogenic factors themselves can also modulate luteal secretory function. Previously, Otzen et al. (67) found that PGE$_2$ stimulates vascular endothelial growth factor (VEGF), which participates in the regeneration and expansion of the equine uterine blood vessel network. Moreover, VEGF has been reported to effectively modulate luteal secretory function of equine CL (P$_4$ and PGE$_2$ production) (66).

In the first experiment, the dose of PGE$_2$ 5 mg/5 ml was chosen as an effective dose based on an increase in P$_4$ concentrations in blood samples after intra-U treatments in mares. We demonstrated that the highest dose of PGE$_2$ administered into the uterus does not affect P$_4$ concentrations in blood plasma. Therefore, we can suspect the possibility of the conversion of PGE$_2$ by the PGE2-9-K into PGF$_{2\alpha}$. It is known that PGE2-9-K enzyme has also a 20 α-HSD activity, and in fact converts P$_4$ into 20α-OH-P$_4$, which may contribute to the decrease of P$_4$ induced by PGF$_{2\alpha}$ (4, 48). To check and confirm this fact, we examined the effect of an intra-U administration of different doses of PGE$_2$ on total PGF$_{2\alpha}$ (sum of PGF$_{2\alpha}$ and its main metabolite—PGFM) concentrations in blood plasma on day 10 of the estrous cycle in mares. Interestingly,
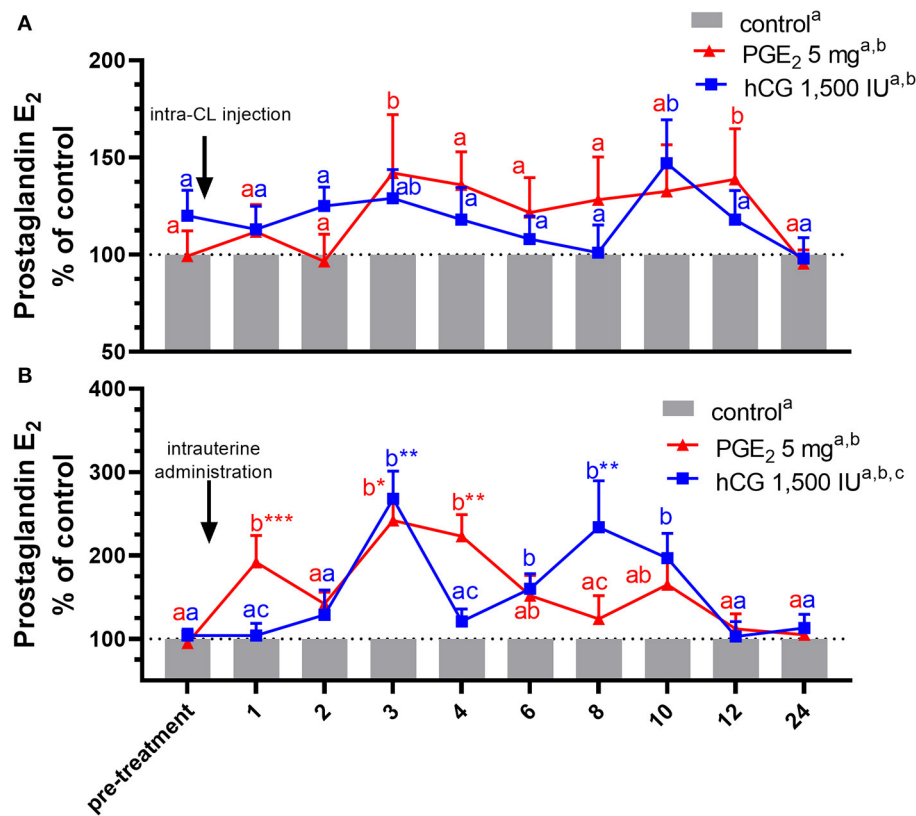
FIGURE 4 | Concentrations of prostaglandin (PG) E$_2$ in the jugular vein blood plasma in mares with one **(A)** intra-CL injection of saline (control; gray bar), PGE$_2$ (5 mg/ml; red line), or human chorionic gonadotropin (hCG, positive control; 1,500 IU/ml, blue line) or **(B)** one intrauterine administration of saline (control; gray bar), PGE$_2$ (5 mg/5 ml; red line), or hCG (positive control; 1,500 IU/5ml, blue line) on day 10 of the estrous cycle. All values are presented as % of the control. Different superscript letters [a,b,c] indicate significant differences between blood PGE$_2$ level in PGE$_2$- or hCG-treated groups of mares vs. control group at specific time points of blood sample collection. Asterisks indicate significant differences in blood PGE$_2$ level within PGE$_2$- or hCG-treated group of mares vs. average concentration of PGE$_2$ in the period before treatment (pre-treatment time: −2 to 0 h). Average concentrations of PGE$_2$ in the blood plasma samples of control mares during the period before treatment (pre-treatment time) were **(A)** 243.39 ng/ml or **(B)** 264.18, respectively. The results were considered significantly different at $p < 0.05$.

TABLE 3 | The effect of one intra-CL injection or one intrauterine administration of prostaglandin (PG) E$_2$ or human chorionic gonadotropin (hCG; positive control) on progesterone (P$_4$) concentrations in mares' blood plasma samples ($n = 6$ per group) at day 10 of the estrous cycle.

Group	Type of administration	Progesterone Total amount (mean ± SEM)
PGE$_2$	Intra-CL	13.55 ± 5.98[a]
5 mg	Intra-U	28.19 ± 6.05[ab]
hCG (positive control)	Intra-CL	23.25 ± 3.8[a]
1,500 IU	Intra-U	49.24 ± 7.47[b]

All values are expressed as total amount of P$_4$ secretion (area under curve). Different superscript letters [a,b] indicate significant differences in P$_4$ concentrations between PGE$_2$ and hCG groups. The results were considered significantly different at $p < 0.05$.

TABLE 4 | The effect of one intra-CL injection or one intrauterine administration of prostaglandin (PG) E$_2$ or human chorionic gonadotropin (hCG; positive control) on PGE$_2$ concentrations in mares' blood plasma samples ($n = 6$ per group) at day 10 of the estrous cycle.

Group	Type of administration	Prostaglandin E$_2$ Total amount (mean ± SEM)
PGE$_2$	Intra-CL	408.9 ± 205.9[a]
5 mg	Intra-U	994.8 ± 155.1[a]
hCG (positive control)	Intra-CL	457.9 ± 132.4[a]
1,500 IU	Intra-U	951.4 ± 176.7[a]

All values are expressed as total amount of PGE$_2$ secretion (area under curve). Different superscript letters [a,b] indicate significant differences in PGE$_2$ concentration between PGE$_2$ and hCG groups. The results were considered significantly different at $p < 0.05$.

we observed higher total PGF$_{2\alpha}$ concentrations in blood plasma between 1 and 3 h after intra-U administration of PGE$_2$ at the highest dose (20 mg/5 ml), compared with its concentrations in

the pre-treatment time. Hence, our *in vivo* results should be interpreted carefully and our hypothesis that the lack of the effect of PGE$_2$ in the highest dose on P$_4$ concentrations may be related

to its conversion into PGF$_{2\alpha}$ by PGE2-9-K needs further studies in mares.

In our study, we assume that intra-CL injection and intra-U administration of PGE$_2$ increased its own concentration in blood plasma. There is evidence that in the endometrium of mare, PGF$_{2\alpha}$ has an auto-amplification system, stimulating its own production (40). Therefore, future study should be planned to assume whether there is a positive PGE$_2$ feedback loop and whether PGE$_2$ has a positive effect on its own production.

There are a large number of *in vivo* studies concerning the effect of hCG on CL function in mare (16–19, 68). Kelly et al. (18) and Watson et al. (19) demonstrated the positive luteotropic effect of hCG on P$_4$ secretion. Therefore, in our study, we decided to assign mares treated with intra-CL injection or intra-U administration of hCG as positive control group. In the present study, we observed an increase in P$_4$ concentration in blood plasma after intra-U administration of 1,500 IU of hCG. No effect on CL function was reported by Brito et al. (68), using one i.v. injection of this same dose-—1,500 IU of hCG at day 10 after ovulation. In agreement with our results, a positive effect on P$_4$ secretion was observed in diestrus mares, using repeated i.m. injections of 1,000 IU of hCG (days 3, 4, 5) (18) or a single i.v. injection of 1,500 IU of hCG (day 8) (19). Interestingly, in our study one intra-U administration of hCG at the doses 3,000 IU or 4,500 IU did not affect P$_4$ secretion from equine CL. Likewise, Köhne et al. (16) did not observe any increase in P$_4$ concentration and luteal size after i.v. administration of 5,000 IU of hCG at day 5 after ovulation. Therefore, it might be suggested that higher doses of hCG are not related to their effectiveness. We have noted that both a single intra-CL injection of hCG and a single intra-U administration of hCG increased blood P$_4$ concentrations, supporting P$_4$ secretion from mare CL. The intra-CL injection of hCG seems to directly influence the luteal steroidogenic cells. An additional *in vitro* study should be conducted to explore molecular mechanisms involved in the CL secretory function in response to intra-CL injection of hCG. Unexpectedly, the intra-U administration of hCG was more effective in increasing P$_4$ secretion by CL (**Table 3**), throughout its indirect effect on equine PGE$_2$ receptors in the uterus, affecting regulation of vasculature events and induction of luteotropic factors involved in luteal support.

Human chorionic gonadotropin has structural and functional similarities with LH, sharing the same receptor with this luteotropic hormone (1). The evidence for the presence of the LH/CGR receptor in the reproductive tract of humans and other domestic animals is well described (11, 12). In mares, LH receptor expression occurs in the CL (69) and in the endometrium and myometrium during the estrous cycle and anestrus (13). Therefore, the presence of LH/CGR receptors in equine reproductive tract could mediate the indirect effect of intra-U administration of hCG and the direct effect of hCG injection into the CL. Interestingly, in the present study, we show that only one intra-U administration of hCG increases PGE$_2$ concentration in blood plasma. We have previously demonstrated that LH stimulated PGE$_2$ secretion by equine endometrium and myometrium (45). We postulate that hCG through LH/CGR receptors in the mare uterus affects the luteotropic PGE$_2$ production. Moreover, PGE$_2$ has a positive effect on P$_4$ secretion. However, further studies are needed to clarify the mechanism of action of hCG on PGE$_2$ production within the equine reproductive tract.

In conclusion, the aforementioned results indicate the importance of proper application site of drugs and may influence drug delivery strategies in veterinary medicine. Application of PGE$_2$ supports equine CL function via augmentation of P$_4$ and PGE$_2$ secretions. Progesterone secretion in response to PGE$_2$ depends on their application site. In the present study, we found more effective increase in P$_4$ secretion after intra-U administration of luteotropic factors (especially hCG) than their intra-CL injections. Therefore, the efficacy of intra-CL site of application warrants further *in vitro* and *in vivo* studies. We confirm that therapeutic use of intra-U administration of luteotropic factors is an easily applicable, valuable method in veterinary practice that may be used to support early pregnancy in mares. However, this knowledge is still insufficient and needs better understanding of the endocrine, cellular, receptor, and molecular mechanism action of luteotropic factors on equine CL function.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Local Ethics Committee for Experiments on Animals, University of Warmia and Mazury in Olsztyn, Poland (Approval No. 51/2011). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

KKP-T: conceptualization, investigation, methodology, formal analysis, visualization, writing—original draft, and writing—review and editing. AWJ: investigation, methodology, formal analysis, visualization, writing—original draft, and writing—review and editing. AZS-M: conceptualization, investigation, methodology, formal analysis, writing—original draft, and writing—review and editing. EŻ: formal analysis. GF-D: supervision and writing—review and editing. DJS: conceptualization, investigation, formal analysis, supervision, funding acquisition, and writing—review and editing. All authors have read, critically revised, and approved the final version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.753796/full#supplementary-material

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