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Early Maturation of Corpus Luteum in Rabbits - Effect of Sildenafil Citrate on Luteolytic Capacity in the Early Luteal Period

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ABSTRACT

***Background*:** Prostaglandin F_2 alpha ($PGF_2\alpha$) binds to the specific receptor (PTGFR) on the *corpus luteum* (CL) in mammals, inducing regression of the CL structure (luteolysis) and initiating a new cycle. While $PGF_2\alpha$ is effective only on mature CL, the immature CL structure (early luteal phase) resists $PGF_2\alpha$. In this study, sildenafil citrate, which is used to increase blood flow in the genital organs for treating specific pregnancy issues in women, was administered during the early luteal phase in a rabbit model to test the hypothesis of enhancing blood flow to the CL, thereby promoting earlier maturation and enabling a response to $PGF_2\alpha$.

***Materials, Methods & Results*:** The study was conducted in 2 sub-studies: clinical and molecular. A large number of rabbits were initially included in the sub-studies to ensure a sufficient number of pseudo-pregnant rabbits. Ovulation in rabbits was induced with buserelin acetate and was considered as day 0 of the study. The sub-studies were continued with rabbits whose pseudo-pregnancies were confirmed according to progesterone (P_4) results. As a result, the studies were continued with a total of 41 pseudo-pregnant New Zealand female rabbits, 21 of which were included in the clinical sub-study and 20 in the molecular sub-study. In both sub-studies, on day 3 of the luteal period, rabbits in the treatment group received 5 mg/kg sildenafil citrate and all rabbits received a single dose of exogenous $PGF_2\alpha$ on day 4 to induce luteolysis. In the clinical sub-study, echotexture and intraovarian blood flow changes in the ovaries were determined by ultrasonography (USG) examination. In the molecular sub-study, the expression changes of Hypoxia Inducible Factor 1 Alpha (*HIF1A*) and Vascular Endothelial Growth Factor (*VEGF*) related to angiogenesis, Steroidogenic Acute Regulatory Protein (*StAR*) related to P_4 metabolism, Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*) related to prostaglandin (PG) mechanism and 15-Hydroxyprostaglandin Dehydrogenase (*HPGD*) genes at mRNA level were determined using Real Time Polymerase Chain Reaction (RT-PCR) in CL tissues obtained with ovariohysterectomy (OVH) at 1 and 12 h after $PGF_2\alpha$ injection. In addition, blood samples were collected for determine P_4 levels from all rabbits. In the clinical sub-study; there was no difference between the groups in mean gray values (MGV), whereas there was a significant decrease in both pulsatile index (PI) and resistance index (RI) values at 40 min after $PGF_2\alpha$ injection ($P < 0.05$). In the molecular sub-study, it was determined that sildenafil citrate had no significant effect ($P > 0.05$) on the expression levels 1 and 12 h after $PGF_2\alpha$ injection. According to the results of the molecular sub-study, no significant effect of sildenafil citrate on the mRNA expression levels in the investigated genes was detected ($P > 0.05$). However, within each group, differences were found according to OVH time after $PGF_2\alpha$ injection. It was observed that *PTGS2* and *HPGD* mRNA expressions decreased at the 12th h compared to the 1st h, while *HIF1A* expression increased ($P < 0.05$).

***Discussion*:** According to the results obtained from clinical and molecular sub-studies, it was determined that a single dose of sildenafil citrate (5 mg/kg) applied on the 3rd day of the luteal period did not contribute to the maturation process of the CL, did not increase blood flow, and was insufficient to break the resistance of the CL against $PGF_2\alpha$ applied on the 4th day of the luteal period. However, a significant decrease in the PI value at the 40th min after $PGF_2\alpha$ injection suggests that sildenafil citrate has a supportive effect, and that this decrease is also seen in the RI value, suggesting that its effect is insufficient against the vasoconstrictive effect of $PGF_2\alpha$.

Keywords: sildenafil citrate, PGF_2 alpha, corpus luteum, early luteal period, rabbit.

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INTRODUCTION

Prostaglandin F_2 alpha ($PGF_2\alpha$) acts as the main luteolytic factor that promotes structural and functional regression of the corpus lutem (CL) and exerts its effect by binding to its specific receptor (PTGFR) [25]. However, $PGF_2\alpha$ can't show its luteolytic effect on developing CL in the early luteal period. Although the CL has sufficient PTGFR in the early luteal period, indicating the capacity to respond to $PGF_2\alpha$, the absence of a luteolytic response suggests that the CL should mature for the luteolytic response. Previous studies showed that the CL needs optimum blood flow to form, develop, and maintain function [45]. Therefore, with increased blood flow in the early luteal period, the CL may mature earlier than expected and become sensitive to exogenous $PGF_2\alpha$. Sildenafil citrate, has a short half-life, is commonly used to increase blood flow in organs/tissue. The increasing blood flow effect of sildenafil citrate relates to the inhibition of a type-5 specific phosphodiesterase (PDE5) that lowers intracellular calcium (Ca^{+2}) levels by activating guanylate cyclase via nitric oxide (NO) to produce cyclic guanosine monophosphate (sGMP) [22]. This leads to vasodilation and smooth muscle relaxation.

In the present study, we tested a hypothesis that the administration of sildenafil citrate at the early luteal period can cause increasing the blood flow to the CL, leading to the earlier maturation of the CL and gaining luteolytic capacity.

MATERIALS AND METHODS

Animals

The study was conducted with healthy, non-breeding New Zealand female rabbits aged 6-18 months, with an average weight of 4 kg. Rabbits were fed *ad libitum* water and pellets. During the study, they were kept in individual wire mesh cages under controlled heat (18-24°C) and light (14 h light, 10 h dark).

Procedures and studies designs

The study was carried out as 2 sub-studies, clinical and molecular. In order to reach sufficient number of pseudo-pregnant rabbits in the groups, studies were started with a large number of rabbits. Firstly blood samples were taken to determine P_4 levels to confirm that the rabbits were in the follicular stage before starting the study, then ovulations were induced according to Dal Bosco *et al*. [9] by intramuscular (IM) injection of 0.8 µg buserelin acetate[1] [Receptal®]. On the 3rd day of the luteal phase, before the administration of sildenafil citrate/placebo to rabbit's blood samples were taken again. Studies were continued with rabbits who were found to be pseudopregnancy according to the P_4 results obtained from the blood samples taken before and after ovulation induction. As a result, the studies were continued with a total of 41 pseudo-pregnant rabbits, 21 in the clinical sub-study and 20 in the molecular sub-study.

Study design of clinical sub-study

In the clinical sub-study, Ultrasonography (USG) examinations were performed on the 3rd day of the luteal period. Afterwards, the rabbits were randomly divided into Sildenafil (S; n=10) and Control (C; n=11) main groups. Rabbits in the S group were given 5 mg/kg sildenafil citrate[2] [Viagra®] dissolved in 1 mL 0.9 % isotonic sodium chloride (NaCl) by nasogastric tube. The dose of sildenafil citrate was determined according to previous studies in rabbits [13,31] and rats [7,15]. The rabbits in the C group were given the same amount of 0.9 % NaCl as a placebo. On the 4th day of the luteal period, USG examinations were repeated in all rabbits and luteolysis was induced with 200 µg $PGF_2\alpha$[3] [Alfaprostol, Gabbrostim®]. Various studies have shown that Alfaprostol, a $PGF_2\alpha$ analogue, induced luteolysis in rabbits with the dose applied in the present study [51,57]. The USG examinations were repeated at 40, 80, 120 min and 6, 12 and 24 h following the $PGF_2\alpha$ injection.

In the clinical sub-study, B-mode, Color and Pulsed USG examinations were performed to determine changes in luteal perfusion and echotexture in both ovaries. During USG examinations, the left ovary was first detected, and B-mode image samples were taken for computer-assisted image analyses for echotextural evaluations. The ImageJ[4] (Version 1.42q, America) program was used to determine the mean grey value (MGV) in the B-mode images taken from the ovaries. Then, following the creation of ovarian blood flow via the device's color Doppler mode, the Pulsatile Index (PI) and Resistance Index (RI) of CL were measured using the pulsed Doppler mode. All USG examinations were performed by the same clinician using an ultrasound device equipped with an 18 MHz-linear probe[5] (My Lab 30, Esaote®) as described by Polisca *et al*. [41].

Study design of molecular sub-study

In the molecular sub-study, rabbits were first randomly assigned to Sildenafil (S; n=10) and Control (C; n=10) main groups as in the clinical sub-study. Similar to the clinical sub-study, rabbits were treated with sildenafil citrate (5 mg/kg) or placebo and $PGF_2\alpha$ on days 3 and 4, respectively. Rabbits in groups (S and C groups) were spayed at 1 and 12 h after $PGF_2\alpha$ injection. For this purpose, SP1 and SP12 groups in the S group, CP1 and CP12 groups in the C group were formed, with 5 rabbits in each group (n=5), according to the ovariohysterectomy (OVH) times performed after $PGF_2\alpha$ injection.

Ovariohysterectomy operations in rabbits were performed under general anaesthesia. For premedication, anaesthesia was achieved by Xylazine hydrochloridum[6] [Xylazinbio® 2% - injecting 5 mg/kg] followed by Ketamine hydrochloride[7] [Ketasol® 10% - 35 mg/kg, IM]. The operation was performed on the median line. Chlorhexidine was used for disinfection of the operation area. Postoperatively, a single dose of Meloxicam[8] [Maxicam® - 0.5 mg/kg, administered subcutaneously] and Enrofloxacin[9] [Baytril K 5%® - 10 mg/kg for 3 days] [28,52].

In the molecular sub-study, the expression changes of Hypoxia Inducible Factor 1 Alpha (*HIF1A*) and Vascular Endothelial Growth Factor (*VEGF*) related to angiogenesis, Steroidogenic Acute Regulatory Protein (*StAR*) related to P_4 metabolism, Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*) related to prostaglandin (*PG*) mechanism and 15-Hydroxyprostaglandin Dehydrogenase (*HPGD*) genes at mRNA level were determined using Real Time Polymerase Chain Reaction (RT-PCR) in CL tissues obtained with ovariohysterectomy (OVH) at 1 and 12 h after $PGF_2\alpha$ injection.

Collection of samples for molecular sub-study

After OVH operations, CLs were taken from the ovaries and washed with phosphate-buffered solution (PBS). Then, CL tissues were cut into small pieces with a clean scalpel and placed in 2 mL steril cryo tubes[10]. The cryotubes were quickly frozen in liquid nitrogen and stored until RT-PCR analysis.

RNA isolation, RT reaction and quantitative PCR

About 50 mg of CL tissue was homogenized in TRIzol®[11] by using a homogenizer[12] [Slientcruzer M]. Total RNA was then extracted using the manufacturer's protocol. The integrity of RNA was evaluated by agarose gel (1%) electrophoresis and optical density at 260/280 nm of 2 ± 0.1 with NanoDrop ND-2000[13]. Two µg of total RNA was treated with DNAse I to eliminate possible genomic DNA contamination. RNA was then reverse transcribed in the presence of oligodT primers using the Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's protocol. To clarify RT specificity, analyses were also done with all components of the RT kit without RNA (RT negative).

Oligonucleotide primers for *StAR, PTGS2, HPGD, HIF1A, VEGF* and housekeeping gene (*18S*) were designed using Primer[3] from the NCBI gene database or published primer sequence. The primer pair sequences and product sizes are shown in Table 1.

All PCR reactions were set up: 5 µL SYBR Green Master Mix (2X)[14], 2.5 pmol of each primer, 0.5 µL cDNA, and ddH_2O to bring the final volume to 10 µL. Thermal cycling was done by initially incubating the mixture at 50°C for 2 min with subsequent denaturation at 95°C for 10 min. This was followed by 40 cycles of denaturation, annealing, and amplification (95°C 15 s, 60°C 30 s, 72°C 30 s). All reactions were done on an Stepone Plus RT-PCR System[15]. Melting curve analysis was performed: 95°C for 1 min followed by fluorescence measurement at every 1-degree increments between 60°C and 95°C. Negative controls with no cDNA template and RT negative controls were included in each run. Amplification products were evaluated after separation on a 2 % agarose gel to verify reaction specificity. All samples have been assessed in duplicate for each cDNA. The whole procedure was performed twice as a technical replicate, from the RNA extraction to the RT- PCR. Amplifications were carried out with specific primers in duplicate on a serial dilution of specific pooled cDNA to determine the efficiency for each primer pair. The efficiency of qPCR for specific gene product amplifications was between 95 and 105%. The amplified PCR products were confirmed by restriction endonuclease digestion.

The Ct values for the housekeeping gene were used to normalize the Ct values of specific genes.

Serum progesterone (P_4) analysis

Serum progesterone (P_4) levels were used as a marker for luteal activity. Blood samples were obtained from each rabbit before induction of ovulation, sildenafil citrate/placebo applications, injection of $PGF_2\alpha$ and 6, 12 and 24 h after $PGF_2\alpha$ injection through a

puncture to ear vein into BD Vacutainer SST tubes. Sera were collected by centrifugation, and serum P_4 was measured by ADVIA Centaur XP Immunoassay System[16]. Before quantification, the kit was validated physiologically by testing sera samples collected from male and pregnant rabbits.

Statistical analysis

Statistical analysis of the data obtained in the clinical sub-study were performed using the Statistical Package for the Social Sciences (SPSS) version 19.0[17]. In the control of significance of all analysis results, the $P < 0.05$ level was considered statistically significant. Arithmetic means, standard error of the mean (SEM), medians, and interquartile ranges (IQR) were calculated using descriptive statistics at each sampling time and for each parameter. The distribution of numerical data was checked with the Kolmogorov-Smirnov test, and it was determined that all data showed normal distribution. The differences between the groups regarding the evaluated parameters were compared with the t-test for independent groups. In addition, time-dependent changes of the parameters evaluated in the S and C groups were assessed with parametric repeated measures analysis of variance.

In the molecular sub-study, qPCR data were normalized according to Livak & Schmittgen [30], and the 2^-ddCt method was used (http://sabiosciences.com). The data were analyzed by One-way ANOVA. Differences among the groups were evaluated by the post hoc Fisher method. The results were given as mean ± SD, and $P < 0.05$ was considered for significance.

RESULTS

Progesterone (P_4) levels of clinical and molecular sub-studies

The study was initiated with a total of 52 rabbits, with 27 in the clinical sub-study and 25 in the molecular sub-study. Based on the P_4 values obtained from blood samples taken before ovulation, it was determined that one rabbit in the clinical sub-study was in the luteal phase with a P_4 level of 2.21 ng/mL, and it was excluded from the study. According to the P_4 values obtained from blood samples taken on the 3rd day of the luteal phase, it was found that ovulation could not be stimulated in 5 rabbits in the clinical sub-study and 5 rabbits in the molecular sub-study. Their P_4 levels remained below 1 ng/mL, and they were also excluded from the study. Consequently, pseudopregnancies were confirmed in 21 rabbits in the clinical sub-study and 20 rabbits in the molecular sub-study, and the study continued with a total of 41 rabbits.

In the clinical sub-study, there was no difference between groups in P_4 values. Depending on the time within the group, the difference between the mean P_4 levels before and 24 h after the placebo administration was found to be significant in the C group ($P < 0.05$). This significant difference was not detected in the S group. The mean P_4 levels of the rabbits in the clinical sub-study groups at times determined in the study are shown in Figure 1.

Ultrasonographic examination measurement results

In the USG examinations, the MGV, PI and RI values of the right and left ovaries of the rabbits in the study groups were measured and recorded separately at the specified time intervals. Considering that the experiment would have a systemic effect, the mean values of each rabbit were used in the analysis results.

When the MGV values between groups were compared, it was determined that there was no statistical difference ($P > 0.05$). On the 3rd day of the luteal period, MGV values were close to each other at 32,383 ± 4,587 in the S group and 33,050 ± 4,764 in the C group. In the S group, this value was relative to the MGV value taken at the 24th h (4th-day 0 h) after sildenafil administration. In group C, it was determined that there was an increase in the MGV value measured simultaneously after the placebo administration. It was determined that this increase continued until 40 min after the $PGF_2\alpha$ injection in both groups, remained stable until the 2 h after the $PGF_2\alpha$ injection, and decreased towards the 12 h after the $PGF_2\alpha$ injection. It was determined that there was an increase in MGV values taken at the 24 h after the $PGF_2\alpha$ injection, which coincided with the 5th day of the luteal period in both groups. It was determined that MGV values were higher in group C. However, no statistical significance was found in these within-group changes ($P > 0.05$). MGV values according to measurement times are shown in Table 2 and Figure 2A.

According to the results obtained from Doppler USG examinations, it was determined that there was no statistical difference between the measurement times within the group in PI and RI values. However, there was a difference between the groups in PI and RI values at 40 min after $PGF_2\alpha$ injection. The mean PI

and RI values of the measurement times in the groups are shown in Table 3, Figure 2B & 2C.

Molecular sub-study results

The amplified products of studied genes were at the predicted size (not shown). No amplification was observed in the negative control samples, indicating the absence of genomic DNA contamination and primer dimers.

The steady-state mRNA levels of *StAR* in luteal tissues taken during the $PGF_2\alpha$ treatment are shown in Figure 3A. The *StAR* gene expression values were 1.10 ± 0.27 in the CP1 group, 1.22 ± 0.31 in the SP1 group, 0.94 ± 0.20 in the CP12 group, and 1.20 ± 0.27 in the SP12 group measured. It was determined that *StAR* mRNA expression did not change between the 1st and 12th h after $PGF_2\alpha$ injection in both groups (S and C groups), and sildenafil citrate administered in the early luteal period had no effect ($P > 0.05$).

The steady-state mRNA levels of *PTGS2* in luteal tissues taken during the $PGF_2\alpha$ treatment are shown in Figure 3B. The *PTGS2* gene expression values were 2.07 ± 0.86 in the CP1 group, 1.24 ± 0.46 in the SP1 group, 0.18 ± 0.10 in the CP12 group and 0.17 ± 0.16 in the SP12 group. It was determined that *PTGS2* gene expression increased at 12 h after $PGF_2\alpha$ injection in both C and S groups. It was determined that there was no difference between S and C groups ($P > 0.05$), but there was a statistical difference in the expression levels of the groups according to the OVH time after $PGF_2\alpha$ injection. ($P < 0.05$).

The steady-state mRNA levels of *HPGD* in luteal tissues taken during the $PGF_2\alpha$ treatment are shown in Figure 3C. The *HPGD* gene expression 1.51 ± 0.54 in the CP1 group, 1.54 ± 0.50 in the SP1 group, 0.47 ± 0.10 in the CP12 group and 0.24 ± 0.02 in the SP12 group was measured. It was determined that there was no difference between S and C groups ($P > 0.05$), but there was a difference between the OVH times after $PGF_2\alpha$ injection in the groups. ($P < 0.05$)

The steady-state mRNA levels of *HIF1A* in luteal tissues taken during the $PGF_2\alpha$ treatment are shown in Figure 3D. The *HIF1A* gene expression values were 1.15 ± 0.38 in the CP1 group, 1.60 ± 0.47 in the SP1 group, 8.28 ± 0.80 in the CP12 group, and 5.73 ± 2.10 in the SP12 group determined. There was no difference between the groups ($P > 0.05$). However, in both groups (S and C groups), *HIF1A* mRNA expression increased significantly at 12 h after $PGF_2\alpha$ injection compared to the 1st h ($P < 0.05$).

The steady-state mRNA levels of *VEGF* in luteal tissues taken during the $PGF_2\alpha$ treatment are shown in Figure 3E. The *VEGF* gene expression was measured as 0.94 ± 0.31 in the CP1 group, 1.09 ± 0.28 in the SP1 group, 0.64 ± 0.30 in the CP12 group and 0.98 ± 0.44 in the SP12 group. Sildenafil citrate administered in the early luteal period did not have a significant effect on *VEGF* mRNA expression ($P > 0.05$). In addition, it was determined that *VEGF* mRNA expression did not change according to the OVH time after $PGF_2\alpha$ injection. ($P > 0.05$).

Table 1. Rabbit target and reference (housekeeping) gene primer information.

	Rabbit primers		Base pair (bp)
StAR	Forward	GGCTGCCAAAGACCATCATC	209
	Reverse	CAGCTGAGATCCTAGACGGG	
PTGS2	Forward	CCTCACTGATGGGCTGTTTT	121
	Reverse	GGTGAAAGCAATGCCTGAA	
HPGD	Forward	GCGATGGCTGCTAATCTCAT	177
	Reverse	CATTGATGGGTCCAAAATCC	
HIF1A	Forward	CCACAGGACAGTACAGGATG	150
	Reverse	TCAAGTCGTGCTGAATAATACC	
VEGF	Forward	AACGAACGTACTTGCAGATGT	200
	Reverse	GCTCACGCAGTCTCCTCTTC	
Reference (Housekeeping) Gene			
18S	Forward	CGATCAGATACCGTCGTAGT	148
	Reverse	TTCCTTTAAGTTTCAGCTTTGC	

Table 2. Time-dependent mean grey value (MGV) values of the ovaries.

B-mod USG Measurement	MGV value ± S_h	MGV value ± S_h
Measurement time	CG n=11	SG n=10
Before Sildenafil/Placebo treatment (day 3)	33,050 ± 4,764	32,383 ± 4,587
24th h after Sildenafil/Placebo treatment (shortly before $PGF_2\alpha$ injection - day 4)	39,050 ± 4,988	31,405 ± 4,550
40th min after $PGF_2\alpha$ injection	46,326 ± 5,235	38,983 ± 5,908
80th min after $PGF_2\alpha$ injection	44,970 ± 5,751	38,313 ± 5,968
120th min after $PGF_2\alpha$ injection	46,604 ± 4,347	35,415 ± 5,895
6th h after $PGF_2\alpha$ injection	36,190 ± 5,629	31,356 ± 4,922
12th h after $PGF_2\alpha$ injection	33,170 ± 5,747	28,337 ± 5,092
24th h after $PGF_2\alpha$ injection	39,507 ± 5,178	30,111 ± 3,644

MGV: Mean Grey Value; CG: Control Group; SG: Sildenafil Group.

Table 3. The mean pulsatile index (PI) and resistance index (RI) values of the rabbits in the clinical sub-study at the time of measurement.

Doppler USG Measurement	PI		RI	
Measurement time	CG n=11	SG n=10	CG n=11	SG n=10
Before Sildenafil/Placebo treatment (day 3)	0.68 ± 0.04	0.77 ± 0.05	0.46 ± 0.02	0.49 ± 0.02
24th h after Sildenafil/Placebo treatment (shortly before $PGF_2\alpha$ injection - day 4)	0.69 ± 0.02	0.78 ± 0.06	0.46 ± 0.03	0.51 ± 0.02
40th min after $PGF_2\alpha$ injection	0.62 ± 0.02*	0.74 ± 0.05*	0.43 ± 0.01**	0.49 ± 0.02**
80th min after $PGF_2\alpha$ injection	0.62 ± 0.03	0.67 ± 0.04	0.46 ± 0.03	0.46 ± 0.02
120th min after $PGF_2\alpha$ injection	0.61 ± 0.03	0.65 ± 0.04	0.43 ± 0.02	0.45 ± 0.02
6th h after $PGF_2\alpha$ injection	0.66 ± 0.03	0.81 ± 0.07	0.46 ± 0.02	0.52 ± 0.03
12th h after $PGF_2\alpha$ injection	0.69 ± 0.05	0.78 ± 0.05	0.46 ± 0.02	0.50 ± 0.02
24th h after $PGF_2\alpha$ injection	0.69 ± 0.04	0.80 ± 0.05	0.45 ± 0.03	0.52 ± 0.02

PI: Pulsatile Index; RI: Resistance Index; CG: Control group. SG: Sildenafil group. *It indicates a difference between the groups in the PI values ($P < 0.05$). **It indicates a difference between the groups in the RI values ($P < 0.05$).

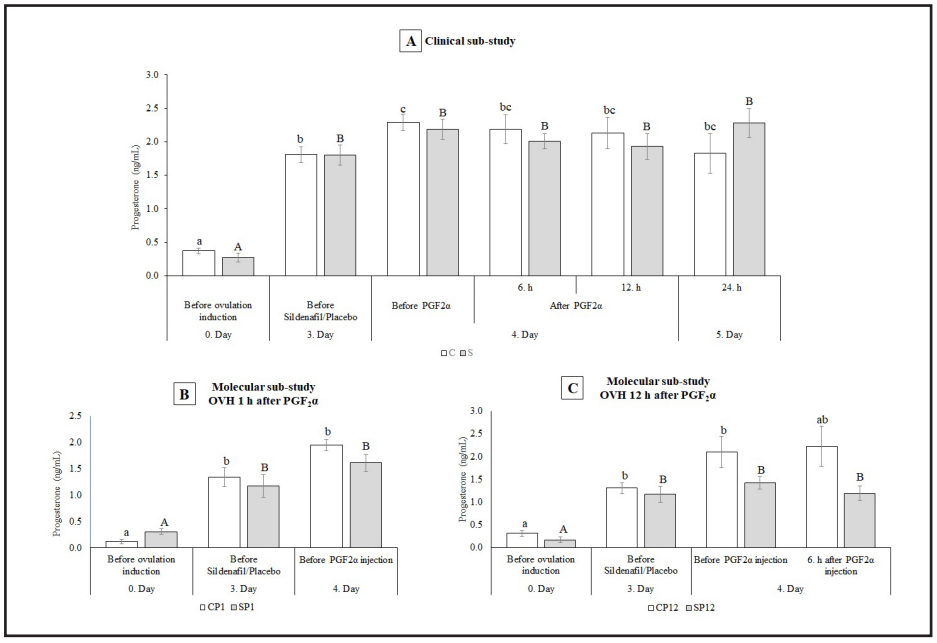

Figure 1. Serum progesterone (P_4) concentrations from blood samples taken on days 0, 3, 4 and 5 of the study. A- Serum P_4 levels of clinical sub-study. B- Serum P_4 levels of rabbits with OVH 1 h after $PGF_2\alpha$ injection in the molecular sub-study. C- Serum P_4 levels of rabbits with OVH 12 h after $PGF_2\alpha$ injection in the molecular sub-study. S: Sildenafil group; C: Control group. Letter indicates statistical differences ($P < 0.05$).

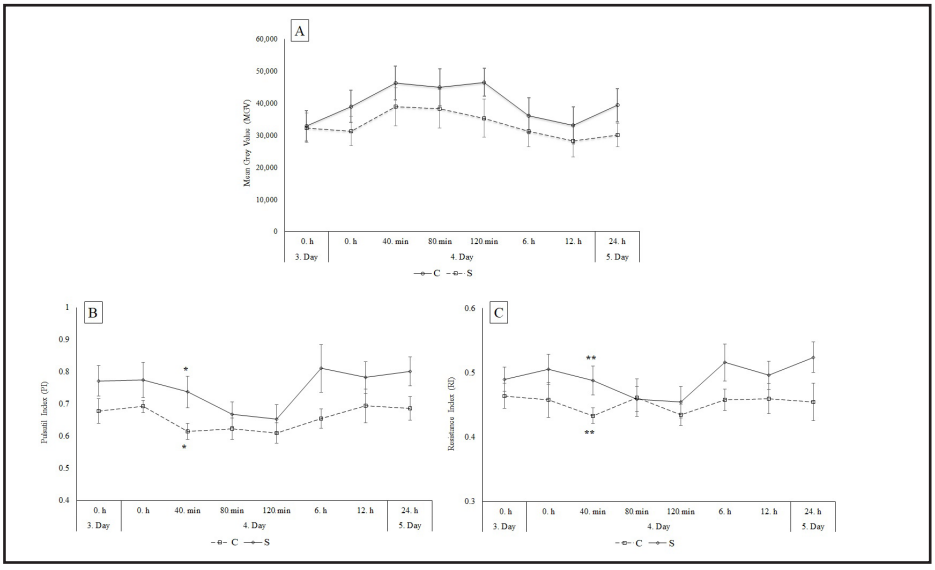

Figure 2. A- Time-dependent mean grey value (MGV) values of the ovaries. B- The mean pulsatile index (PI) values of the rabbits at the time of measurement. C- The mean resistance index (RI) values of the rabbits at the time of measurement. [* and ** indicates the statistical difference between groups ($P < 0.05$)].

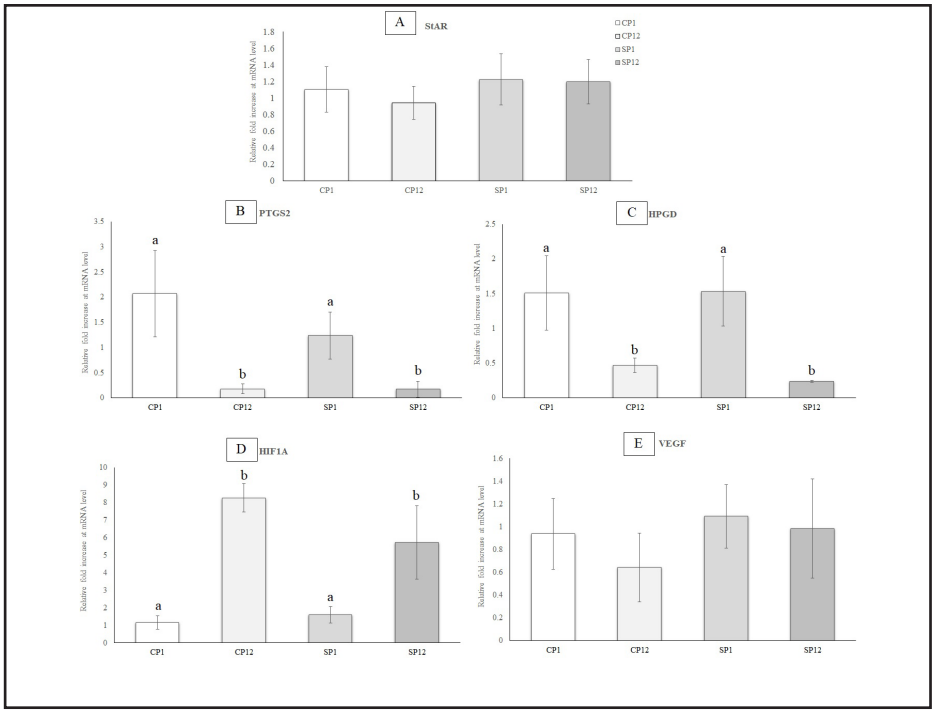

Figure 3. mRNA expression of some genes in luteal tissue in molecular sub-study groups by RT-PCR. A- *StAR*, B- *PTGS2*, C- *HPGD*, D- *HIF1A*, E- *VEGF*. CP1: OVH 1 h after $PGF_2\alpha$ injection in C group; CP12: OVH 12 h after $PGF_2\alpha$ injection in C group; SP1: OVH 1 h after $PGF_2\alpha$ injection in S group; SP12: OVH 12 h after $PGF_2\alpha$ injection in S group. [The data are shown as fold change ± SEM. Columns with different letters (a, b) at each biopsy indicate differences ($P < 0.05$)]

DISCUSSION

The luteolytic effect of $PGF_2\alpha$ is related to the age-dependent luteolytic capacity of the CL. Luteolytic capacity gain varies according to the species. In most animal species, including cows, rabbits, rats, mares and pigs, at the beginning of the luteal period (the first 6

days), CL does not regress with $PGF_2\alpha$ administration [11]. Earlier gaining luteolytic capacity in CL aims to induce a new estrous cycle which may help start resynchronization protocol or assisted reproductive technology. Therefore, in this present study, we aimed to use sildenafil citrate, that causes increasing the blood flow to the CL, leading to the earlier maturation of the CL and thus may lead to a luteolytic response to $PGF_2\alpha$. During the study, plasma P_4 levels were repeatedly measured to follow the ovulation and the luteolytic responses to GnRH and $PGF_2\alpha$, respectively. Similarly, we evaluated the luteal blood flow using Doppler USG, if any, an increase in blood flow to response in sildenafil citrate. Moreover, the luteolytic effects of $PGF_2\alpha$ on CL were also shown in the expressing levels of the genes, which have critical roles in developing regressing mechanisms in CL.

The presented study used the rabbit as an animal model, the 3rd most widely used experimental mammal species in the European Union [43]. Pseudopregnancy in rabbits was induced to establish a functional CL. Rabbit is also one of the most suitable model animals for humans, which can be used effectively in studies on the CL and luteolysis mechanism. The P_4 level increases on the 4th and 5th days after ovulation and peaks on the 11th day. Then, it decreased to 19-20th day [6]. Moreover, evidence of CL structure and secretions and the uterine-derived luteolytic signal has been demonstrated in rabbits [36]. According to those similarities, at the end of the present study, we hoped to provide some valuable information for earlier gaining luteolytic capacity in CL to both livestock and human studies.

At the beginning of the early luteal period, during the formation of the CL, active angiogenesis starts with the ovulatory LH secretion during ovulation, and the CL becomes one of the organs with the highest vascularization in the body [17]. The blood flow in the CL is essential for the development and continuity of this structure [1]. The high amount of cholesterol required for P_4 synthesis is supplied to the luteal cells with blood flow, and the produced P_4 is released into circulation. In a study in which changes in blood flow in the CL were monitored during the luteal phase in women [50], it was reported that there is a close relationship between luteal blood flow and luteal function. The present study aimed to accelerate the luteal-follicular transition process by increasing the blood flow to the CL by using sildenafil citrate in the early luteal period and to shorten the maturation period of the CL. It has been reported that sildenafil citrate improves radial artery RI by increasing blood flow in women with weak endometrium [49]. In a study investigating the effect of sildenafil citrate on feto-maternal circulation in pregnant rabbits before anesthesia, it was reported that sildenafil citrate increased fetal blood circulation by decreasing uterine vascular resistance for 24 h [13]. In our previous report, in which we applied sildenafil citrate with a smaller number of rabbits regardless of their cycle status [39], no significant effect of sildenafil citrate on uterine perfusion was detected when the measurements at the 1st h after the application was evaluated.

Determination of P_4 concentration is the gold standard in evaluating luteal function [18]. Considering the possibility of not stimulating ovulation to reach the required number of pseudo-pregnant rabbits in the present study, many rabbits (n = 52) were initially included in the sub-study groups. Ovulation was induced by IM administration of buserelin acetate (GnRH agonist) in 52 rabbits, 27 in the clinical sub-study and 25 in the molecular sub-study. Control of ovulation stimulation and evaluation of luteal function were made according to serum P_4 levels. In the clinical sub-study, 5 of 27 rabbits and the molecular sub-study, 5 of 25 rabbits, ovulation was not stimulated, and P_4 levels remained below 1 ng/mL. One rabbit in the clinical sub-study group was determined to be in the luteal period before ovulation stimulation and was excluded from the study. Ovulation was successfully achieved in 41 (80.3%) of the 51 rabbits (clinical and molecular sub-studies) included in the whole study. Ovulations were induced in 21 (80.7%) of 26 rabbits in the clinical sub-study and 20 (80%) of 25 rabbits in the molecular sub-study. A study comparing GnRH analogues in rabbits' ovulation stimulation reported an 87.9% ovulation rate with buserelin acetate [9].

Prostaglandin $F_2\alpha$ is vasoconstrictive and reduces luteal blood flow [1]. It has been reported that its luteolytic effect may occur due to its constricting effect on the utero-ovarian vein [40]. Previous studies also examine whether specific vascular mechanisms in the ovaries are involved in the luteolytic process following the administration of $PGF_2\alpha$ to pregnant and pseudo-pregnant rabbits [5,20,37]. Blood flow regulation in rabbit CL and its relationship to steroidogenesis has

also been investigated [55]. In all these studies, ovarian or luteal blood flow was measured with tracer-labelled microspheres, a technique that requires catheterization, general anesthesia, and subsequent killing of animals.

Doppler USG is a non-invasive technique in evaluating the ovaries' vascular functions, enabling visual observation of blood flow in the CL and real-time study [51]. Blood flow in the CL measured by this technique is related to luteal function [50]. This technique characterized local blood flow changes in follicles and CL [1] of cows [1] and mares [56]. It is known that there are a limited number of similar study findings in laboratory animals. In recent years, advanced USG techniques in veterinary reproduction have been widespread [32,34]. It was used to determine the vascular indices of the umbilical cord, aorta and caudal vena cava of fetuses as well as uteroplacental arterial vessels during pregnancy in rabbits [2,41]. A recent study [51] characterized dynamic intra-ovarian blood flow changes during $PGF_2\alpha$-induced luteolysis in early and mid-luteal stages of pseudo-pregnant rabbits. In this study, although an invasive technique, such as entering the abdomen and fixing the tissue to the abdominal wall, was recommended for USG examination and evaluation of the ovary in rabbits, the risk of this application changing the perfusion on the ovarian surface was not mentioned. In the present study, the ovaries were easily visualized on the lateral surface of the caudal abdomen without any invasive procedures, and measurements of B-mode and Doppler were completed without any problems.

Computer-assisted USG image analysis is a practical application in evaluating the structure and functions of various tissues. It allows quantitative and objective evaluation of multiple parameters, especially the greyness ratio [29]. The grey value will be expected to increase in the advanced luteolysis stage, as functional tissue is replaced by structural tissue with luteolysis and tissues with high perfusions, such as follicular-luteal structures, and, therefore, less grey ratio, are eliminated. However, the fact that our study was conducted in a narrower time frame in the early luteal stage may reduce the likelihood of this effect.

Promising and contradictory information regarding the relationship between MGV and P_4 is available. Davies *et al*. [10] found a positive correlation between MGV and P_4 (r = 0.52 - 0.69) in sheep. Despite this evidence favouring a positive correlation, Siqueira *et al*. [47] found a negative correlation (r = -0.63) between MGV and P_4 levels in cattle. In the present study, a positive correlation (r = 0.27) between the MGV and P_4 levels in the follicular stage might be due to follicular luteinization, which decreased contrast between follicles and ovarian connective tissue. With increasing luteinization, the homogeneity of images was increased, and the shade of grey was switched to hyperechogenic grey tones.

However, at the end of the study, there was no difference between the S and C groups regarding the MGV values of rabbits. Erdogan *et al*. [12] detected significant MGV changes in ovarian images taken during follicular and luteal periods in dogs. This is thought to be related to the early luteal phase in both groups in the present study. In addition, no significant difference was found in the main groups depending on the measurement times within the group. Although no significant time-dependent change was detected in both main group, there was an increase in MGV value from the 3rd day to the 4th day in the C group, while this increase was not observed in the S group. It is seen that the higher P_4 level obtained in the C group compared to the S group is reflected in the MGV values. In this context, it is the first study to include USG monitoring and echotextural examination findings of the ovaries in non-pregnant rabbits.

At the end of our study, the pulsatility value in the CL arterioles decreased at the 40th min in the C group and the 80th min in the S group following $PGF_2\alpha$ injection. This situation parallels the decrease observed by Troisi *et al*. [51] at the 40th min. It was determined that there was a statistical difference between the groups at the 40th min after $PGF_2\alpha$ injection. It shows that sildenafil citrate application has a possible supportive effect on pulsatility in the treatment group. Similarly, a statistical difference was detected in the RI values at the 40th min after $PGF_2\alpha$ injection. The higher RI value in the S group indicates that sildenafil citrate does not provide sufficient effect in preventing the vasoconstriction caused by $PGF_2\alpha$ injection and, thus, the development of reactive hyperemia.

In the molecular sub-study, the expressions of genes involved in angiogenesis (*HIF1A*, *VEGF*), steroidogenesis (*StAR*) and prostaglandin (*HPGD*, *PTGS2*) mechanisms were determined at the 1st and 12th h after $PGF_2\alpha$ injection to investigate the effect of sildenafil citrate administration on luteolytic capacity in the early

luteal period at the molecular level. The substrate for the production of P_4 is cholesterol. *StAR* is responsible for cholesterol transport into the mitochondrial membrane [48]. It was stated that *StAR* mRNA increased in the early and middle luteal phases and decreased significantly in the late luteal phase. In luteal cells and tissues, $PGF_2\alpha$ has been shown to reduce the *StAR* gene at mRNA and protein levels in rats [44], pigs [11], humans [8], sheep [16], cows [3] and dogs [53]. The presented study observed that $PGF_2\alpha$ didn't affect the *StAR* gene at the mRNA level on the 4th day of the luteal period in rabbits in the S and C groups ($P > 0.05$). Since the P_4 value did not decrease after $PGF_2\alpha$ injection in either the C or S groups, the absence of a change in *StAR* expression can be considered a compatible finding for the present study. Like our study, Troisi *et al*. [51] stated that rabbit CL on the 4th day of the luteal period showed resistance to $PGF_2\alpha$. It can be said that sildenafil citrate applied on the 3rd day of the luteal period did not change the luteolytic gain through *StAR* gene expression since *StAR* expression did not change in the treatment group. It has been stated that low oxygen (O_2) concentration in bovine luteal cells inhibits P_4 production by suppressing P450 Side-Chain Cleavage enzyme (P450scc) activity, does not affect *StAR* and 3 beta hydroxysteroid dehydrogenase (3βHSD) expressions, and O_2 deficiency caused by decreased blood flow is an essential condition for the functioning of the luteolytic mechanism in cattle [35]. This explanation supports that P_4 and *StAR* gene expressions did not change in both groups after $PGF_2\alpha$ injection in the present study.

Prostaglandin endoperoxide synthase 2 (*PTGS2*), which is the rate-limiting enzyme in prostaglandin (PG) biosynthesis, is also formerly known as cyclooxygenase 2 (COX-2). It has been reported that this enzyme, responsible for converting arachidonic acid to PGH_2, increases during the luteal development stage [26], and its expression is positively correlated with the PGES enzyme responsible for the conversion of PGH_2 to PGE_2 and PGT [23]. It has been reported that its expression is high in the early luteal period in dogs and decreases in the luteal regression process [24,26]. In a study [53], in which repeated low-dose administrations of $PGF_2\alpha$ were studied in dogs in the mature CL period, it was stated that *PTGS2* expression did not change after the 1st 2 injections of $PGF_2\alpha$ but decreased after the 3rd injection ($P < 0.05$). In the presented study, it was observed that *PTGS2* expression was high in both groups (S and C groups) after $PGF_2\alpha$ injection, and expression levels decreased significantly ($P < 0.05$) 12 h after $PGF_2\alpha$ injection. It was reported that *PTGS2* mRNA expression increased between 1.5 and 24 h after $PGF_2\alpha$ injection in the 4th day CL of pseudo-pregnant rabbits [57]. Similarly, the presented study showed a significant increase in *PTGS2* mRNA expression ($P < 0.05$) between the 1st and 12th h after $PGF_2\alpha$ injection. There was no difference between the S group and the C group. In this respect, it can be concluded that $PGF_2\alpha$ injection increased the expression at the *PTGS2* gene level in the 1st h in both groups, but this increase decreased significantly at the 12th h compared to the 1st h.

The enzyme 15-hydroxyprostaglandin dehydrogenase is encoded by the *hydroxyprostaglandin dehydrogenase* (*HPGD*) gene that converts PGE_2 to 15-keto-prostaglandin, eventually leading to the inactivation of PGE_2 [54]. This enzyme performs the rate-limiting enzymatic step in prostaglandin E and F inactivation [38]. Many tissues are noted to express *HPGD* to provide local protection from the biological effects of $PGF_2\alpha$. Resistance to $PGF_2\alpha$ in the early luteal period and increased *HPGD* enzymatic activity in this period indicate the existence of such a mechanism [46]. The presented study determined that *HPGD* mRNA expression was high at the 1st h after $PGF_2\alpha$ injection in both groups (groups S and C), and expression increased significantly at the 12th h. This supports the resistance of CL to $PGF_2\alpha$ in the early luteal period with the mechanisms described above [36,46]. The fact that sildenafil citrate application did not have a different effect on *HPGD* gene expression compared to the C was interpreted as the application of sildenafil citrate did not have a positive effect on luteolytic capacity in terms of *HPGD* gene expression.

It has been stated that *VEGF* mRNA, one of the factors that play a role in the angiogenesis mechanism during the development of the CL, is present throughout the estrous cycle in sheep. Its expression is relatively high in the early cycle period when luteal vascularization occurs [42]. It has been reported that the expression of *VEGF* in the CL increases in the early luteal phase, where the luteal structure occurs, and decreases towards the mid-luteal phase when its formation is completed [33]. In the present study, there was no change in *VEGF* mRNA expression in both groups between 1st and 12th h after $PGF_2\alpha$ injection in the early luteal period. This can be interpreted as

mRNA expression of *VEGF* is not affected by sildenafil citrate or $PGF_2\alpha$.

In the study in which the expression model of *HIF1A*, which is the proangiogenic regulatory factor in the angiogenesis process, was determined before and after LH secretion according to the developmental stage of the follicle, as well as after ovulation [4], it was stated that *HIF1A* expression decreased before ovulation and increased during the CL formation process. On the other hand, a cow CL study showed that *HIF1A* expression peaks in the early luteal phase of the estrous cycle (period of angiogenesis) and then drops significantly to a lower plateau during the middle and late luteal phases [27]. In addition, increased expression of *HIF1A* was detected during the early luteal phase in monkeys [14] and humans [19] and has been implicated in the regulation of luteal angiogenesis [42]. The expression of *HIF1A* in cows has been determined in the early luteal period and has been reported to be active in the luteal formation process [21]. In the presented study, an increase in *HIF1A* mRNA expression, determined after $PGF_2\alpha$ injection on the 4th day of pseudopregnancy in rabbits, was observed at the 12th h ($P < 0.05$). The fact that this increase was seen in both study groups suggests that the relevant change is independent of sildenafil citrate administration.

CONCLUSION

This study suggests that measuring the serum P_4 level is an excellent indicator to monitor ovulation and $PGF_2\alpha$ response in rabbits. According to collected data, we concluded that a single dose of sildenafil citrate (5 mg/kg) at the early luteal stage (on day 3 after ovulation) does not induce/increase the blood flow, decrease P_4 levels or change gene expression in CL and thus failed to maturate CL than earlier expected time. Finally, we concluded that new studies are needed to involve the trial of sildenafil citrate at different application doses and times.

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