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—Original Article—

Disrupting the circadian photo-period alters the release of follicle-stimulating hormone, luteinizing hormone, progesterone, and estradiol in maternal and fetal sheep

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Abstract. Although a large number of studies show that photo-period disruption potentially affects hormone secretion in mammals, information about the effects of circadian photo-period disruption during pregnancy on fetal blood reproductive hormone levels is scarce. This study used ewes and their fetuses to determine the effects of circadian photo-period disruption (deprivation of darkness) on follicle-stimulating hormone, luteinizing hormone, estradiol, and progesterone in maternal and fetal circulation at late gestation. Pregnant ewes (gestational age: 135 ± 3 days) were randomly placed into control and dark deprivation groups. The control (N = 5) and dark deprivation (N = 5) groups were exposed to a fixed 12 h light/12 h dark cycle and a 24 h constant light cycle, respectively, for 2 days. Dark deprivation up-regulated follicle-stimulating hormone and estradiol levels and down-regulated progesterone levels in both maternal and fetal circulation, and up-regulated luteinizing hormone levels in fetal but not maternal circulation. These results provide new information about how circadian photo-period disruption during pregnancy could alter the release of certain reproductive hormones into fetal blood, which may influence the development of fetal organs *in utero*, as well as long-term health.
Key words: Fetuses, Photo-period disruption, Reproductive hormones

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In mammals, the photo-period is a crucial environmental cue that drives the circadian time-keeping system in the suprachiasmatic nucleus of the hypothalamus. Circadian photo-period is vital to many animals, since a number of physiological activities are related to photo-period signals [1–3]. For example, in mammals, disrupting circadian photo-period could alter the secretion of numerous hormones [4–10], and recent studies on dairy heifers and sheep showed that prolactin concentrations were changed by photo-period manipulation [4, 5]. The circadian photo-period is also involved in regulating the secretion of many important reproductive hormones including luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and progesterone in non-pregnant mammals [6–10]. In addition, recent studies have shown that expression of the clock and clock-controlled ovarian stromal genes synchronized by the environmental photo-period play a role in ovarian hormone synthesis, suggesting that the circadian photo-period manipulates hormone secretion, potentially *via* the molecular clockwork mechanism [11, 12]. While it has been confirmed that circadian photo-period can manipulate hormone secretion, the exact mechanism(s) remains unknown.

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Previous studies have shown dynamic changes in reproductive hormones in pregnant women [13, 14]. Pulsatile secretion of LH and FSH in maternal circulation suggested these hormones might be involved in maintaining pregnancy. In the absence of pregnancy, estradiol and progesterone are produced in high amounts in mammalian ovaries. In pregnant women and sheep, estradiol and progesterone are increased because of placental secretion [15, 16]. The concentration of estradiol and progesterone in the peripheral plasma of maternal and fetal sheep gradually increases during pregnancy progression [17, 18]. Studies on baboons and humans have demonstrated that estradiol also promotes uterine blood flow, cervical softening at term and that it is essential for initiating labor in the third trimester of pregnancy [19, 20]. In addition, studies on pregnant women have indicated that a decrease in progesterone levels is critical for the initiation of labor [21, 22]. Together, the pulsatile secretion of these reproductive hormones seems to play an important role in pregnancy.

In mammals, hormone secretion throughout pregnancy is also closely related to the circadian photo-period [23, 24]. For example, disrupting the circadian photo-period in sheep perturbs glucose metabolism and prolactin secretion during pregnancy [23, 24]. However, to date, there is limited information regarding the effects of photo-period disruption in pregnancy on maternal and fetal reproductive hormone secretion. This study aimed to determine the changes in FSH, LH, estradiol, and progesterone levels upon disruption of the circadian photo-period in both maternal and fetal sheep. The findings provide new information regarding the relationship between circadian photo-period and fetal hormone secretion *in utero* in late pregnancy.

Materials and Methods

Animals

All of the pregnant ewes (gestational age: 121–136 days, term: 147 ± 3 days, all singleton) used in this study had histories of normal pregnancy and healthy offspring. The ewes were housed in a light-controlled room with standard food and water, and allowed to rest for 2 days prior to surgery. All surgical and experimental procedures were approved by the Institutional Animal Care Committee of the First Affiliated Hospital of Soochow University, and conformed to the Guide for the Care and Use of Laboratory Animals.

Surgical operation

Pregnant sheep were given general anesthesia, which was initiated by intramuscular administration of 20 mg/kg of ketamine hydrochloride and maintained with 1 l/min of oxygen and 3% isoflurane. Polyethylene catheters were inserted into the maternal femoral artery and vein, and subsequently tunneled to an incision on the left flank. The uterus was opened *via* a small hysterotomy to provide access to a fetal hindlimb. After insertion of polyethylene catheters into the femoral artery of female fetuses, the uterine incisions were closed in layers with silk sutures. All polyethylene catheters were tunneled to the flank incision and placed in a cloth pouch attached to the flank of the ewe. After surgery, pregnant sheep were allowed to recover for a minimum of 4–5 days before experimental manipulation of environmental light. During post-surgery recovery, a daily dose of antibiotics was administered intravenously to both the ewes (70 mg gentamicin and 1g oxacillin) and the fetuses (10 mg gentamicin and 20 mg oxacillin).

Experimental design

The pregnant ewes (gestational age: 135 ± 3 days) were randomly placed into the control and dark deprivation groups. The control (N = 5, LD) were kept indoors and exposed to a fixed cycle of 12 h light (0800–2000 h) and 12 h dark (2000–0800 h). The dark deprivation group (N = 5, LL) was exposed to constant light from 0800 h of the first day to 2000 h of third day, having been deprived of two nights. Blood samples were collected from 2100 h on the second day until 1800 h on the third day. Maternal (4 ml for each sample) and fetal (3 ml for each sample) arterial blood samples were collected in test tubes with anticoagulant from the implanted catheters every 3 h. Blood samples were centrifuged at 3,000 rpm for 10 min, and plasma was transferred to new tubes, shock frozen in liquid nitrogen, and stored at –80°C until further analysis. For the control group, blood samples were collected in the night using a flashlight covered with red cloth. Figure 1 shows the pattern of light-dark cycling and the timing of blood sampling for the two groups.

Blood values analysis

Blood samples (1 ml for each sampling) were withdrawn from the fetal and maternal arterial catheters to measure blood oxygen partial pressure (PO_2), carbon dioxide partial pressure (PCO_2), lactate (Lac), hematocrit (Hct), electrolyte concentrations (Na^+, K^+ and Ca^{++}), and pH on a blood gas analyzer GEM Premier 3000 (Instrumentation Laboratory Company, Lexington, U.S.A.). All experiments were performed on the animals with fetal blood at pH ≥ 7.30.

Radioimmunoassay

A radioimmunoassay (RIA) was used to measure plasma hormone concentrations. Plasma levels of LH, FSH, progesterone, and estradiol were determined by double antibody RIA using a RIA kit (HY-10286, HY-10176, HY-10028 and HY-10029), according to the manufacturer's instructions (Beijing Huaying Biotechnology Research Institute, China). Sensitivity for FSH, LH, progesterone, and estradiol was 0.01 mIU/ml, 0.01 mIU/ml, 0.05 ng/ml, and 0.01 pg/ml, respectively. The intra-assay and inter-assay coefficient of variation (CV) were approximately 3.0–6.7% and 7.5–9.9%, respectively. The samples and data were handled in a blind manner.

Statistical analyses

Statistical analyses were conducted using Graph Pad Prism 5 (Graph Pad Software, San Diego, CA, USA). Significance ($P < 0.05$) was determined by t-test or two-way analysis of variance (ANOVA), where appropriate. The effects of dark deprivation and time on maternal and fetal reproductive hormones between the LD and LL groups were analyzed by two-way ANOVA with Bonferroni post-tests. The mean values of maternal or fetal reproductive hormones in "Day" and "Night" between the two groups were also analyzed by two-way ANOVA followed by Bonferroni post-tests. The mean values of maternal or fetal reproductive hormones over 24 h were compared between the two groups using t-tests. Maternal and fetal blood values were analyzed by two-way ANOVA with Bonferroni post-tests. Statistical significance was accepted when $P < 0.05$. All data are presented as mean ± standard deviation.

Results

Maternal and fetal arterial blood values

There was no significant difference in the maternal and fetal arterial blood values (including PO_2, PCO_2, Lac, Hct, Na^+, K^+, Ca^{++}, and pH) between the control and dark deprivation groups (Tables 1 and 2, N = 10, all $P > 0.05$). There was also no significant change in maternal and fetal arterial blood values at various time points. All maternal and fetal arterial values were within normal ranges and did not vary significantly between the control and dark deprivation groups (all $P > 0.05$).

Effect of night deprivation on maternal and fetal FSH levels

Although FSH levels were not significantly increased at each testing point in both maternal and fetal blood (Fig. 2(A) and (D), $P > 0.05$), mean FSH levels over 24 h were significantly higher in the dark deprivation group than in the control group (Fig. 2(C), $P < 0.05$; Fig. 2(F), $P < 0.01$). FSH levels in both the maternal ewes and fetuses during "Day" or "Night" periods were not significantly different between the LD and LL groups (Fig. 2(B) and (E), $P > 0.05$).

Effect of night deprivation on maternal and fetal LH levels

In females, LH is necessary to maintain luteal functions and it acts synergistically with FSH to regulate estrogen levels. Here, there were no significant differences in maternal blood LH levels at each time point, during "Day" and "Night", or over a 24 h period between the control and dark deprivation groups (Fig. 3(A), (B) and (C), $P > 0.05$). However, compared with the control group, fetal blood LH

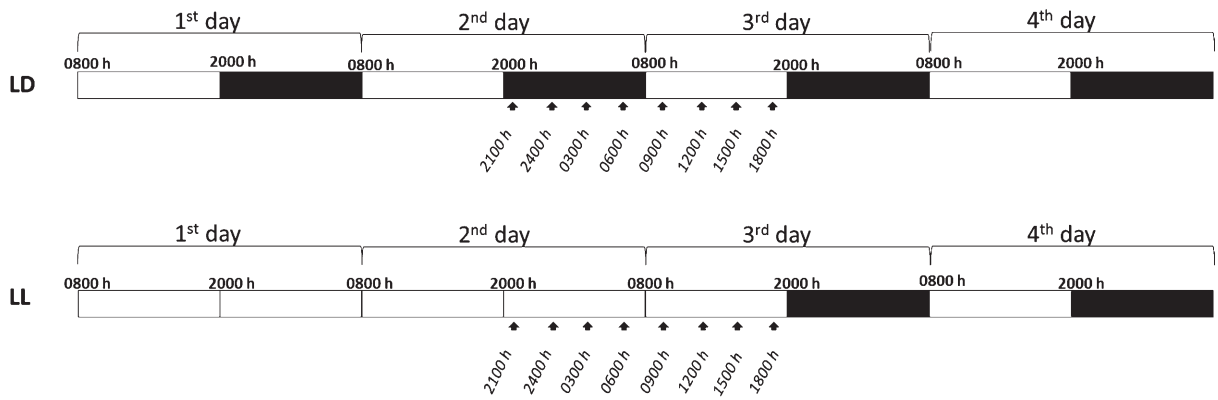

Fig. 1. Pattern of the light-dark cycle and timing of blood collection (black arrows) for the control (LD) and dark deprivation (LL) groups. Maternal and fetal blood samples were collected every 3 h from 2100 h of the second day to 1800 h of the third day.

Table 1. Maternal blood values

	2400 h		0600 h		1200 h		1800 h	
	LL	LD	LL	LD	LL	LD	LL	LD
pH	7.42 ± 0.03	7.40 ± 0.02	7.45 ± 0.02	7.45 ± 0.03	7.46 ± 0.02	7.45 ± 0.03	7.40 ± 0.02	7.43 ± 0.02
PCO_2 (mmHg)	25.03 ± 3.12	24.20 ± 3.52	25.08 ± 4.32	24.43 ± 4.20	23.80 ± 5.00	25.00 ± 3.30	23.46 ± 4.00	24.17 ± 4.08
PO_2 (mmHg)	118.00 ± 4.00	116.00 ± 3.00	117.00 ± 3.00	119.00 ± 2.00	119.00 ± 4.00	119.00 ± 4.00	120.00 ± 2.00	121.00 ± 2.00
Na^+ (mmol/l)	142.00 ± 3.00	141.00 ± 3.00	143.00 ± 5.00	140.00 ± 5.00	141.00 ± 4.00	140.00 ± 3.00	144.00 ± 3.00	143.00 ± 3.00
K^+ (mmol/l)	3.92 ± 0.30	3.84 ± 0.32	4.07 ± 0.41	3.82 ± 0.52	3.81 ± 0.48	3.90 ± 0.20	3.88 ± 0.26	3.98 ± 0.28
Ca^{++} (mmol/l)	1.99 ± 0.06	1.97 ± 0.05	1.97 ± 0.05	1.94 ± 0.06	1.96 ± 0.05	1.98 ± 0.05	1.92 ± 0.04	1.98 ± 0.03
Lac (mmol/l)	1.33 ± 0.30	1.40 ± 0.29	1.31 ± 0.22	1.33 ± 0.31	1.28 ± 0.47	1.29 ± 0.42	1.34 ± 0.45	1.36 ± 0.42
Hct (%)	35.43 ± 4.86	34.67 ± 4.78	33.28 ± 6.12	35.15 ± 6.09	34.12 ± 4.56	35.20 ± 3.34	35.30 ± 5.09	33.87 ± 4.28

Table 2. Fetal blood values

	2400 h		0600 h		1200 h		1800 h	
	LL	LD	LL	LD	LL	LD	LL	LD
pH	7.38 ± 0.03	7.36 ± 0.02	7.36 ± 0.01	7.37 ± 0.02	7.35 ± 0.02	7.36 ± 0.02	7.37 ± 0.03	7.35 ± 0.03
PCO_2 (mmHg)	53.50 ± 4.08	54.30 ± 3.15	53.50 ± 3.59	52.80 ± 4.62	52.92 ± 4.03	52.71 ± 4.34	53.11 ± 3.17	53.06 ± 4.90
PO_2 (mmHg)	22.45 ± 3.19	20.30 ± 4.06	21.80 ± 3.68	22.42 ± 2.80	22.30 ± 3.59	22.55 ± 2.78	21.82 ± 3.05	23.16 ± 5.09
Na^+ (mmol/l)	142.00 ± 2.00	145.00 ± 3.00	143.00 ± 4.00	145.00 ± 3.00	144.00 ± 2.00	144.00 ± 3.00	143.00 ± 3.00	142.00 ± 3.00
K^+ (mmol/l)	3.74 ± 0.32	3.80 ± 0.39	3.90 ± 0.28	3.99 ± 0.29	3.80 ± 0.36	3.89 ± 0.20	3.87 ± 0.27	3.84 ± 0.20
Ca^{++} (mmol/l)	1.29 ± 0.05	1.32 ± 0.04	1.29 ± 0.04	1.29 ± 0.05	1.28 ± 0.05	1.31 ± 0.04	1.28 ± 0.04	1.29 ± 0.05
Lac (mmol/l)	2.24 ± 0.28	2.10 ± 0.34	1.99 ± 0.44	1.80 ± 0.20	2.20 ± 0.39	2.10 ± 0.43	1.90 ± 0.43	2.07 ± 0.27
Hct (%)	25.15 ± 3.45	24.08 ± 3.56	23.20 ± 4.71	25.39 ± 5.42	24.28 ± 3.64	25.28 ± 3.09	23.50 ± 4.17	24.45 ± 5.10

was significantly increased at 2100 h ($P < 0.01$), 0300 h ($P < 0.05$), and 1800 h ($P < 0.05$) in the dark deprivation group (Fig. 3(D)). LH levels in the fetuses were increased during the "Night" phase (Fig. 3(E), $P < 0.05$), as well as over a 24 h period (Fig. 3(F), $P < 0.01$).

Effect of night deprivation on maternal and fetal estradiol levels

Compared with the control group, maternal blood estradiol levels in the dark deprivation group were significantly increased at 0300 h, 0600 h, and 0900 h (Fig 4(A), all $P < 0.05$). Fetal blood estradiol levels were also significantly elevated in the dark deprivation group at 0300 h (Fig. 4(D), $P < 0.01$) and 1200 h (Fig. 4(D), $P < 0.05$). There was no significant difference in maternal and fetal blood estradiol levels during "Day" or "Night" periods (Fig. 4(B) and (E), $P > 0.05$) between the two groups. Maternal and fetal blood estradiol levels were elevated over a 24 h period in the dark deprivation group (Fig. 4(C) and (F), $P < 0.05$).

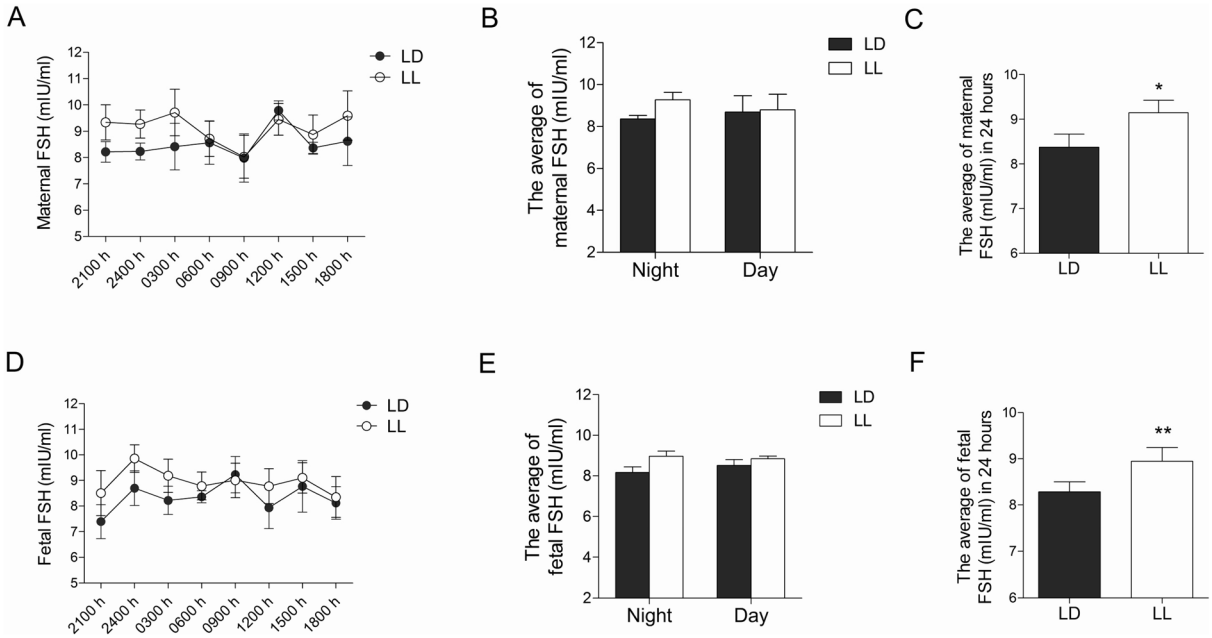

Fig. 2. Effect of dark deprivation on both maternal and fetal blood FSH levels (A–C), and on fetal blood FSH levels (D–F). "Day" and "Night" refer to 12 h of light and 12 h of darkness for the LD group, and two 12 h periods of light for the LL group. N = 10; * P < 0.05; ** P < 0.01.

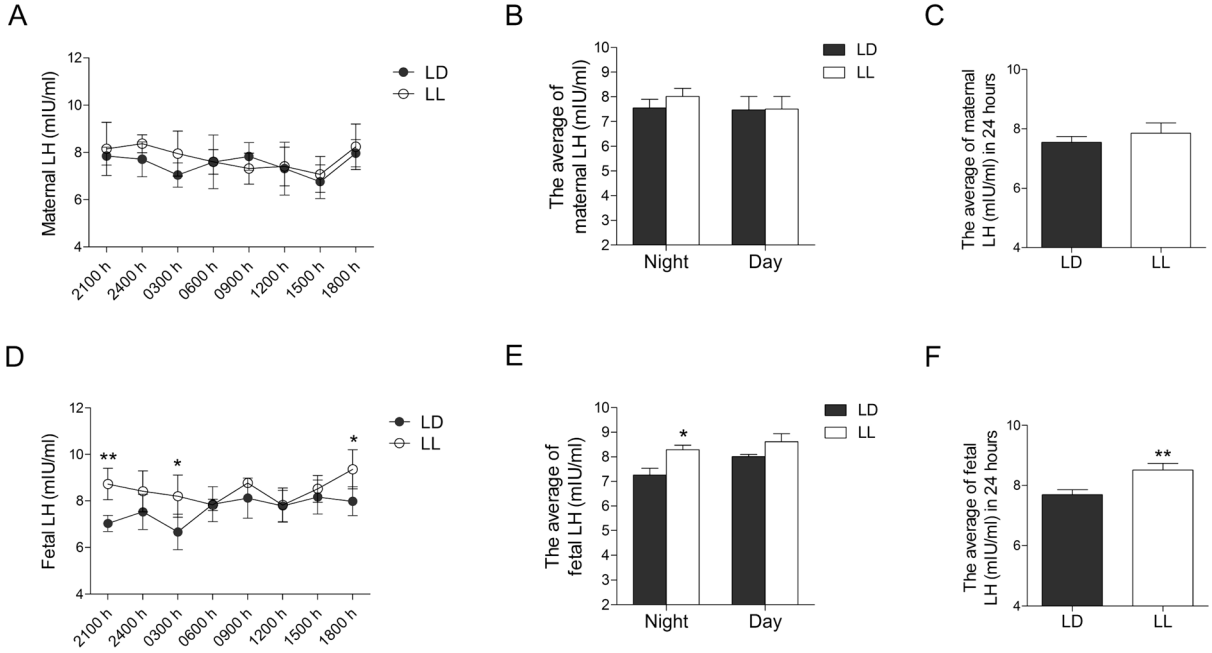

Fig. 3. Effect of dark deprivation on both maternal and fetal blood LH levels (A–C) and on fetal blood LH levels (D–F). N = 10; * P < 0.05; ** P < 0.01.

Effect of night deprivation on blood progesterone levels

Plasma progesterone levels were markedly changed in both ewes and fetuses in the LL group (Fig. 5). Compared with the control group, maternal blood progesterone levels were significantly decreased at 2100 h (P < 0.001), 0300 h (P < 0.05), and 0600 h (P < 0.01) (Fig. 5(A)). Dark-deprived fetal blood progesterone levels were also significantly decreased at 2100 h (P < 0.001), 2400 h (P < 0.001), 0300 h (P < 0.01), 0600 h (P < 0.001), and 0900 h (P < 0.01) (Fig.

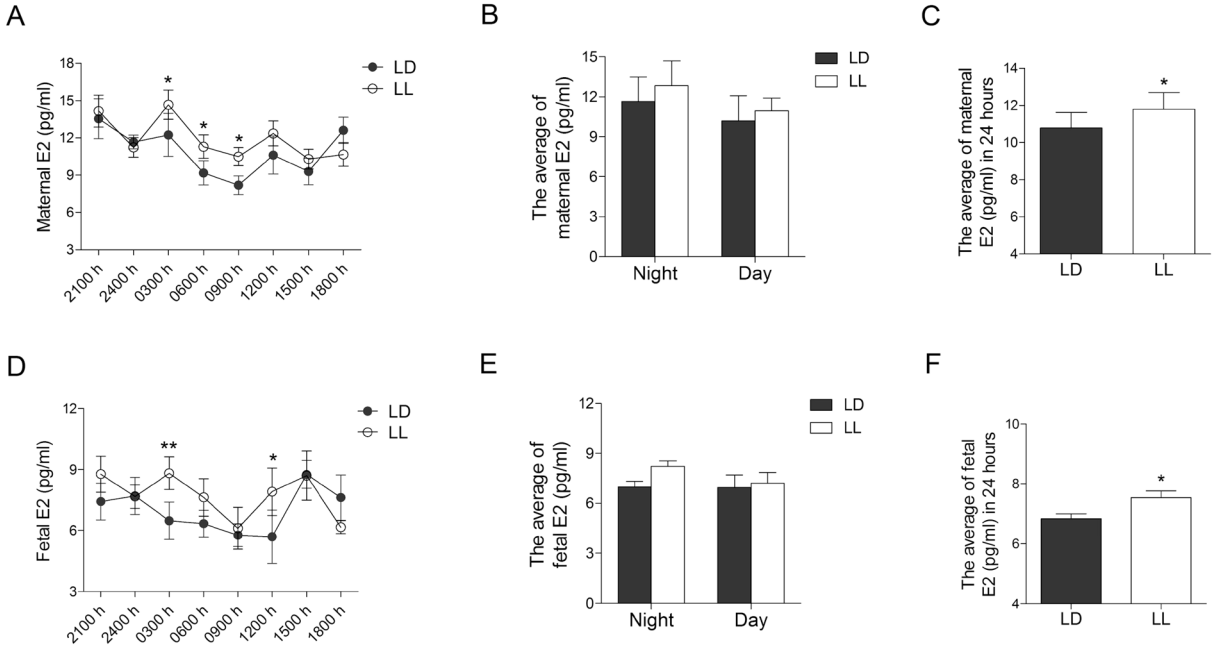

Fig. 4. Effect of dark deprivation on both maternal and fetal blood estradiol (E2) levels (A–C) and on fetal blood estradiol levels (D–F). N = 10; * P < 0.05; ** P < 0.01.

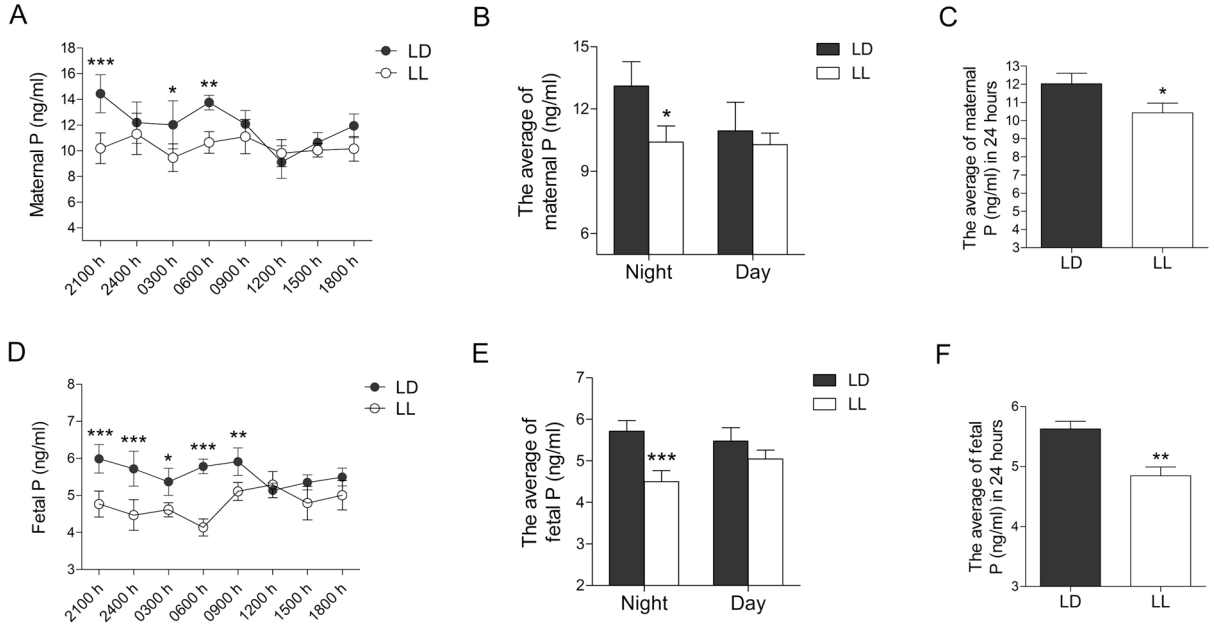

Fig. 5. Effect of dark deprivation on both maternal and fetal blood progesterone (P) levels (A–C), and on fetal blood progesterone levels (D–F). N = 10; * P < 0.05; ** P < 0.01; *** P < 0.001.

5(D)). Fig. 5(B) and (C) show that in the dark deprivation group, maternal blood progesterone was decreased during the "Night" period, and the average of progesterone levels were lower over a 24 h period than that of the control group. Fetal blood progesterone was also decreased during the "Night" period (Fig. 5(E), P < 0.001), as well as over a 24 h period (Fig. 5(F), P < 0.01) in the dark deprivation group.

Discussion

Although disruption of circadian photo-period has been reported to affect hormone secretion in mammals, little is known about the effects of circadian photo-period disruption during pregnancy on the secretion of reproductive hormones in fetal blood. Deprivation of darkness resulted in FSH and estradiol up-regulation, and down-regulation of progesterone levels in both maternal and fetal circulation. Dark deprivation also up-regulated LH levels in fetal blood, but did not influence maternal LH levels. This study indicates that disruption of normal environmental light patterns during pregnancy alters the release of some reproductive hormones in maternal and/or fetal circulation.

In this study, there was no significant change in maternal and fetal arterial blood values throughout the various time points. These remained at a stable physiological status, demonstrating that manipulation of photo-period during pregnancy had no effect on basic arterial values. However, in the dark deprivation group, mean fetal FSH and LH levels and maternal LH levels were significantly increased over 24 h, suggesting that the release of FSH and/or LH may be regulated by the circadian photo-period. This was the first study to demonstrate the effect of circadian photo-period disruption on FSH and/or LH release in maternal and fetal sheep during pregnancy, although other works have studied this in non-pregnant mammals [25, 26]. Although the actions of fetal FSH and LH during pregnancy are not fully understood, these disrupted photo-period-induced changes may have adverse effects on maternal and fetal endocrine system homeostasis, as well as the development of fetal organs in the third trimester of pregnancy. Notably, evidence accumulated in last three decades has demonstrated that prenatal insults may increase the risk of developing disease in later life [27, 28]. Therefore, the novel data in this study may not only increase our understanding of environmental or maternal influence on fetal reproductive system development related to circadian regulation, but also offer new avenues for further investigation of diseases with fetal origins.

In mammals, estradiol is important in regulating female reproductive cycles, and in the development and maintenance of female reproductive functions. In both pregnant women and sheep, plasma estradiol concentrations increase significantly, peaking during late pregnancy [15, 16, 29]. In late pregnancy, estradiol may promote uterine blood flow, stimulate cervical softening at term, and be essential for initiation of labor [19–21]. Estradiol also plays an important role in regulating fetal ovarian development in baboons [30]. In this study, mean estradiol levels in the circulation of ewes and fetuses over 24 h were significantly increased following dark deprivation. This suggests that photo-period disruption in late gestation may promote the initiation of labor *via* changes in estradiol levels, influencing fetal reproductive organ development and function.

Like estradiol, progesterone is also involved in the menstrual cycle, successful conception, embryogenesis, and continuation of pregnancy in humans and other species. Plasma concentrations of progesterone increase abruptly in the early weeks of pregnancy and remain at high levels throughout pregnancy [15, 16, 29]. Recent studies have suggested that a reduction in progesterone is critical for the initiation of labor [21, 22]. Progesterone also plays an important role in regulating fetal development [31]. In this study, both maternal and fetal plasma progesterone levels were remarkably decreased during the "Night" period in the dark deprivation group. This suggests that disruption of environmental photo-period in late gestation alters hormone concentrations in a way that may impact fetal development *in utero* potentially by promoting initiation of labor, resulting in preterm birth.

Although factors and mechanisms governing estradiol and progesterone secretion and release are complex during pregnancy, concentrations of these hormones in maternal and fetal blood may partially reflect placental functions, since previous studies have demonstrated that the placenta produces these hormones [15, 16]. Here, both estradiol and progesterone levels were altered by dark deprivation. The altered levels of these hormones in circulation may reflect the effect of dark deprivation on placental functions if there was a proportion of estradiol and progesterone originating from the placenta in the blood. Our study suggests that disrupting the photo-period may lead to placental endocrine dysfunctions, which may have adverse effects on maintenance of pregnancy and fetal development *in utero*.

Notably, accumulating evidence has shown that the circadian photo-period is initiated in the fetus *in utero* [32, 33]. Surgical disconnection of the fetal hypothalamus and pituitary result in abolished daily fetal plasma melatonin and prolactin rhythms [34]. Although it is difficult to discern whether the circulation of reproductive hormones in fetuses originated from the ewes, the placenta, or the fetus itself, the hormone curve similarities between the ewes and fetuses in both the control and experimental groups suggests that the main influence is maternal.

In conclusion, to the best of our knowledge, this study was the first to show that dark deprivation not only influenced FSH and LH levels, but also affected estradiol and progesterone secretion at late gestation in fetal sheep, indicating that disruption of the photo-period during pregnancy could influence fetal reproductive hormone secretion. Further investigation is needed to determine whether those changes during pregnancy cause problems in fetal development as well as long-term health or the development of disease.

Acknowledgments

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