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Evidence of a molecular clock in the ovine ovary and the influence of photoperiod

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ABSTRACT

The influence of the central circadian clock on reproductive timing is well established. Much less is known about the role of peripheral oscillators such as those in the ovary. We investigated the influence of photoperiod and timing of the LH surge on expression of circadian clock genes and genes involved in steroidogenesis in ovine ovarian stroma. Seventy-two Suffolk cross ewes were divided into two groups, and their estrous cycles were synchronized. Progestagen sponge removal was staggered by 12 hours between the groups such that expected LH peak would occur midway through either the light or dark phase of the photoperiodic cycle. Four animals from each group were killed, and their ovaries were harvested beginning 36 hours after sponge removal, at 6-hour intervals for 48 hours. Blood was sampled every 3 hours for the period 24 to 48 hours after sponge removal to detect the LH surge. The interval to peak LH did not differ between the groups (36.2 ± 1.2 and 35.6 ± 1.1 hours, respectively). There was an interaction between group and the time of sponge removal on the expression of the core clock genes *ARNTL*, *PER1*, *CRY1*, *CLOCK*, and *DBP* ($P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively). As no significant interaction between group and time of day was detected, the datasets were combined. Statistically significant rhythmic oscillation was observed for *ARNTL*, *CLOCK*, *CRY1* ($P < 0.01$, respectively), *PTGS2*, *DBP*, *PTGER2*, and *CYP17A1* ($P < 0.05$, respectively), confirming the existence of a time-sensitive functionality within the ovary, which may influence steroidogenesis and is independent of the ovulatory cycle.

1. Introduction

Circadian (24-hour) oscillations are present in virtually all mammalian tissues with “clock” genes rhythmically expressed in multiple peripheral tissues including the liver, muscle, kidney, heart, and adipose tissue [1–5]. The suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian clock in mammals, receiving photoperiodic information from the retina and coordinating cellular oscillations of peripheral clocks throughout the organism *via* humoral and neural outputs [6,7]. In this way, physiological alignment with the solar day is maintained. In many species, day length and seasonality play important roles in regulating reproduction [8]. Studies in rodents have established the SCN clock as having a vital role in regulating the LH surge, which is required for ovulation. Suprachiasmatic nucleus–lesioned rats lack the preovulatory LH surge and fail to ovulate [9]. Anatomical, physiological, and pharmacologic data reveal links between SCN neurons, GnRH, and estradiol-receptive areas of the brain [10,11].

Cellular circadian rhythms are generated by a highly conserved molecular mechanism comprised of a core group of clock genes: *aryl hydrocarbon receptor nuclear translocator-like* (*Arntl*), *circadian locomotor output cycles kaput*

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(*Clock*), *period* genes (*Per1*, *Per2*, *Per3*), *cryptochrome* genes (*Cry1*, *Cry1*), *nuclear receptor subfamily 1*, *group D*, *member 1* (*Nr1d1*), and *RAR-related orphan receptor A* (*Rora*), and their protein products [12,13]. These molecular components form interlocking transcriptional and/or translational autoregulatory feedback loops that oscillate approximately every 24 hours [14–17]. Each cycle of the molecular clock simultaneously gives rise to cyclic transcriptional activation of an array of clock-controlled genes (CCGs), contributing to tissue-specific rhythmical biological processes outside of the clockwork mechanism [18]. Up to 10% of all genes in a tissue are reported to exhibit circadian oscillations [19].

Evidence of a molecular clock has previously been reported in a variety of reproductive tissues of rodents. In mice, rhythmic expression of *Per2*, *Cry1*, and *Arntl* transcripts was reported in RNA isolated from whole uteri [20]. In rats, *Per1* and *Per2* displayed cyclic circadian expression in homogenized whole ovaries, which was independent of the stage of estrous [21]. Furthermore, Karman and Tischkau discovered that hCG treatment induced a circadian expression of both *Arntl* and *Per2* genes in the ovaries of hypophysectomized immature rats, leading to the hypothesis that the preovulatory LH surge may regulate expression of one or more clock genes [22]. These results indicate that, similar to other peripheral tissues, the rodent ovary is equipped with a molecular clock mechanism. Importantly, impaired fertility is observed in genetically altered mice with deficiencies in genes of the molecular clock, suggesting a critical role for these genes in reproduction [23–26].

To understand the role of the circadian clock in ovarian physiology, we investigated the presence of a temporally synchronized molecular clockwork mechanism in ovarian stromal tissue of sheep during the periovulatory period and examined its interaction with the timing of the ovulatory LH surge and the photoperiod. The aim of this experiment is to determine whether clock gene expression in stromal tissue is primarily regulated by the prevailing photoperiod or by the local hormonal signals during the periovulatory period.

2. Materials and methods

2.1. Animals

The estrous cycles of 72 Suffolk cross ewes were synchronized using a 12-day progestagen pessary (Chronogest intravaginal sponges, 30-mg Cronolone; Intervet, New Zealand) and 400 IU of intramuscular injection of PMSG (Folligon; Intervet UK Ltd.) at sponge withdrawal. Sheep were randomly assigned to group A or B ($n = 36$ per group), and sponge removal was staggered by 12 hours between the groups such that expected LH peak (36 hours post sponge removal [HPSR]) would occur midway through either the light phase (group A) or the dark phase (group B) of the light–dark (LD) cycle. This experiment was conducted during the autumnal equinox, a time of year when the natural environmental LD cycle provides 12 hours of light and 12 hours of dark (LD 12:12) at longitude W6.8°, latitude N53.2° (Newcastle, County Dublin, Ireland). The experiment was scheduled such that the expected time of ovulation (60 HPSR) for both groups coincided with the day of the equinox (September 24) where sunrise and sunset occurred at 7.16 AM and 7.16 PM, respectively. All animals were maintained outdoors for 1 month before the experiment to ensure entrainment to the natural photoperiod. Figure 1 provides a diagrammatic representation of the synchronization and sampling protocol for the experiment.

2.2. Blood sampling

Beginning 24 hours after sponge removal, blood was collected for serum LH analysis *via* jugular venipuncture every 3 hours until slaughter. Sampling that coincided with nighttime hours was conducted with the aid of dim red light to prevent disruption to the photoperiod signal. Blood was allowed to clot at room temperature for 1 hour and then stored at 4 °C for a further 24 hours at which point samples were centrifuged at 16,000 × *g* for 20 minutes and serum decanted. Serum samples were stored at −20 °C until assayed.

2.3. Ovarian tissue collection

Beginning 36 hours after sponge removal, four animals from each group were slaughtered (captive bolt) at 6-hour intervals with the last group of four animals being slaughtered at 84 hours after sponge removal. Tissue collection from group A began at 1 PM on Day 1 and continued for 48 hours until 1 PM on Day 3. Tissue collection from group B began at 1 AM on Day 2 and continued for 48 hours until 1 AM on Day 4 (Fig. 1). Ovaries were collected within 5 minutes of euthanasia and were kept on ice until processed. Samples of ovarian stroma were dissected from each ovary, with care taken to avoid inclusion of any follicular or luteal tissue, placed in 1.5-mL Eppendorf tubes, and snap frozen in liquid nitrogen before storage at −80 °C until RNA isolation. During the tissue collection period, animals were maintained under LD conditions that mimicked the natural external photoperiod (LD 12:12), and all handling procedures during dark hours were conducted using dim red light to avoid disruption to endogenous circadian rhythmicity.

2.4. RNA extraction and cDNA synthesis

Total RNA was isolated from approximately 20 mg of each ovarian stroma sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and on-column DNAse digestion and RNA clean-up was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer V 3.5.2 was used to quantify RNA (NanoDrop Technologies, Wilmington, DE, USA). The Agilent 2100 Bioanalyzer was used to assess RNA integrity (Agilent Technologies, Palo Alto, CA, USA). The 260:280 nm ration for each sample was greater than 2, and the RNA integrity number for each sample was greater than 7.5. For each sample, 500-ng RNA was converted to complementary DNA (cDNA) in a 20.0-μL reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer's