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ORIGINAL PAPERS

Continuous artificial light potentially disrupts central and peripheral reproductive clocks leading to altered uterine physiology and reduced pregnancy success in albino mice

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Abstract

Aims The mechanism behind clock coordination in female reproductive disorders is poorly understood despite the known importance of coordinated and synchronized timing of central and clocks in reproductive organs. We investigated the effect of continuous artificial light (LL) on the central and peripheral reproductive clock gene (*Bmal1*, *Clock*, *Per1*, *Per2* and *Cry1*) and its downstream regulators (*Hgf*, PR-A and HOXA10) during non-pregnancy and pregnancy phases of female mice.

Main methods Mice ($n=60$) in two sets, were maintained under continuous light (LL) and natural day cycle (LD;12L: 12D) for both non-pregnant and pregnant study. Tissues from hypothalamus-containing SCN, ovary, uterus and serum were collected at different zeitgeber time points (ZT; at 4-h intervals across 24-h periods).

Key findings LL exposure desynchronized the expressions of the clock mRNAs (*Bmal1*, *Clock*, *Per1*, *Per2* and *Cry1*) in SCN, ovary, and uterus along with *Hgf* mRNA rhythm. LL significantly increased the thickness of endometrial tissues. Furthermore, the pregnant study revealed lower serum progesterone level during peri- and post-implantation under LL along with downregulated expression of progesterone receptor (PR) as well as progesterone dependent uterine Homeobox A-10 (Hoxa10) proteins with lowered pregnancy outcomes.

Significance Our result suggests that LL disrupted the circadian coordination between central and clock genes in reproductive tissue leading to interrupted uterine physiology and altered pregnancy in mice. This led us to propose that duration of light exposure at work-places or home for females is very important in prevention of pregnancy anomalies.

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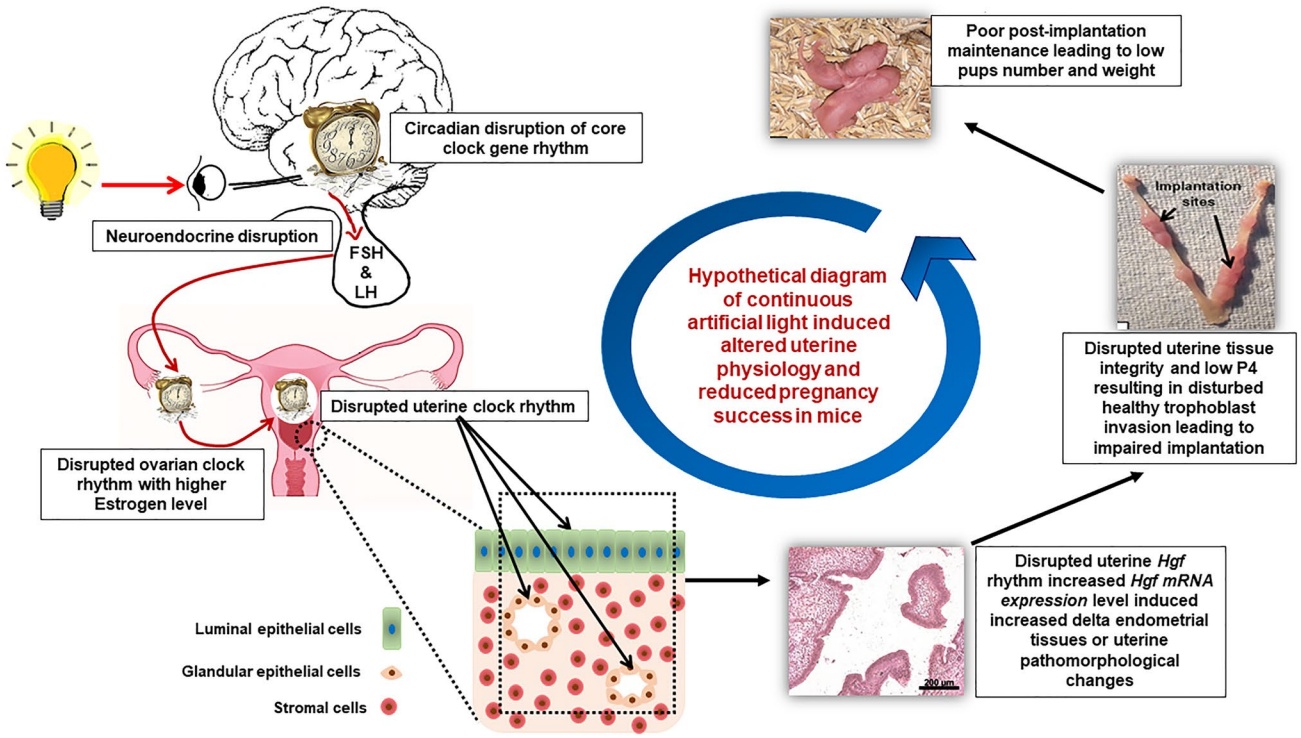
Graphical Abstract



Keywords Clock genes · Chronodisruption · Estradiol · Hepatocyte growth factor · Decidualization

1 Introduction

Circadian rhythm coordinates many physiological functions in mammals including human beings. These circadian rhythms are essential for sleep, cognitive functions, gene expressions, metabolic regulations, hormone production and for the maintenance of female health including implantation and parturition [1]. In mammals, the biological rhythm is regulated by the Suprachiasmatic Nucleus (SCN) of the anterior hypothalamus which is coordinated with peripheral clocks located in other than brain centers, including pancreas, liver, muscle, kidney, adipose tissue, ovary, uterus, etc. [2, 3]. Several studies have shown that clock gene synchronization is critical for reproductive success in females [4–7]. Studies on female night shift workers have shown that circadian dysregulation by extra artificial light severed reproductive function leading to low pregnancy rates, low birth weight and multiple miscarriages [8–10]. It has been suggested that any disturbances in the light and dark cycle disturbed the sleep pattern of the mother, early stage of embryo development, postnatal weight gain and visual development of the fetus [11, 12]. In contrary, repeated light/dark shift or jet lag like conditions have no effect on uterine receptivity and early gestation in female mice [13–16].

Pregnancy is a normal physiological process that requires the synchronized coordination between the central and peripheral clocks for physiological homeostasis [17]. Growing evidence suggest the presence of peripheral molecular clocks in the ovary, oviduct, and uterus for integrating and coordinating various physiological processes [2]. Light is the most important photo-entraining agent regulating 24 h periodicity, seasonal cycles, and neuroendocrine responses in many species including humans [18]. The duration and timing of artificial light exposure are important for circadian system homeostasis [19]. The widespread use of 24 h light and frequent abortion has become an alarming factor for chronodisruption related human reproductive disorders [5, 8]. Despite the known importance of coordinated and synchronized timing of central and peripheral reproductive clocks in female reproductive disorders and infertility, the mechanism between clock coordination is poorly understood.

Recently with increased urbanization and untimely use of artificial light exposure has led us to study its influence on female reproductive health. We propose in the present study that LL might influence or affect the coordination between central and peripheral reproductive clock genes during pre- and post-implantation maintenance and the progress of the

pregnancy. Hence, we examined the effect of LL on clock genes (*Bmal1, Clock, Per1, Per2* and *Cry1)* expression in hypothalamic with SCN, ovary and uterus along with important downstream markers of uterine physiology such as, hepatocyte growth factor (*Hgf*), progesterone receptor (PR-A) and HOXA10 that are important in the maintenance of uterine tissue integrity during pre- and post-pregnancy.

2 Material and methods

2.1 Animal housing

Experiments were performed on 10–12-week-old adult female Swiss albino mice (*Mus musculus*). All the mice were maintained in animal house facility of Department of Zoology, Banaras Hindu University, Varanasi, India. All the institutional practices were done according to CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal), Government of India, 2007 (CPCSEA Registration No.-1802/GO/Re/S/15/ CPCSEA) approved rules. The samplings were obtained in accordance with international chronobiological standards [20]. Animals had free access to food and water ad libitum. They were maintained under 12 h light: 12 h dark cycle and 25 ± 2 °C temperature. All animals were acclimated in the above condition for 2 weeks prior to the experiment [21].

2.2 Experimental design

Mus musculus ($n = 60$) were exposed to following different photoperiodic regimes (Fig. 1):

(i) Control: $n = 30$ mice; LD-12L:12D (12 h light: 12 h dark).
(ii) Continuous light*:* $n = 30$ mice; LL- (24 h light).

Mice of each group were reared under white LED light (light intensity was 50 ± 0.5 lx, measured at the level of cage base) for 30 days (Fig. 1) [20]. Estrous cycle was checked throughout the experimental period.

For the non-pregnancy study, 18 mice/group (3 animals/ZTs) were sacrificed under total ether anesthesia at 6 different zeitgeber time points (ZT0: time of light on, 7.30 am for LD group; ZT12: time of light off, 7.30 pm for LD group). For the gene expression studies, tissue was taken from the hypothalamic region of brain containing SCN as described previously [22], uterus and ovary tissues were collected over

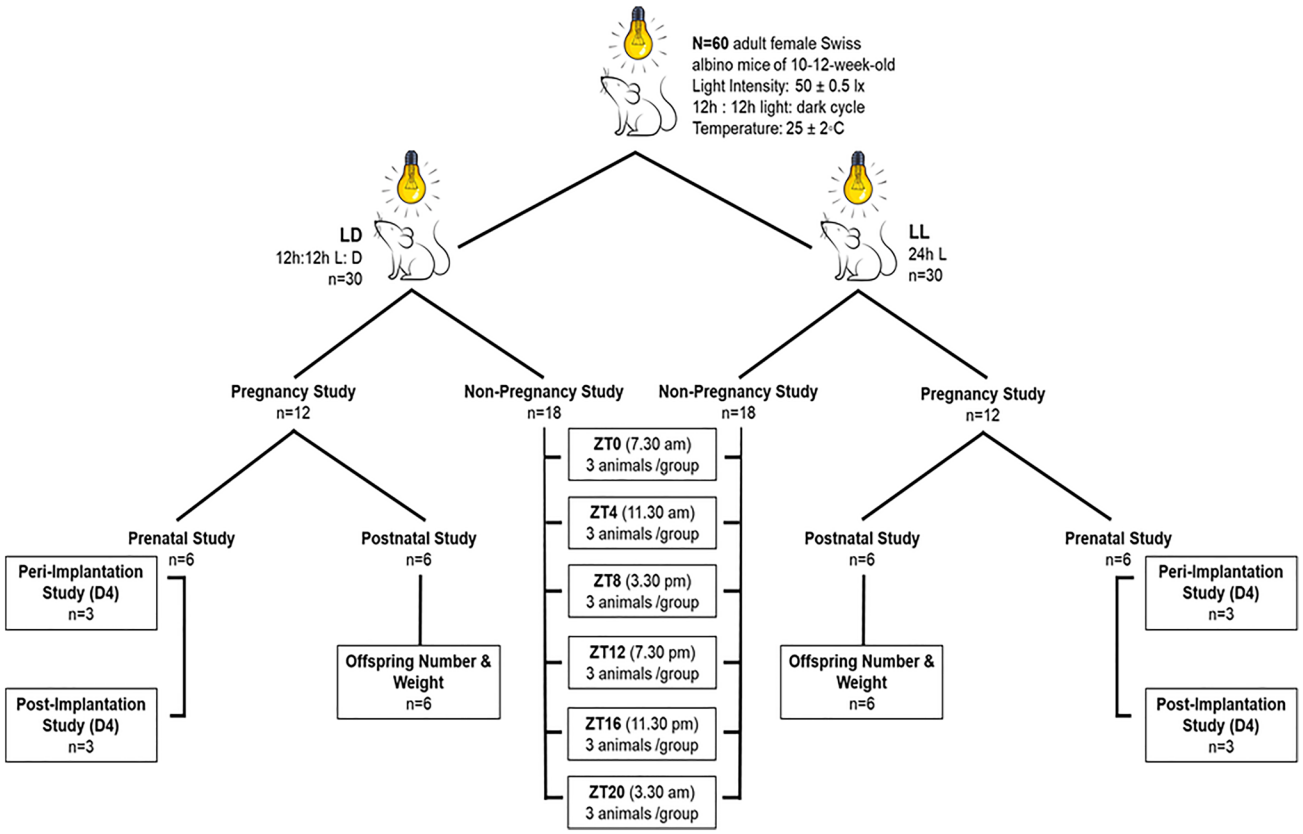


Fig. 1 Experimental plan showing studies under LL and LD conditions

ice for RNA isolation (for *Bmal1, Clock, Per1, Per2*, *Cry1 and Hgf*), western blot analysis (PR-A and Hoxa10) as well as fixed in bouin's solution for histology.

Due to limitation for recorder in measurement of circadian locomotors activity, we could not perform the free-running activity or circadian phase measurements. Therefore, we are unable to determine the degree to which mice have aligned to circadian phase following the continuous light exposure. However, in continuous light, animals were known to become arrhythmic or free running [23].

For the pregnancy study, each female mouse of remaining group ($n = 12$/group) was paired with single male mouse in separate cages under respective LL and LD conditions. Mating behavior was monitored by analysis of cohabitation period for sexual receptivity [24]. Pregnancy was confirmed by checking the presence of a vaginal plug and counted as pregnancy day 1(D1). The male mouse was then separated from the female mouse on the same day. Pregnancy study was further divided into two group's ($n = 6$) prenatal and ($n = 6$) postnatal study under LL conditions. For prenatal study pregnant animals ($n = 6$/group) were sacrificed on D4 and D7 for peri-implantation and post-implantation analysis, respectively. Uteri were collected for implantation site analysis and western blot for PR-A and HOXA10. Serum was collected for hormonal analysis of progesterone. Gestation time was also noted from the day of copulation plug till delivery. For postnatal study, rest of the mice ($n = 6$ animals/group) were kept under respective LL and LD conditions till delivery. Finally, the offspring number and average pup (single) weight/delivery/group was recorded.

2.3 Estrous cycle study

The estrous cycle of each mouse was scrutinized via vaginal smears taken at 8.30 am (IST). Each mouse vaginal smear slide was stained in trypan blue. Slides were classified cytologically based on majority of leucocytes, nucleated epithelial cells, cornified cells). We prepared five smears slides/animal and the averages of those slides were considered. The average estrous cycle phases were calculated using following formula [25].

$$\frac{\text{Estrous phase obtained in each day} \times 100}{\text{Experimental period}} \%$$

Estrous phases were also checked at different ZT before animals were sacrificed for the non-pregnancy study (Supplementary Table 1).

2.4 Histological study

For histological analysis, uterine tissues were fixed in bouin's solution, dehydrated, and processed for hematoxylin and eosin staining (HE) [26, 27]. It was strictly observed that uterine sections were not oblique. Every fifth section of each slide was measured for the different uterine layer thicknesses and was then documented under an inverted bright field microscope (Nikon E 200, Japan).

2.5 RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was isolated using Trizol reagent (Qiagen, USA, CAT No. 79306, LOT. 54,506,831), from the hypothalamus-containing SCN, ovary and uterine horns of each zeitgeber time point. Total RNA concentrations were measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific). A high-capacity cDNA reverse transcription kit (Thermo Scientific, Waltham, MA, USA, REF. K1622, LOT. 00,799,576) was used to transcribe total RNA into cDNA in a PCR machine (Bio-Rad). The 10 µl reaction mixtures for amplification were prepared using SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA, REF. K1622, LOT. 00,799,576), with forward and reverse primers (Table 1) [28] and cDNA as a template (10 µg/ul). Finally, quantitative Real-Time PCR (qPCR) was performed in a qPCR machine (Applied Biosystems, 7500 Real-Time PCR System). The endogenous *β-actin* of each zeitgeber time point were used for the calculation of ΔCt of each gene of respective zeitgeber time points [e.g., Ct (target gene at ZT0 to 24)-Ct (*β-actin* at ZT0 to 24)]. ΔΔCt was calculated in two different ways; first, each ΔCt was normalized against the ΔCt value of ZT0 for rhythmicity study [29] and secondly, ΔCt of each ZT of LL group was normalized against the ΔCt value of the respective ZT of LD group to study the fold changes of each gene after light exposure. Relative mRNA expression levels were measured as $2^{-\Delta\Delta Ct}$ [30]. We have collected samples at six time points starting from 7:30 am (ZT 0) to 3.30 am (ZT20). We have not sacrificed any animals twice at ZT 0/ZT 24 (7:30 am) to minimizing the animal sacrifice. We have used same data of ZT 0 (7:30 am) to plot the graph at ZT 24.

2.6 Progesterone (P_4) ELISA

Progesterone (P_4) level pregnant mice was measured using highly sensitive and specific commercial immunoassay kits (17OH Progesterone: DiaMetra, Italy, REF. DKO004, LOT. 4249C) according to the manufacturer's instructions where the intra and inter-assay variations were ≤ 9% and ≤ 10%, respectively, and the sensitivity was 8.7 pg/mL. In a single assay, all the samples were estimated [31]. For sample preparation, serum was isolated and pooled from the blood of each animal from each experimental group by centrifugation (Thermo Scientific) at 2500 rpm at 4 °C for 30 min. Following the manufacturers' protocol, samples, standards, and

Table 1 qPCR conditions for quantification of gene expressions

Gene	Primer sequence (5′ To 3′)	Annealing temperature (°C)	Amplicon size
β-actin	F: GGCAGGCAAAGGTTACTCTG R: TGGTGACAGGTGGACAAGAT	60	167
Bmal1	F: CGTGCTAAGGATGGCTGTTC R: CTTCCCTCGGTCACATCCTA	60	166
Clock	F: ACAACGCACACATAGGCCTTC R: TGGTGGTGCCCTGTGATCTA	60	175
Per1	F: TGCACTTCGGGAGCTCAAACTTC R: GTCCATGGCACAAGGCTCACC	59	169
Per2	F: AACAAATCCACCGGC R: CTCCGGTGAGACTCC	60	145
Cry1	F: AACGTCCCGAGCTGTAGCGGT R: GACGCTTCCCACTGCTGAGGC	60	139
Hgf	F: CCTGTCACCATCCCCTATGC R: CACACTCATCTGCCGAGTTCA	60	193

monoclonal biotinylated secondary antibodies were added per well to 96-well microplates coated with monoclonal antibodies against P_4. Wells were washed after binding to the immobilized antibodies and Streptavidin-HRP conjugate solutions were added to each well. Finally, the colorimetric analysis was done to visualize the reactions and the optical density of each well was measured at 450 nm on a microplate reader (Bio-Rad). Sample values were determined by extrapolations from standard curves for each of the hormonal analyses. All pooled samples and standards of each experimental group were replicated in triplicates.

2.7 Western blot analysis

Uterine tissues were homogenized in freshly prepared lysate buffer [50 mM Tris buffer (pH8), 150 mM NaCl, 1% NP-40, 0.5% $C_{24}H_{39}NaO_4$ and protease inhibitor {1 µg/ml Aprotinin (Roche, REF. 10,236,624,001, LOT. 25,869,127), 1 mM EDTA (Hi-Media, RM678), 100 µg/ml PMSF (Roche, REF.10837091001, LOT. 25,416,924) and 1 mM Sodium orthovandate (Sigma Aldrich, USA, CAT No. 450243-10 g, LOT. MKBZ6930V)}]. Homogenized tissues were kept at 4 °C for 1 h and then centrifuged (12,000 rpm, 4 °C) for 20 min. Supernatants were collected and kept at − 80 °C for protein estimation. Uterine tissue protein concentration was measured by Bradford Protein Assay (SRL, India, CAT No: 19219, LOT. 4,404,227). 50 µg proteins sample was loaded in 2 × laemmli buffer [Constituents: 10% SDS, 20% Glycerol, 25 mM Tris pH 6.8 buffer, 0.02% Bromophenol blue and 10% β-mercaptoethanol]. Samples were resolved with 12% SDS-PAGE for and Homeobox A-10 (HOXA10) and 10% SDS-PAGE for Progesterone Receptor-A (PR-A) followed by electrotransfer (Biometra, Gottingen, Germany) to PVDF membrane (Millipore, USA, LOT. BM7PA8500A). Membranes were then blocked by 5% non-fat milk in TBS-T (10 mM pH8 Tris–HCL buffer, 0.05% tween20 and 150 mM NaCl) for 1–2 h at room temperature (RT). PVDF membranes were incubated at 4 °C overnight with primary antibodies, HOXA10 (rabbit polyclonal, Santa Cruz Biotechnology, sc-271428, dilution 1:500) and PR-A (rabbit polyclonal, Cell Signaling Technology, 8757 T, dilution 1:800). Furthermore, membrane incubated with secondary anti-rabbit IgG PR-A and HOXA10 at the concentration of 1:2000 in 5% blocking buffer (non-fat milk) for 2 h at room temperature. After those membranes were washed with twin-20 wash-buffer and subjected to ECL reagent (Millipore, USA, CAT No. WBKLS0100, LOT. 1,900,901). Finally, membranes were developed at x-ray film. β-actin (Puregene, PG-13002) was used as the loading control. Image J Software (NIH) was used to quantify the band intensity. Values were expressed as the ratio of the density of the specific signal to the β-actin signal [32].

2.8 Sexual responsiveness (cohabitation period), implantation site number, gestation period and offspring number analysis

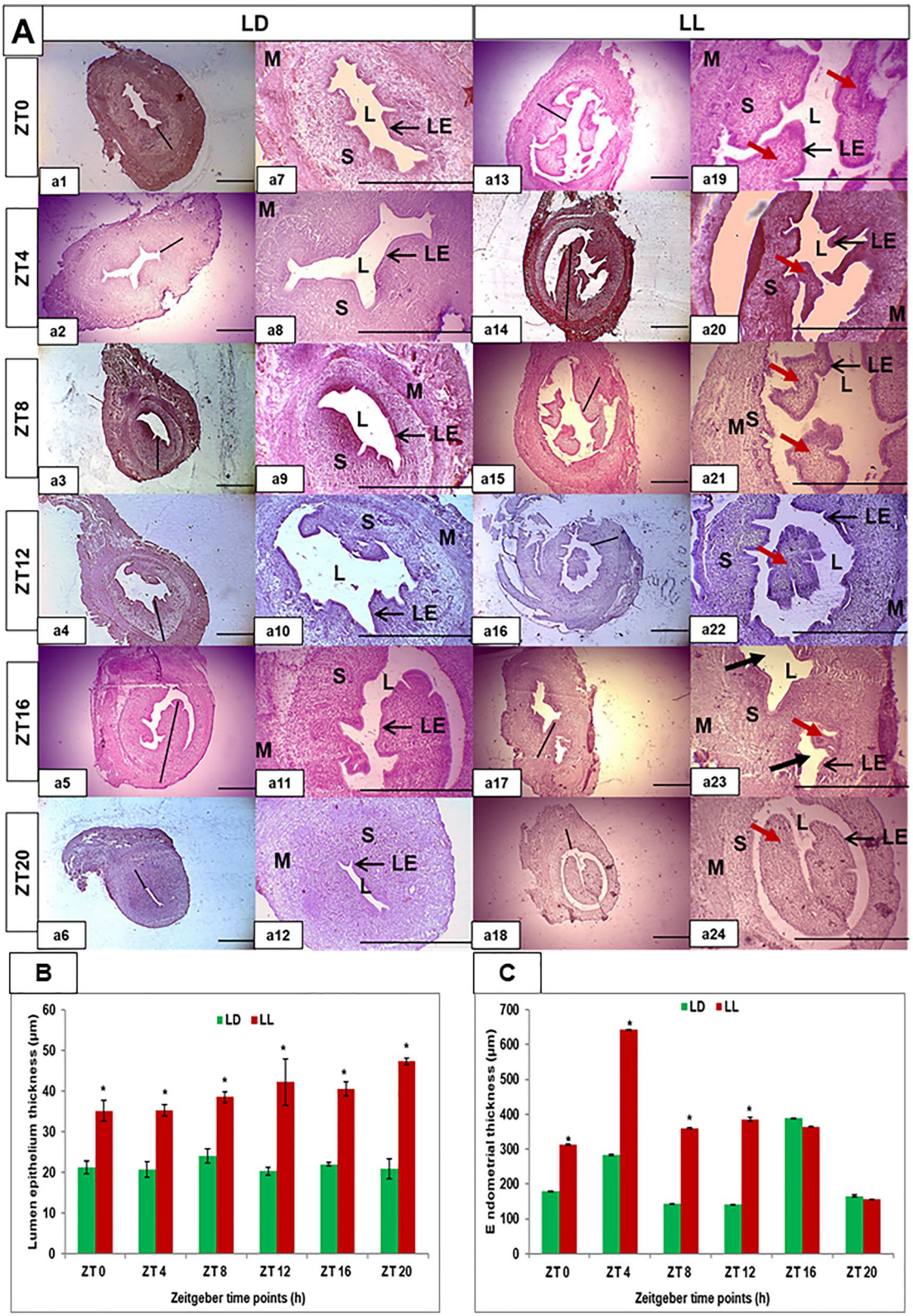
A standard index of the relative sexual receptivity, gestation period, implantation frequency, offspring number and offspring weight of each female mouse of each group was evaluated by the following methods [24, 33]:

2.8.1 Cohabitation period

Average number of days of the visualization of the vaginal plug after pair formation.

2.8.2 Gestation time

Average period from copulation to delivery (days).


A
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LL
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Lumen epithelium thickness (µm)
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◂**Fig. 2** Transverse section (TS) showing morphometric changes in uterine tissues of *M. musculus* under LD and LL conditions. **A** Hematoxylin and eosin stained (HE staining) uterus showing endometrial polyp (red arrows) like structure inside the lumen of LL experiencing mice [a13–a18 is under 4 × and a19–a24 is under 10 ×], under LD conditions [a1–a6 is under 4 × and a7–a12 is under 10 ×] respectively. Abbreviation: *L* lumen, *LE* luminal epithelium, *S* stroma, *M* myometrium and *EP* endometrial polyp. Black line showing endometrium thickness and black arrows (a23) showing bifurcated lumen. **B** Morphometric analysis of uterine tissue of mice under LL and LD condition showing epithelium thickness of lumen (mean ± SEM). **C** Endometrium thickness (mean ± SEM). All data analyzed by students' *t* test and one-way ANOVA followed by Tukey post hoc test. Data were considered statistically significant as * for $P < 0.05$

2.8.3 Implantation site number

Average number of implantation sites counted at D7 uterus of each animal/group.

2.8.4 Offspring number

Average number of offspring/groups.

2.8.5 Offspring weight

Average weight of single pups/group.

2.9 Statistical analysis

The effects of continuous light (LL; factor 1), *zeitgeber* time points (factor 2) and their interaction (factor 1 × factor 2) on normalized gene expressions and hormonal levels were assessed using two-way analysis of variance (2-way ANOVA). The data of fold change of gene expression, histological parameters and ELISA were analyzed by one-way ANOVA (analysis of variance) followed by Tukey post hoc test. Student's *t* test was applied when two values were compared during western blot; immunohistochemical studies, implantation offspring number and the total weight of offspring were recorded. All results are given as means ± SEM. Statistical significance was mentioned only when $P \leq 0.05$. The analyses were carried out with SPCC Statistics v. 16.0 (IBM, Armonk, NY, USA).

Daily rhythmicity of clock mRNA expressions and hormone levels were also evaluated by unimodal cosinor regression analysis using the formula.

$$Y(t) = M + \left[A.\cos\{2\pi(t-\Phi)/\tau\}\right],$$

where Y is the level of gene expression/hormone level at a given time point t, M is mesor (rhythm-adjusted mean), A is the amplitude (half the difference between maximum and minimum values of the best fitted curve) and Φ is the acrophase (time of peak) and τ is period (in hours), 24-h length of a cycle. The rhythm was considered as significant if $P < 0.05$ and was considered statistically different from zero (the waveform versus a straight line of zero amplitude (null hypothesis) (F-test of variance). Cosinor analyses were performed by TSA-Time Series Analysis Serial Cosinor 6.3 software (Expert Soft Technologies, Esvres, France). All data were expressed as mean ± SEM (standard error of the mean).

3 Results

3.1 Variation in estrous cycle under LL and LD

Estrous cycle was analyzed for 30 days. LL group mice showed 25.8%, 15.2%, 45.4% and 13.6% of diestrus, proestrus, estrus and metestrus, respectively. On the other hand, LD group mice maintained estrous cyclicity with 51.3%, 15.6%, 17.4% and 15.7% of diestrus, proestrus, estrus and metestrus, respectively (Supplementary Table 1).

3.2 Histopathological changes in the uterus

Histopathological observations of uterus under LL presented significant increase in the endometrial tissues thickness when compared with control mice ($P < 0.05$) (Fig. 2A a1–a24, Fig. 2 C). The serial sections of all LL uterus of mice showed the phenomenon of polyp-like structure (red arrows, Fig. 2A a1–a24). Morphometric analysis suggest LL significantly increased the thickness of lumen epithelium when compared with control mice with maximum thickness at ZT20 (one-way ANOVA, Tukey Post hoc, $P < 0.05$) (Fig. 2B). On the other hand, higher thickness of endometrium was observed under LL condition with maximum increased thickness at ZT4 (one-way ANOVA, Tukey Post hoc, $P < 0.05$) (Fig. 2C).

3.3 Quantitative real-time PCR (qPCR) analysis

3.3.1 3.3.a. Daily rhythmic variation of *Bmal1*, *Clock*, *Per1*, *Per2 Cry1*, mRNAs in mice hypothalamus-containing SCN, ovary and uterus during non-pregnancy

Two-way ANOVA presented significant interaction between the effect of continuous light and the different zeitgeber time points for all studied clock genes (*Bmal1, Clock, Per1, Per2* and *Cry1*) in Hypothalamus-containing SCN (*Bmal1*: $F_{6,28} = 25.140.$, $P < 0.0001$; *Clock*: $F_{6,28} = 98.195$, $P < 0.0001$; *Per1*: $F_{6,28} = 19.71$, $P < 0.0001$; *Per2*: $F_{6,28} = 122.54$, $P < 0.0001$; *Cry1*: $F_{6,28} = 137.885$, $P < 0.0001$, Supplementary Table 2). Cosinor analysis demonstrated statistically robust of 24-h rhythms for all the clock

Table 2 Cosinor summary of relative mRNA expression in SCN, ovary and uterus of mice under natural (LD) and constant artificial light condition (LL)

Tissue	Gene/hormone	Mesor		Amplitude		Acrophase (hour: min: sec)		Cosinor *P* value (24 h)	
		LD	LL	LD	LL	LD	LL	LD	LL
SCN	*Bmal1*	0.88	1.03	1.02	0.86	12:02:38	00:05:16	0.01	0.39
	Clock	0.77	1.45	0.86	1.43	11:38:46	16:07:09	0.02	0.13
	Per1	1.69	0.91	1.53	0.61	23:42:19	03:45:48	0.03	0.39
	Per2	1.88	0.34	1.21	0.49	18:27:15	06:30:40	0.01	0.05
	Cry1	15.4	0.69	18.87	0.39	20:43:22	10:08:42	0.02	0.16
Ovary	*Bmal1*	1.64	1.01	1.25	0.43	15:17:25	04:50:51	0.01	0.68
	Clock	1.54	3.11	1.44	3.22	13:56:23	22:37:58	0.04	0.23
	Per1	12.56	4.06	17.25	1.37	23:19:59	03:19:54	0.02	0.88
	Per2	2.0	0.81	1.42	0.98	17:46:53	04:04:31	0.02	0.35
	Cry1	3.41	2.54	3.12	1.23	22:00:57	06:52:09	0.05	0.79
Uterus	*Bmal1*	3.03	1.3	3.24	1.47	16:47:44	03:41:23	0.03	0.36
	Clock	2.77	1.67	2.87	1.10	15:46:49	11:37:43	0.02	0.63
	Per1	1.14	0.36	1.51	0.33	02:01:13	07:32:30	0.03	0.34
	Per2	30.38	0.59	44.12	0.53	23:07:18	11:47:34	0.01	0.11
	Cry1	17.49	0.83	16.29	0.09	21:37:48	09:34:57	0.01	0.66
	Hgf	6.43	4.52	5.34	8.50	21:14:34	02:40:20	0.02	0.44

Data are represented as mean for the best fitting 24-h single component cosine curve

$P < 0.05$ was considered as statistically significant

genes ($P < 0.05$) of the LD exposed mice (Table 2, Fig. 3A a1–c1, Fig. 3B d1–e1). Under LL, a robust 24-h expression levels of all clock genes were arrhythmic ($P > 0.05$, Table 2, Fig. 3A a2, b2, c2, and Fig. 3B e2), except *Per2* where a significant oscillation persisted ($P = 0.05$, Table 2, Fig. 3B d2). Relative expressions of *Per2* mRNA were showed inversion of phase at LL (*Per2*: 06:30:40, Table 2, Fig. 3B d2) exposed SCN compared to LD (*Per2*: 18:27:15, Table 2, Fig. 3B d1). Additionally, mesor and amplitude of *Per2* mRNA in LL reared mice decreased from observation of *Per2* mRNA in LD raised mice (Table 2). The relative *Bmal1* mRNA expression significantly increased by 2.71 folds at ZT16 ($P < 0.05$) and no significant changes were found at other ZTs ($P > 0.05$) in LL mice when compared to *Bmal1* expressions at respective ZTs in LD mice. Relative *Clock* mRNA expression showed a significant increase in all ZTs ($P < 0.05$) as compared to respective ZTs in LD mice. Compared to LD, relative *Per1* mRNA in LL mice was significantly increased at ZT0, 4, 8, 12, 20 and 24 of more than 1.3 -fold but decreased at ZT16 ($P < 0.05$), whereas *Per2* relative expression was significantly decreased in all ZTs except ZT0, 20 and 24 ($P > 0.05$). Finally, the relative expression of *Cry1* in LL mice significantly increased at all ZTs as compared to LD.

Two-way ANOVA of ovary showed significant interaction of continuous light and the different *zeitgeber* time points for all studied clock genes (*Bmal1*: $F_{6,28} = 21.962.$, $P < 0.0001$; *Clock*: $F_{6,28} = 218.717$, $P < 0.0001$; *Per1*: $F_{6,28} = 40.09$, $P < 0.0001$; *Per2*: $F_{6,28} = 33.05$, $P < 0.0001$; *Cry1*: $F_{6,28} = 39.512$, $P < 0.0001$, Supplementary Table 2). Under LL the relative expressions of all clock genes in the ovary were arhythmic ($P > 0.05$, Cosinor analysis, F-test, Table 2, Fig. 4A a2–c2, Fig. 4B d2–e2), whereas under LD, the relative expressions of all clock genes showed significant oscillations ($P = 0.05$, Cosinor analysis, F-test, Table 2, Fig. 4A a1–c1, Fig. 4B d1–e1). The relative *Bmal1* mRNA significantly increased at ZT0, 4, 12, 20 and 24 by almost 1.3–threefold but decreased at ZT16 ($P < 0.05$), whereas *Clock* mRNA relative expression was significantly increased by 1.5–threefold only at ZT12 and 16, respectively, ($P < 0.05$) as compared to LD. Relative *Per1* significantly increased almost twofold only at ZT4 and decreased at ZT12 and 16 ($P < 0.05$). Relative *Per2* mRNA expression showed a significant increase of relative expression at all ZTs ($P < 0.05$) except ZT12 and 16 ($P > 0.05$). Finally, the relative expression of *Cry1* significantly increased only at ZT4 and 20 but decreased at ZT 8, 12 and 16 ($P < 0.05$) as compared to LD.

Two-way ANOVA of uterus had significant interaction with continuous light and the different *zeitgeber* time points for all studied clock genes (*Bmal1*: $F_{6,28} = 107.71$, $P < 0.0001$; *Clock*: $F_{6,28} = 259.717$, $P < 0.0001$; *Per1*: $F_{6,28} = 56.09$, $P < 0.0001$; *Per2*: $F_{6,28} = 7620.97.$, $P < 0.0001$; *Cry1*: $F_{6,28} = 534.4.512$, $P < 0.0001$, Supplementary Table 2). Furthermore, the relative expressions of all clock genes were arhythmic ($P > 0.05$, Cosinor analysis, F-test, Table 2, Fig. 5A a2–c2, Fig. 5B d2–e2), whereas under LD the relative expressions of all clock

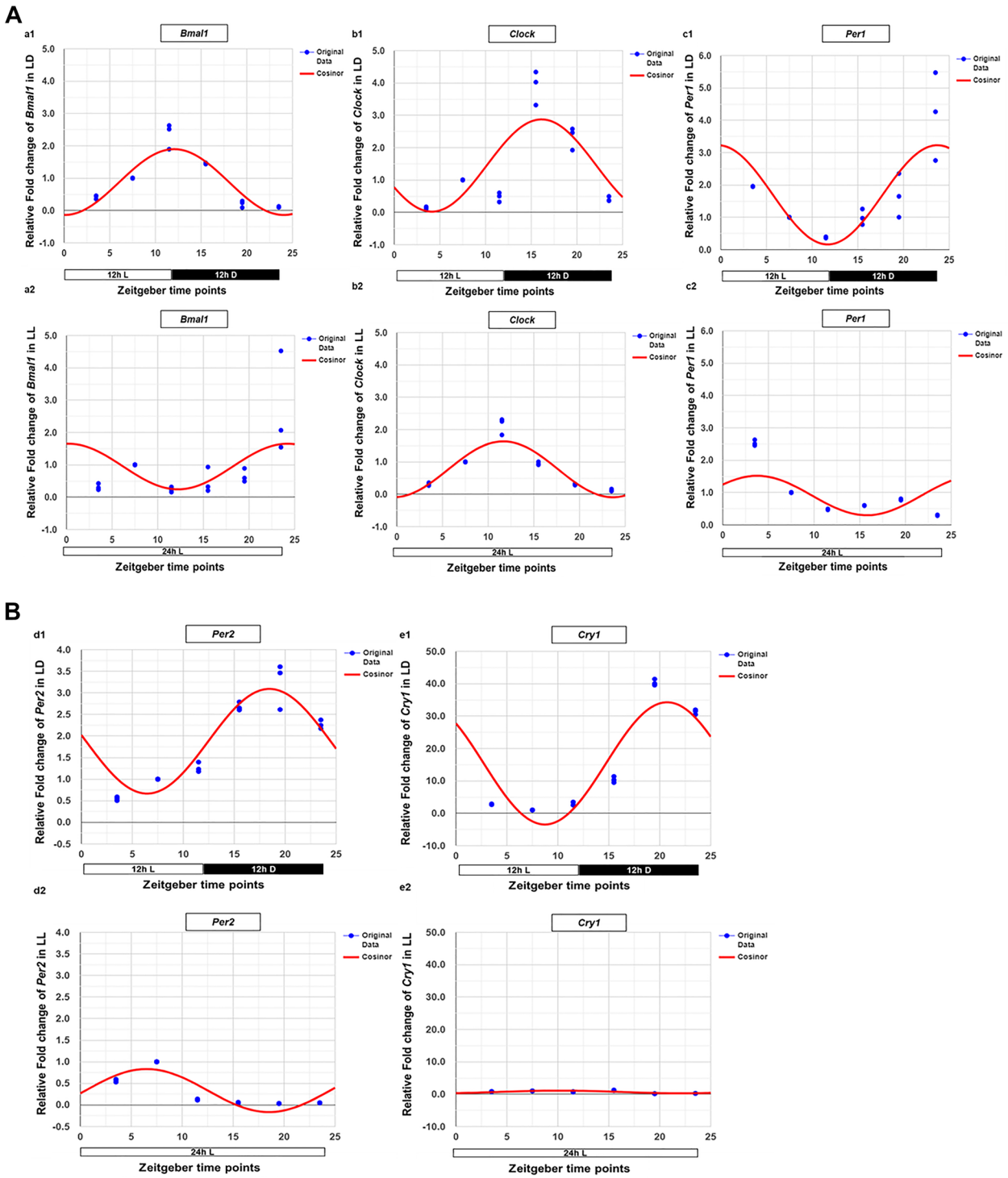

Fig. 3 **A** & **B** Cosinor analysis depicting temporal variation of core clock genes (*Bmal*, *Clock*, *Per1*, *Per2* and *Cry1*) over a 24-h circadian cycle in suprachiasmatic nuclei (SCN) of mice under LL (a1–e1) and under LD (a2–e2) conditions. Relative expressions of clock gene data are represented as mean for the best fitting 24-h single component cosine curve and considered statistically significant if $P < 0.05$

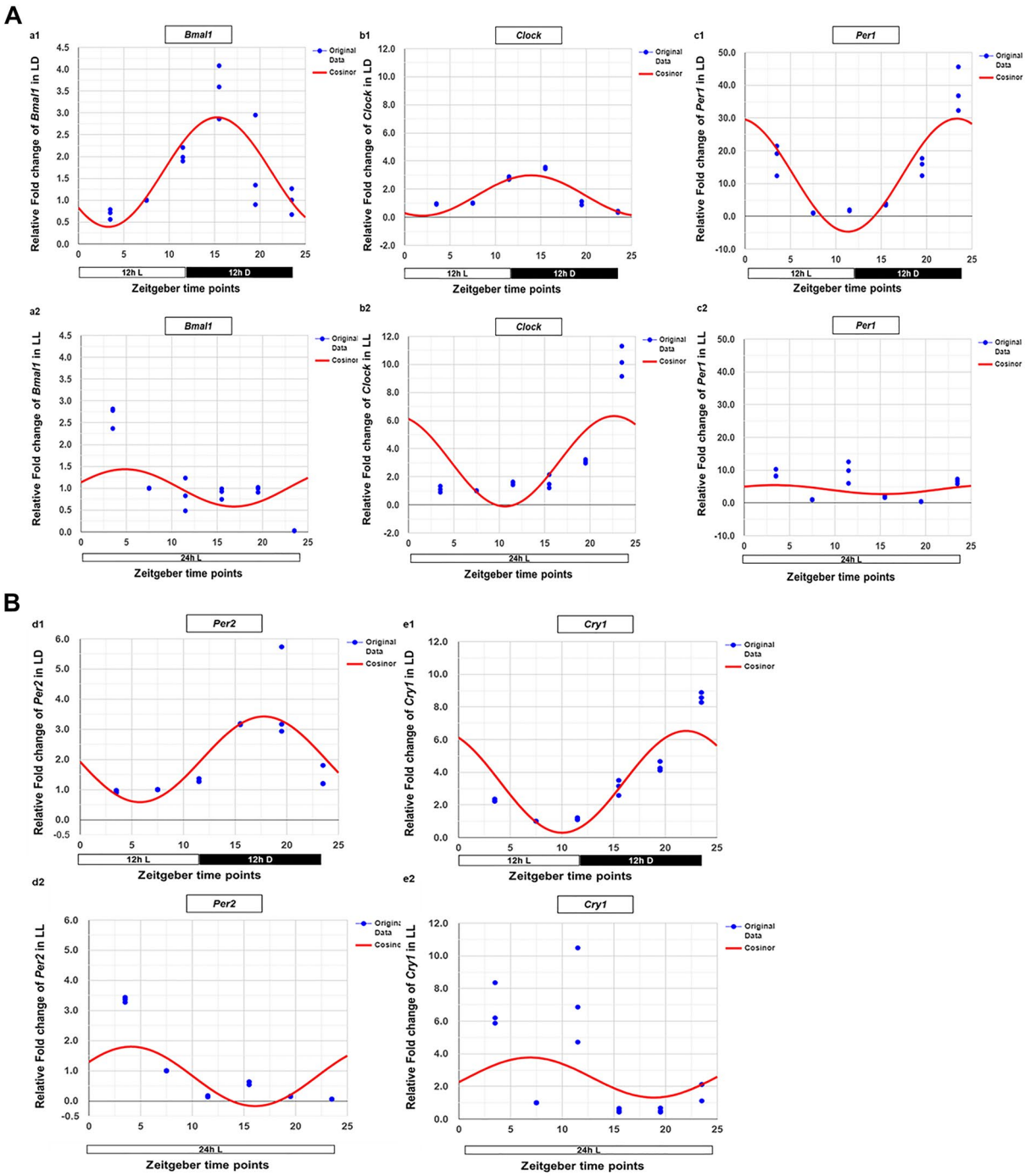

Fig. 4 **A** & **B** Cosinor analysis depicting temporal variation of core clock genes (*Bmal*, *Clock*, *Per1*, *Per2* and *Cry1*) over a 24-h circadian cycle in ovary of mice under LL (a1–e1) and under LD (a2–e2) conditions. Relative expressions of clock gene data are represented as mean for the best fitting 24-h single component cosine curve and considered statistically significant if $P < 0.05$

genes showed significant oscillations ($P = 0.05$, Cosinor analysis, F-test, Table 2, Fig. 5A a1–c1, Fig. 5B d1–e1). The relative *Bmal1* mRNA significantly increased at all ZTs except ZT8, 12 where a significant decrease of relative expressions was found ($P < 0.05$) and at ZT16 no changes were observed ($P > 0.05$). Expression of *Clock* relatively increased significantly at every ZTs by almost 2–eightfold ($P < 0.05$). Relative expressions of *Per1* and *Per2* mRNA were significantly increased at all ZTs ($P < 0.05$) except ZT16 and 20 ($P > 0.05$). Finally, *Cry1* expression relatively increased only at ZT0 and 24 and decreased only at ZT8 ($P < 0.05$).

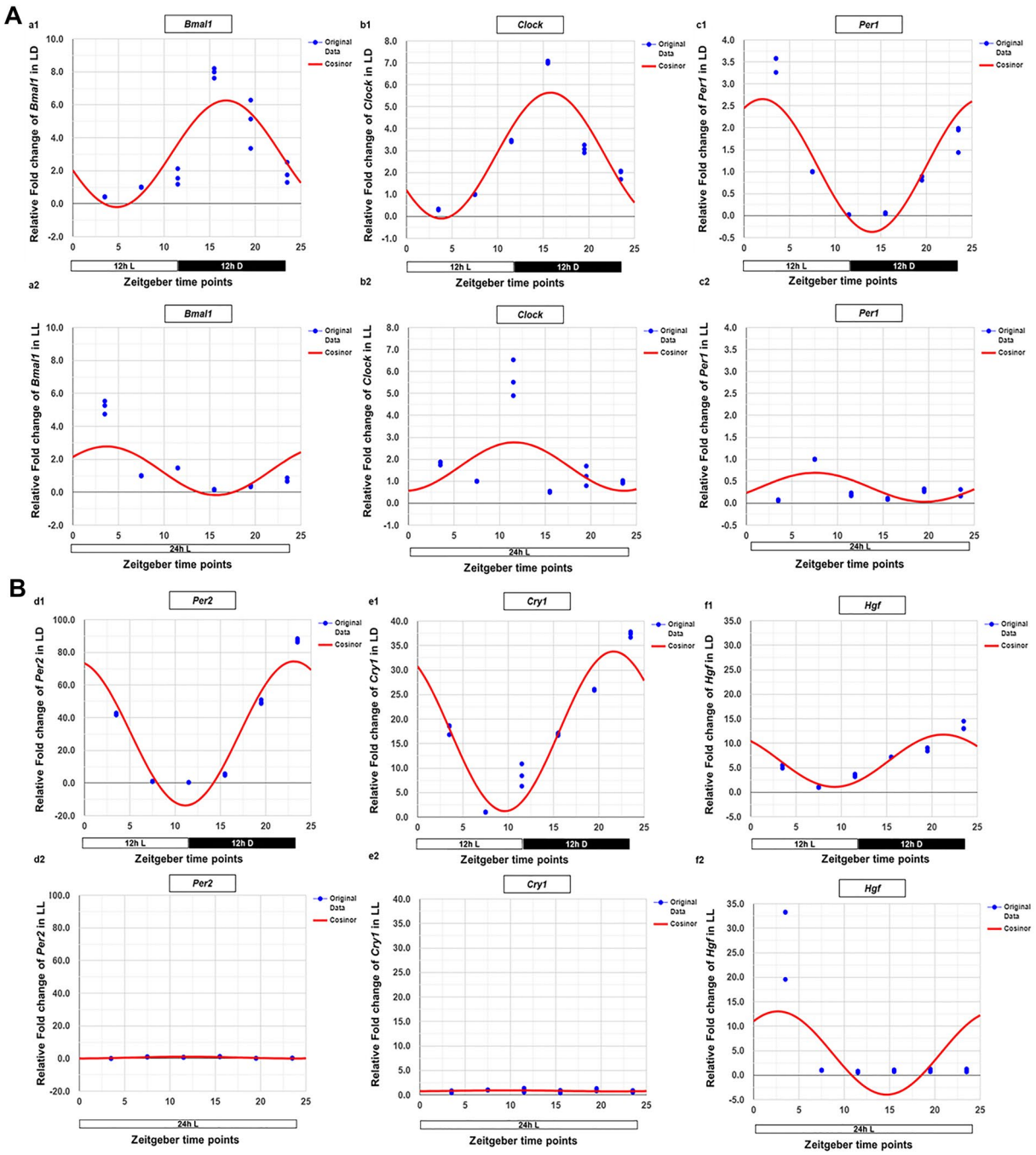

Fig. 5 **A** & **B** Cosinor analysis depicting temporal variation of core clock genes (*Bmal*, *Clock*, *Per1*, *Per2* and *Cry1*) and *Hgf* over a 24-h circadian cycle in uterus of mice under LL (a1–f1) and under LD (a2–f2) conditions. Relative expressions of clock gene data are represented as mean for the best fitting 24-h single component cosine curve and considered statistically significant if $P < 0.05$

3.3.2 b. Daily rhythmic variation of *Hgf* mRNA in mice uterus during non-pregnancy

Two-way ANOVA showed significant effect of continuous light and the different zeitgeber time points for *Hgf* mRNA expression ($F_{6,28} = 519.185$, $P < 0.0001$, Supplementary Table 2). Cosinor analysis demonstrated statistically robust 24-h rhythms of *Hgf* relative expressions under LD exposed mice ($P = 0.05$, Table 2, Fig. 5B f1) with peak expression at 21:14:34 h. However, during LL conditions, relative expressions of *Hgf* were significantly arhythmic ($P > 0.05$, Cosinor analysis, F-test, Table 2, Fig. 5B f2). The relative fold change of *Hgf* mRNA significantly increased at ZT0, 20 and 24 whereas decreased folds at ZT8, 12 and 16 ($P < 0.05$).

3.4 Hormonal analysis (ELISA) of progesterone (P_4) level during pregnancy (implantation and post-implantation)

LL exposure significantly decreased the serum progesterone level on D4 and D7, compared to LD exposed control mice (One-way ANOVA followed by Tukey post hoc, $P<0.05$, Fig. 6A).

3.5 Western blot analysis of PR-A and HOXA10 receptor expression during pregnancy

LL conditions significantly downregulated the progesterone receptor-A (PR-A) and HOXA10 in the uterus on day 4 (D4) or peri-implantation (implantation window) stage as compared to LD control mice ($P<0.0001$ One-way ANOVA followed by Tukey post hoc) (Fig. 6B b1 and b2, respectively).

3.6 Effect on sexual responsiveness (mating frequency), implantation frequency and offspring number

The average cohabitation period of male–female till copulation was approximately 3 days in LD mice (2.5 ± 0.51), whereas 6 days (5.9 ± 0.42) in LL reared mice (Table 3). After successful copulation, LL mice showed a significantly lesser number of implantation sites (5.7 ± 0.32) as compared to LD mice (10.8 ± 0.33, $P<0.05$, Table 3, Fig. 6C). The average gestation period of LL exposed mice was significantly high (25.89 ± 0.52, $P<0.05$) in comparison with LD reared mice (19.72 ± 0.31, Table 3).

A direct effect of continuous light was noted on offspring number (2.11 ± 0.34, $P<0.05$), when compared to the offspring number (10.11 ± 0.34) of the LD control mice (Table 3, Fig. 6D). The average weight of a single pup was 0.666 g in the LL group and 1.203 g in the LD group (Table 3, Fig. 6D). Cage inspection was done manually

Table 3 Reproductive performance of LD and LL reared mice

Pregnancy parameters	LD	LL
Cohabitation period (in days)	2.5 ± 0.51	5.9 ± 0.42
Implantation site number (in number)	10.8 ± 0.33	5.7 ± 0.32
Gestation time (in days)	19.72 ± 0.31	25.89 ± 0.52
Offspring number (in number)	10.11 ± 0.34	2.11 ± 0.34
Average weight of the pups (g)	1.203 ± 0.22	0.666 ± 0.58

Data are represented as mean ± SE

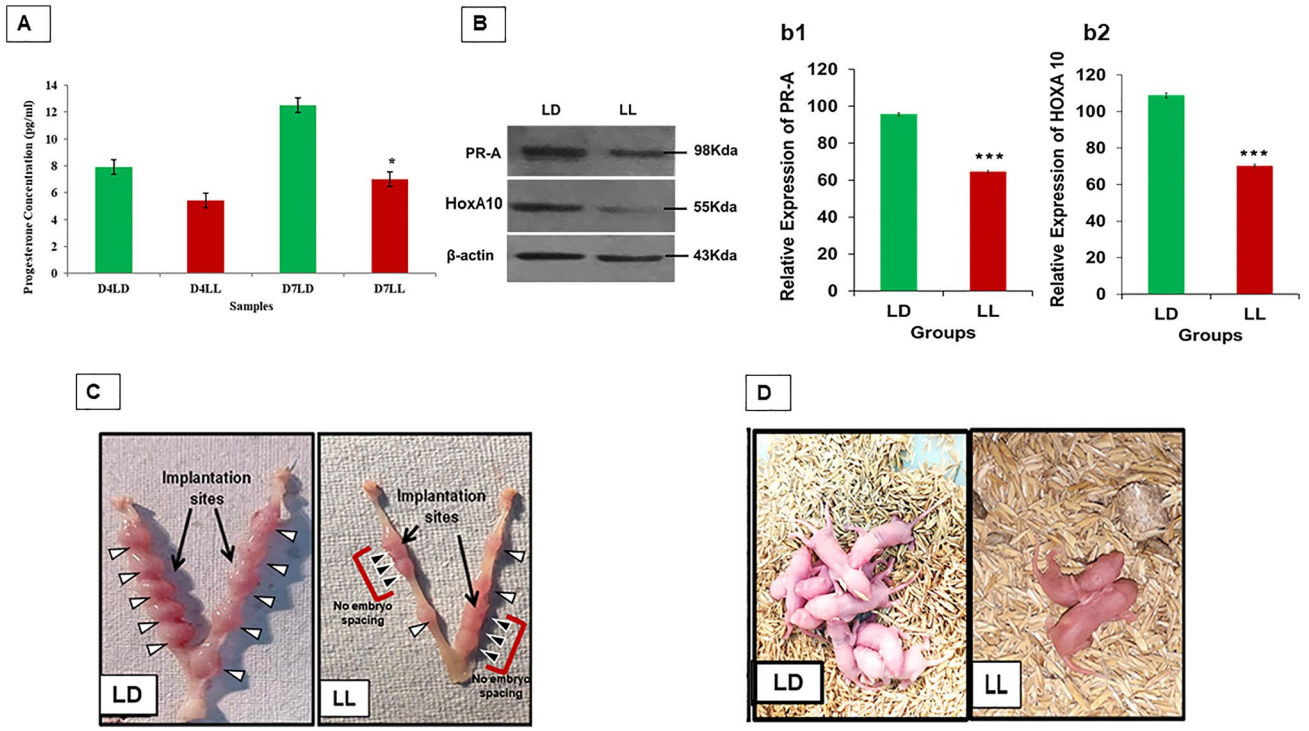

Fig. 6 **A** Histogram presenting progesterone levels during different days. Values expressed as mean ± SEM ($n=3$) for different days. **B** Effect of LD and LL condition on expression of PR-A and HOXA10 in uterus samples of LD and LL. Histogram showing level of PR-A and HOXA10 analyzed using Image J. Values expressed as mean ± SEM for LD and LL groups. Data were considered significant as *** for $P<0.001$ and * for $P<0.05$. **C** Implantation site. Note the large number of implantation site under LD (**D**) Offspring number. Note the high number of offspring under LD

during the experimental period and no cannibalism was observed.

4 Discussion

Successful pregnancy is one of the most challenging phases of the female life-time. There are growing evidences in animal model that suggest a circadian disruption during pregnancy leads to severe reproductive abnormalities such as preterm birth, abortions, low offspring number etc. [28, 29]. In the present study, we have demonstrated that continuous light (LL) disturbed the robustness of central and peripheral (uterus and ovary) clock genes leading to altered estrous cyclicity and progesterone levels and dysregulation of key markers of uterine physiology which are essential for maintenance of cyclicity and pregnancy. These changes then disturbed female uterine homeostasis, resulting in increased gestation time along with poor prenatal and postnatal maintenance in adult female mice.

Light is the most important photo-entraining agent of the biological clock located in suprachiasmatic nuclei (SCN) [18]. Previous studies suggest the presence of peripheral circadian clock genes in the ovary, uterus, and oviduct significantly contribute to the success of female reproductive physiology [2, 6, 35]. Our clock gene expression data showed that LL significantly altered the coordination between 24-h cycle of clock gene transcripts in SCN, ovary and uterus except for SCN *Per2* gene as compared to control mice. Previous reports supported that constant light dampen *Per2 circadian* rhythm in SCN of mice [36] which was parallel to our present qPCR result. Further LL exposure in SCN showed maximum relative fold changes of *Bmal1* and *Clock* mRNA at ZT16 and ZT8 suggesting possible phase shift of the peak expressions from morning time to night/day-night transition time (Fig. 3A). Given the increasing evidence that clock genes regulate the process of timing of ovulation and the early pregnancy [37–44], we expected LL light might disrupted the expression levels of clock genes that have significant influence on central and peripheral clock genes circadian control coordination in non-pregnant female mice.

Reports suggest that progesterone is known to regulate the cyclic regeneration of endometrial lining of the uterus [45–47]. Therefore, we measured the important marker gene of uterine physiology (*Hgf*,) to check its rhythmic variation. Our cosinor results suggest LL disturb the rhythmicity of uterine *Hgf* gene as compared to LD mice (Fig. 5B). Surprisingly, *Hgf* expression showed a similar rhythmic pattern as well as peak expression with uterine *Per1*, *Per2* and *Cry1* at night time (ZT12-ZT20). Thus, our results suggest that continuous light also disturbs the *Hgf* rhythm which is an important modulator of uterine health.

Furthermore, LL disturbed the estrous cyclicity in mice with significantly reduced diestrus phase and longer estrus phase over the 30 days duration. Our results get support from the studies of Hardy D. F. (1970) [24] where it was suggested that constant light disturbs the natural estrous cyclicity, levels of estrogen and timing of the release of progesterone in female rat. Additionally, to evaluate the sexual responsiveness, we have also examined the cohabitation period of LL exposed mice which was longer as compared with LD hence we may suggest constant light increasing the time for receptivity in female mice. But this may result in loss of rhythmic functions such as estrus cycle and uterine perceptibility during pregnancy in female mice.

Uterine histopathological observation showed that LL condition significantly changed the endometrial lumen having significantly increased thickness of epithelium as compared to control mice. Furthermore, LL exposed mice showed presence of polyp-like structure in the lumen with thickened endometrium as compared to control mice. Previous histopathological reports on uterus suggest that uterine alterations of endometrial outgrowth could be endometrial polyp [48, 49]. Reports suggest that uterine epithelial cell proliferation is regulated by steroid hormones estrogen (E_2) and progesterone (P_4) [31, 50], hence alteration of these two hormones under LL condition might have led to polyp formation. Therefore, histological results clearly suggest that LL does affect female reproductive physiology through uterine tissue and health (Fig. 2).

During prenatal days or early pregnancy, endometrial receptivity and implantation is highly coordinated process that requires synchronized timing between circadian clock systems with neuroendocrine hormones of hypothalamus–pituitary–gonadal (HPG) axis [42, 44]. Progesterone (P_4) is the key regulators of endometrial proliferation and is associated with circadian oscillations [43]. Therefore, we recorded the effect of continuous LL on P_4 levels during the pregnant phase (Fig. 6). LL significantly lowered the serum P_4 level at both D4 and D7 suggesting that the peripheral reproductive clock system can influence the P_4 concentration being crucial for implantation and maintenance of pregnancy. We also measured PR-A receptor expression by western blot analysis during pregnancy phase (Fig. 6) and LL downregulated the expression of PR-A protein as compared to control mice. Thus, our results strongly indicate transcriptional–translational interactions between central and peripheral reproductive (ovarian and uterine) clock gene expression with the ovarian synthesis of steroids hormone as well as during early pregnancy the endometrial receptivity and implantation stage of mice.

Previous reports suggest that uterine health and ovarian functions is correlated to the process of embryo development, decidualization and implantation [51–57]. Further prenatal HOXA10 protein was reported as an important uterine

implantation marker gene [55, 56]. For the first time, we have checked the influence of constant LL on HOXA10 protein expression to understand the circadian clock control on the process decidualization in pregnancy. LL significantly downregulated the expression of prenatal HOXA10 uterine protein as compared to control mice suggesting constant LL might have disturbed the coordination between SCN and peripheral clocks which disrupted decidualization process in female mice (Fig. 6). Furthermore, the earlier investigation on circadian control of ovulation with rhythmic presence of clock genes in uterus and ovary supported our view [58, 59].

For postnatal effect of continuous LL, we continued our study to next generation. We measured implantation site number, gestation period, offspring number and weight. We found that LL significantly increased the gestation, delivery time and lowered the implantation number along with offspring number (Fig. 6) and decreased litter weight. Previous studies reported that maternal circadian disruption can negatively affect early fetal loss [13]. This suggest that upstream gene regulation gets altered by continuous light and then it is disturbed the circadian coordination between prenatal and postnatal uterine health of female mice.

5 Conclusion

In conclusion, we may propose that continuous artificial light desynchronized the central and peripheral, ovarian, and uterine core clock gene *Bmal1*, *Clock*, *Per1*, *Per2* and *Cry1* expression resulting in altered estrous cyclicity, serum progesterone levels and its downstream regulators of uterine physiology (*Hgf*, PR-A and HOXA10) during non-pregnancy and pregnancy phases of female mice which are necessary for maintenance of cyclicity and pregnancy. Furthermore, during pregnancy, we may propose that LL reduced progesterone level downregulated HOXA10 protein, hence it impaired the process of decidualization and lowered the implantation rate. This becomes the cause of reduced offspring number. Our study, therefore, provides strong evidences for abnormal uterine functions in both non-pregnant and pregnant females who are getting exposed to longer duration of light during both normal and work from home conditions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s43630-022-00210-6.

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Declarations

Conflict of interest The authors have no conflict of interest that could be distinguished as discriminating the impartiality of the review reported.

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