TC.
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF HISTOLOGY AND EMBRYOLOGY

DETECTION OF THE EFFECTS OF CIRCADIAN RHYTHM DISRUPTION DUE TO CHRONIC CONSTANT LIGHT ON OOCYTE AGING BY mTOR SIGNALING PATHWAY

MASTER OF HISTOLOGY AND EMBRYOLOGY THESIS

GİZEM BORA

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 28/01/2021 and numbered 2021/01-10

Professor Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

25.12.2020

Gizem BORA

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LIST OF SYMBOLS/ABBREVIATIONS

Akt/PKB	Protein kinase B
AMH	Anti-Müllerian hormone
AMPK	Monophosphate-activated protein kinase
BSA	Bovine serum albumin
CLOCK	Circadian locomotor output cycles kaput
COX2	Cyclo-oygenase-2
CRY	Cryptochrome
CT	Circadian time
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
DBP	D-box-related genes
DEPTOR	DEP domain containing mTOR-interacting protein
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
eIF4F	Eukaryotic initiation factor 4F
ERK	Extracellular-signal-regulated kinase
FSH	Follicle stimulating hormone
GAS6	Growth arrest specific 6
GHT	Geniculohypothalamic tract
GnRH	Gonadotropin-releasing hormone
GRE	Glucocorticoid response element
GV	Germinal vesicle
hCG	Human chorionic gonadotropin
H&E	Hematoxylin and eosin
HIFα	Hypoxia inducible factor1α
HLF	Human hepatic leukemia factor
HPO	Hypothalamo-pituitary-ovarian
HRP	Horseradish peroxidase
IGF1	Insulin-like growth factor 1
KITL	Kit ligand
L:D	Light-dark cycle
LH	Luteinizing hormone

L:L	Light-light cycle
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MI	Metaphase I
MII	Metaphase II
NGS	Normal goat serum
NTY	Nitrotyrosine
OFT	Open field test
PAS	Periodic acid schiff
PAS domain	Per-Arnt-Sim
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with tween
PCOS	Polycystic ovarian syndrome
PER	Period
PI3K	Phosphoinositide 3-kinase
POF	Premature ovarian failure
POI	Premature ovarian insufficency
Rheb	Ras homolog enriched in brain
RHT	Retinohypothalamic tract
p-mTOR	Phosphorylated mammalian target of rapamycin
ROR	Retinoic acid receptor-related orphan receptors
ROS	Reactive oxygen species
RSK1	Ribosomal S6 kinase
SCN	Suprachiasmatic nucleus
SREBP1	Streol regulatory element binding protein
S6K1	S6 kinase 1
TBS	Tris-buffered saline
TBS-T	Tris-buffere saline with tween
TEF	Thyrotroph embryonic factor
TNFα	Tumor necrosis factor-α
TSC1	Tuberous sclerosis 1

TTFL	Transitional/post transitional delayed feedback loop
Vip	Vasoactive intestinal peptide
ZP1	Zona pellucida sperm binding protein 1
ZP2	Zona pellucida sperm binding protein 2
ZP3	Zona pellucida sperm binding protein 3
ZT	Zeitgeber time

ABSTRACT

Bora, G. (2020). Detection of the Effects of Circadian Rhythm Disruption due to Chronic Constant Light on Oocyte Aging Through mTOR Signaling Pathway. Yeditepe University, Institute of Health Sciences, Master Thesis. İstanbul

PER2 is one of the core circadian rhythm components and its expression has been demonstrated in ovarian cells. It has been also demonstrated that light exposure at night may cause attenuation in PER2 mRNA and protein levels. Circadian rhythm disruptions are thought to be associated with reproductive diseases. In female reproductive system, mTOR signaling pathway is thought to function in folliculogenesis and oocyte maturation in ovary. Also, it is associated with ovarian and oocyte aging. In a recent study, it has been demonstrated that there might be a relationship between PER2 and mTOR. We hypothesized that circadian rhythm disruption by constant light may alter PER2 protein levels in mouse ovary, and through possible relationship between PER2 and mTOR, circadian rhythm disruption by light may affect the ovarian morphology and function and may cause ovarian and oocyte aging. We aimed to show the effect of constant light on ovary and oocyte morphology. 6-8-week-old female Balb-c mice were used. We housed 12:12h L:L group in constant light and 12:12h L:D group in standard lightening conditions for one week. We performed food intake, body weight change analysis, open field test and vaginal smear. We evaluated ovarian morphology and follicle counting analysis of ovaries by hematoxylin&eosin staining and PAS staining. By immunofluorescence and immunohistochemistry, we evaluated oocyte aging markers. We also performed western blot for PER2, mTOR, p-mTOR and Caspase-3 protein levels. We demonstrated that circadian rhythm disruption caused alteration in their food intake and emotional behaviors and it caused decrease in primordial follicle numbers and increase in atretic follicles. It caused increase in oxidative stress and decrease in ZP3 expression in oocytes. We showed decreased protein levels of PER2, mTOR and p-mTOR. We demonstrated that circadian rhythm disruption by light may cause ovarian and oocyte aging-like consequences. Thus, circadian rhythm-based therapies may be useful for treatment of ovarian or oocyte-aging, at least to relieve their symptoms.

Key words: Circadian rhythm, ovary, oocyte, aging, mTOR

ÖZET

Bora, G. (2020). Sirkadiyen Ritmin Işık ile Bozulmasının mTOR Sinyal Yolağı Üzerinden Oosit Yaşlanmasına Olan Etkisinin Belirlenmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Master Tezi. İstanbul

PER2, sirkadiyen ritmin ana komponentlerinden biridir ve ekspresyonu ovaryum hücrelerinde belirlenmiştir. Akşam döngüsünde ışığa maruz kalmanın PER2 mRNA ve protein seviyelerinde azalmalara sebep olabileceği yapılan çalışmalarda gösterilmiştir. Sirkadiyen ritim bozukluklarının, dişi üreme sistemi hastalıkları ile ilişkilendirilebileceği düşünülmektedir. Dişi üreme sisteminde, mTOR sinyal yolağının ovaryumlarda folikül gelişiminde ve oosit olgunlaşmasında rol aldığı bilinmektedir. Aynı zamanda, mTOR sinyal yolağı ovaryum ve oosit yaşlanması ile ilişkilidir. Yapılan yeni bir çalışmada PER2 ve mTOR arasında bir ilişki olabileceği gösterilmiştir. Bu bilgilerden yola çıkarak, ışık ile sirkadiyen ritmin bozulmasının, fare ovaryumlarında PER2 protein seviyelerini etkileyebileceği ve PER2 ile mTOR arasındaki olası ilişki üzerinden ovaryum morfolojisi ve fonksiyonunu etkileyebileceğini, aynı zamanda ovaryum ve oosit yaşlanmasına sebep olabileceğini hipotez ettik. Devamlı ışığın, ovaryum ve oosit morfolojisi ve fonksiyonu üzerindeki etkilerini belirlemeyi amaçladık. 6-8 haftalık dişi Balb-c fareler kullandık. 12:12h L:D grubu standart ışık döngüsüne maruz kalırken, 12:12h L:L grubunu 1 hafta boyunca devamlı ışığa maruz bıraktık. Besin alımı, vücut ağırlığı değişimi analizleri, açık alan testi ve vajinal smear uyguladık. Ovaryum morfolojisi ve folikül sayımı analizlerini hematoksilen&eosin boyaması ile ve PAS boyaması ile gerçekleştirdik. Immunoflorasan ve immunohistokimya boyamaları ile oosit yaşlanması belirteçlerini değerlendirdik. Western blot ile PER2, m-TOR, p-mTOR ve Caspase-3 protein miktarlarını analiz ettik. Sirkadiyen ritmin ışık ile devamlı bozulmasının, farelerin besin alımlarını ve davranışlarını etkileyebileceğini aynı zamanda primordial folikül sayılarını düşürdüğünü, atretik folikül sayılarını da arttırdığını gösterdik. Oositlerde, oksitatif stresi arttırdığını ve ZP3 ekspresyonunu azalttığını gösterdik. PER2, mTOR ve p-mTOR protein seviyelerinde azalmalar belirledik. Böylece, sirkadiyen ritmin ışık ile bozulmasının ovaryum ve oosit yaşlanmasını tetikleyebileceğini gösterdik. Bu çalışma ile, sirkadiyen ritim bazlı terapilerin, ovaryum yaşlanmasının tedavisinde veya semptomlarının azaltılmasında kullanılabileceğini önermekteyiz.

Anahtar Kelimeler: Sirkadiyen ritim, ovaryum, oosit, yaşlanma, mTOR

1. INTRODUCTION and PURPOSE

Circadian rhythm is an internal oscillator that controls multiple physiological processes through transcriptional-translational feedback loops at the molecular level (1). When adapted to the day-night cycle; it allows organisms to perceive 24-hour daily cycles, to adapt to this cycle, and also to generate behavioral and physiological responses (2). Circadian rhythm regulates the sleep-wake cycle and affects both cells and organ systems such as cardiovascular system, immune system, endocrine and reproductive system at the molecular level (3, 4).

In mammalian cells; Period 1 (*Per1*), Period 2 (*Per2*), Period 3 (*Per3*), Cryptochrome 1 (*Cry1*), Cryptochrome 2 (*Cry2*), and Brain and Muscle ARNT-like Protein 1 (*Bmal1*) genes are known as circadian rhythm regulators and they are thought to play critical roles in some signal pathways (5). When the circadian rhythm is examined at the molecular level, positive and negative feedback mechanisms which are generated by their interactions between these proteins are observed (6). In daylight, RORα provides expression of the *Bmal1* gene through the RRE element of the Bmal1 gene. The CLOCK/BMAL1 complex binds to the E Box in the promoter region and activates transcriptions of *Per* and *Cry* which are the negative regulators of them. In the evening, the PER and CRY combine to form a protein complex and become an inhibitor of the *Clock: Bmal1* complex. At night, REV-ERBα inhibits *Bmal1* expression and also newly formed PER:CRY complex blocks CLOCK/BMAL1 activity. This blockage stops further transcriptions of *Per* and *Cry* genes. At this point, the activated PER/CRY complex begin to degrade and disappears completely. Repeating of this cycle in every 24 hours creates a circadian rhythm (7).

The expressions of these mentioned circadian rhythm genes were determined in the ovaries of rats, mice and ruminants as a result of the previous studies (6). Also, mRNA expressions of *Per1* and *Per2* have been observed in interstitial glandular tissue, granulosa cells, corpora lutea, pre-antral, antral and pre-ovulatory follicles (8). The *Per2* gene is known to play a role in the steroid production and cell proliferation in granulosa cells and it is examined that mRNA expressions of *Per1* and *Per2* have been peaked at night, were rhythmic regardless of the estrogen cycle and have been persisted when rats were left in the dark continuously (6).

It has shown that light exposure at night causes a decrease in the rhythmic expression of *Per2* gene and its mRNA levels in suprachiasmatic nucleus, peripheral tissues such as liver and adipose tissue (7). In another study, again, it has been shown that constant light attenuated the circadian rhythm of *Per2* mRNA and protein expression in suprachiasmatic nucleus of mice (9). In a very recent study, PER2 has been shown to be a scaffold protein that can suppress the activity of the mTORC1 signaling pathway by binding tuberous sclerosis complex 1 (TSC1) to the protein complex consisting of mTORC1 (The Mammalian Target of Rapamycin Complex 1) and Raptor (1).

mTOR is an evolutionarily conserved serine-threonine protein kinase that lies below the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway axis. By activating ribosomal kinases, it regulates cell growth, cell proliferation and cell survival (10). mTOR can create two biochemically and functionally different complexes as mTORC1 and mTORC2 in the cell. The sensitivity of these complexes to Rapamycin is different. Rictor, the element of the mTORC2 complex, is insensitive to Rapamycin (11). The mTORC1 complex, which is sensitive to Rapamycin, inhibits the activation of PI3K/AKT signaling pathway due to the insulin receptor substrate IRS-1 protein by phosphorylating and activating p70S6K kinase (12).

mTOR signaling pathway is thought to be very crucial for female reproductive system. mTOR is known to function in activation and survival of primordial follicles, granulosa cell proliferation, differentiation and meiotic maturation of oocytes (13). Also, studies have shown that mTOR signaling pathway is very important for folliculogenesis (14) and ovarian aging (15). Studies on mTOR signaling pathway showed its role in ovarian neoplasia, polycystic ovarian syndrome, premature ovarian failure, ovarian aging and especially follicular development are also ongoing (10).

In the light of literature, in this purposed study, we hypothesized that the exposure of constant light may alter the PER2 protein level in ovaries and through possible relationship between mTOR and PER2, circadian rhythm disruption by constant light may cause ovarian and oocyte aging by affecting ovarian morphology and function, also follicular development.

With this hypothesis, we aimed to form two groups of 6-8-week-old Balb-c female mice. We aimed to house 12:12h L:D group in standard lightening conditions, 12:12h L:L group in constant light for one week. We aimed to evaluate food intake and body weight

changes during this one-week experimental period. At the end of the one-week experiment, we aimed to evaluate the emotional behavior and locomotor activities with open field test, and to analyze the estrus cycle with vaginal smear. We aimed to evaluate the ovarian morphology by hematoxylin and eosin (H&E) staining and periodic acid schiff (PAS) staining, to count follicles, and to determine PER2, mTOR, p-mTOR and Caspase-3 protein levels in ovaries by western blot. At the same time, we aimed to evaluate oocyte morphology with nitrotyrosine (NTY) and zona pellucida sperm binding protein 3 (ZP3) immunofluorescence and immunohistochemistry staining on ovarian sections.

With this study and other possible further studies, we anticipate that determining the relationship between circadian rhythm disrupted by constant light and ovarian/oocyte aging and explaining the underlying molecular mechanism will help to present new methods for the treatments of disorders related to the female reproductive system.

2. LITERATURE REVIEW

2.1. Circadian Rhythm

Circadian clock which confers a 24-hour structure is known as a temporal organization and found in organisms related to all phyla (most light-sensitive organisms from cyanobacteria to human) (16, 17). Circadian means “approximately a day” which refers to Latin phrase known as “circa diem” (16). Circadian clock is a good way for adaptation to the earth’s rotation (17). This endogenous autonomous circadian rhythm fluctuates with the environment (16, 18). However, it is also known that even there is no daily external signals which are called as “zeitgebers”, circadian clock can be remain as autonomous and produce circa-24-hour rhythms (17). Similar to other every rhythm, circadian rhythm consists phase, amplitude and period (19).

Circadian clocks help organisms to maintain their periodicity and help internal changes to take place in coordination (20). These internal cycles affect their behaviors, physiology and metabolism. By this way, organisms are able to anticipate the 24-hour daily routine of the earth by optimizing the energy harvesting and utilization through light/dark cycles (20). However; circadian clock is not simply a response to the 24-hour changes occurs in earth’s external environment, but also a part of the timekeeping system which is found in organisms (21). This timekeeping system is very critical for organisms’ well-being and survival because it helps organisms to guess possible physical changes in their environment which are associated with day and night. Therefore, organisms are leaded to do the right things at the right time of the day (21).

Circadian clock is not a free-run process, and it is synchronized to the 24 hours as mentioned (17). To synchronize the circadian system to 24-hour sleep/wake cycle, daily adjustments are required. This is known as entrainment and a key characteristic of the biological clock (19). Entrainments is maintained by external stimuli and these stimuli are now as “Zeitgeber” which means time givers such as sleep, temperature, physical activities, social signals, meals etc. Most important zeitgeber is thought to be a light or dark (22). The phases of entrainments which are also known as chronotypes and strongly related to the relationship between body temperature and sleep-wake period, are not fixed and can be maintained with the strengths of zeitgebers and circadian clock (17). These phases of entrainments are generally different from individual to individual and only

entrainment-related selections are principles for shaped circadian programme through evolution (17). Phases of entrainment are generally from larks to owls and the other individuals are distributed in between these groups (23). Genetic polymorphisms in clock genes, age and environment derive this distribution (24-27).

Chronobiology focus on biological timing and high frequency cycles (such as hormone secretion), daily, monthly or annual cycles (such as activity and rest cycles, or reproductive cycles). By improvements on chronobiology, studies on biological rhythms continue for almost 50 years and characteristic properties of circadian rhythms begun to be constructed (21).

First and very critical feature of circadian rhythm is self-sustained nature which is illustrated by De Mairan (21). De Marian observed that the leaves of Mimosa plant continued to open and close every 24 hours even it was in the dark box in 18th century (28). Self-sustained nature shows us that almost all daily rhythms which take place under natural conditions can continue to occur in laboratory conditions which are without any external stimuli (21). Therefore, when the rhythm is persistent under constant conditions, it can be called as circadian because it shows that there is a timekeeping (internal) system. Otherwise, the diurnal rhythms cannot be counted as circadian. By this way, circadian rhythms are distinguished from the other rhythms which are just simply responses to the external environment conditions such as diurnal rhythms (21).

Second characteristic feature of circadian rhythm is the persistence of the cycle with a period of close to, but not exactly, 24 hours because if the rhythms persist with a period of exactly 24 hours, they are generally not internal but exogenously driven (21). It is demonstrated that internal time-keeping system is provided with the deviation from a 24-hour cycle and continuous aligned by and to the light-dark cycles (29). For instance, the intrinsic period of the human biological clock is around 24,2 hours (30).

The third of characteristic property of circadian rhythms is the ability of synchronization by external time stimuli (light-dark cycle). Researchers demonstrated that the response to the advanced, delayed or unchanged light differs according to the phase in the cycle at which it is presented. For example; if the light exposure occurs in the early part of the “normal” dark period generally results in a phase delay, however the light exposure at late part causes phase advance (29). Also, the intensity of light can cause phase shifting. For instance, exposure to the brighter light cause lengthen the period in some species and

shorten in other species. This is also known as Aschoff's rule (21, 31). Thus, it should be noted that even the circadian rhythms persist without any external cues due to its self-sustained nature, it doesn't mean that the environment cannot drive circadian rhythm. Rather, rhythms are generally aligned to these external cues. Circadian rhythms are able to sense the shift of the external cues and they can be aligned to these new cues (21).

The fourth characteristic property of circadian rhythms is ubiquity in nature. Circadian rhythms are found with similar properties and similar phase-response curves in different biological processes and organisms (21).

The last characteristic property of circadian rhythm is generation at the cellular level. Rhythms which are similar to the rhythms found in highly complex mammals are observed in unicellular organisms such as algae (21).

2.1.1. Molecular mechanism of circadian rhythm

The system of circadian clock is known as the totality of all oscillators in organisms which coupled to different physiological events (32). This system is generated by three different parts in mammals: input pathway which sensed the external timing signals such as light/dark, the core circadian clock which receives the information from the input pathway and forms endogenous circadian rhythm and the output pathway which adjust the physiological activities in organs and tissues through neurons or hormones (32).

Some research on unicellular organisms showed that the cellular nature of the system which generates circadian rhythm. However, in higher organisms, the circadian pacemarker is generally found in the cells of specific tissues or organs such as optic and cerebral lobes of brain in insects, eyes in invertebrates and vertebrates and pineal gland in nonmammalian vertebrates (21).

In mammals, SCN (suprachiasmatic nucleus) is known as the core circadian clock which forms the endogenous rhythms both at the cell or tissue levels and leads the outputs to peripheral tissues after synchronizations (16).

Circadian clock is located in the suprachiasmatic nuclei (SCN) which is found at the anterior hypothalamus in the base of the brain (21). SCN became very important for circadian rhythm with the lesion experiments. In the early 1970s, researchers damaged the SCN in rats, and observed the disruption of endocrine and behavioral circadian rhythms. Therefore, they demonstrated the role of the SCN in circadian rhythm (33). Researches

also showed that the SCN which is transplanted to the animals with lesioned SCN lead restoration in some of the circadian rhythms (21).

After similar restorations obtained from the studies on hamsters, SCN was started to be counted as a master pacemaker which control other rhythmic systems (34). It is thought that SCN transfer the information to the organs and tissues of the body with the aid of humoral and neural pathways (35). However, recently, researchers demonstrated with in vitro experiments that circadian rhythm is persistent even in isolated livers, lungs and other tissues without any control of SCN (36). Thus, the characteristics and organization of interaction between SCN and the rest of the body are not well-understood (37). However, it is also known that cyclic changes in the expressions of certain genes underlying SCN (internal pacemarker) maintain the properties of circadian clocks (21).

In 2017, Jeffrey C. Hall, Michael Rosbash and Michael W. Young won the Nobel Prize in Physiology of Medicine for their discoveries of molecular mechanisms controlling the circadian rhythm (38, 39). *Clock* (Circadian Locomotor Output Cycles Kaput), *Bmal1* (Brain and Muscle ARNT-Like 1), *Per1/2/3* (Period), *Cry1/2* (Cryptochrome) genes are known main circadian rhythm genes (Figure 2.1) (32, 39, 40). These genes and proteins encoded by these genes were reported by different experimental approaches and these studies led the identification of molecular circadian clock components (21). By using chemical agents, researchers generate random mutations into DNAs of the fruit fly, Drosophila melanogaster and rhythm abnormalities were reported. Thus, the first circadian clock mutants which are called *Period (Per)* was identified (21). Then in 90s, researchers used similar mutagenesis screening testing in the mouse and reported the first mouse circadian mutation called *Clock* (41). Like Per mutants, Clock mutants also caused free-running rhythm period. Both *Clock* mutation in mouse and *Per* mutation in flies resulted with the altered behavior rather than the altered physiological (21). Short after *Per* gene was found in flies, three per gene and their functions were reported in mammals too (41, 42). With the identification of *Bmal1* and *Cry* genes; the list of mammalian circadian clock genes has grown by time.

These oscillating clock-related genes are simply divided to activators such as CLOCK and BMAL1 and the repressors such as PER 1/2/3 and CRY1/2 (32, 40). The proteins of these genes are known as the core TTFL (transitional/post-transitional delayed feedback loop) to maintain oscillation and this loop takes approximately 24 hours to complete (43).

Besides this core loop, there are also sub-loops. RORs and nuclear REV-ERB receptors form the first sub-loop and the second sub-loop is generated by D-box-related genes (DBP, TEF and HLF) (44, 45). Circadian rhythm regulates biological behaviors in cells by TTFLs or indirectly. Transcriptional activators, BMAL1 and CLOCK generate a heterodimer and this complex binds to the cis-activating element E-box (5'-CACGTG-3'). By this way, transcription of *Per* and *Cry* genes is activated at the beginning of the cycle at circadian dawn (32, 39, 40). Then, PER and CRY proteins start to accumulate and dimerize. Thus, a new complex (PER/CRY) is generated and this complex translocate to the nucleus. By this way, CLOCK/BMAL1 heterodimer is inhibited (46) and also this PER/CRY complexes which accumulated in the nucleus start to repress their own expression (39). mRNA levels of *Per* and *Cry* start to fall and PER/CRY complexes which are already existing start to degrade at circadian night (39).

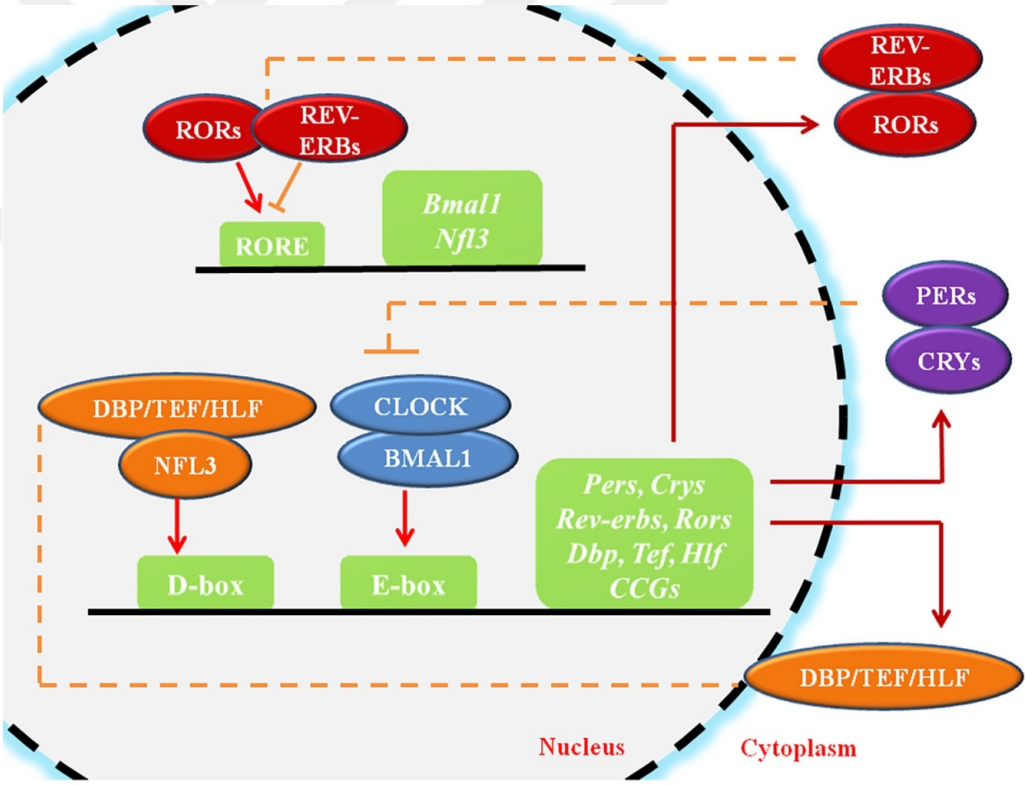


Figure 2.1. Molecular mechanism of circadian rhythm (32).

Other loops which are coupled to the core TTFL lead the completion of the oscillation. RORs and REV-ERB related to first sub-loop are directly targets at CLOCK/BMAL1 or ROREs (44, 45). The repressor NFIL3 (E4BP4) is driven by the REV-ERB/ROR loop and communicate with these proteins at sites containing D-boxes (47-49).

The second sub-loop which contains the PAR-bZip factors DBP, TEF and HLF is known as CLOCK/BMAL1-driven. ROR/REV-ERB and DBP (TEF, HLF)/E4BP4 are thought to act on other cis-acting elements such as RORE and D-box. Thus, they participate in the regulation of the core feedback loop (32). It is also known that PER and CRY levels are high during the day and low at night (50). Clock factors can bind not only other clock factors but also the specific enhancers of other genes outside the core clock mechanism and act as epigenetic regulators. These genes are called as clock-controlled genes (51).

2.1.2. Synchronizers of circadian rhythm

External timing cues are thought to influence 24-hour physiological oscillations and maintain circadian rhythm. These cues are known as “zeitgebers”, “synchronizer” or “entraining agents”. These are the factors found in all cells at the same phase and may reset circadian clock in organisms. This is called as circadian rhythm synchronization (32). “ZT” refers to the external cues when “CT” refers to timing without external signals (52).

2.1.2.1. Light

Light is required for the visual performance and visibility in both in indoor and outdoor environments. Besides; safety and comfort for the organisms is also affected by the light which is a source for relaxation and productivity (53). Light is also reported that it has important impacts on human, animals, plants and ecosystem balance (53). Changing in light intensity during the day is known as “photic entrainment” and lead the reset of the clock (54). Also, light is known to be the strongest of the environmental time cues which can reset the circadian clock in mammals and humans (Figure 2.2) (55, 56). Studies reported that the humans are less sensitive to the light when compared with the other mammals (56).

In chronobiology experiments, to induce responses by the circadian clock, researchers generally use light. In vivo, information about light is received by melanopsin receptors in retinal ganglion cells and then transferred to the SCN through the RHT (57-61) Then with neurohumoral factors, peripheral clocks can be regulated by SCN (62).

Period proteins such as PER1 and PER2 are known to be the important factors in daylight-elicited synchronization of the circadian rhythm because the exposure of the

daylight causes the stimulation of *Per1*/*Per2* transcription and translation (63). Besides their expression in SCN, their expression is also reported in throughout the body. They are known to be the regulators of metabolism and sleep (64). These genes are thought to play a role in sensation of oxygen and light in cells because they are members of the PAS domain superfamily (65).

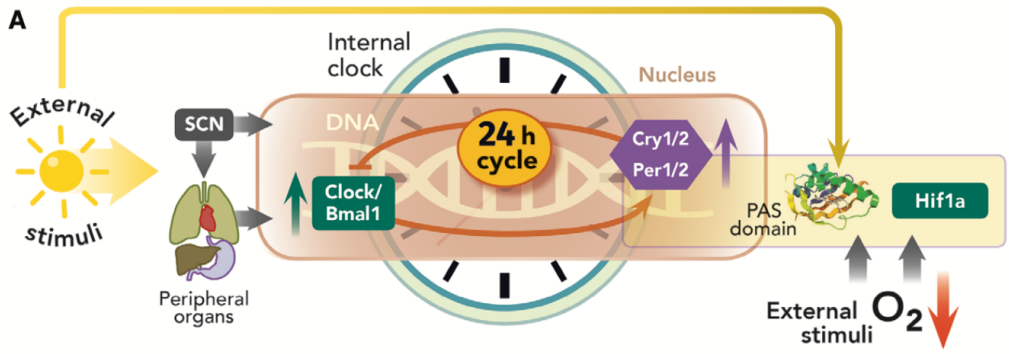


Figure 2.2. Mechanism of circadian rhythm which is setting with the light (5).

2.1.2.2. Arousal stimuli

Arousal stimuli are known as non-photic entrainment and consists of exercise, social interactions, restraint stress etc. (66). Different responses can be generated by different species to the arousal stimuli (32). Arousal stimuli are transferred to the SCN by GHT and the serotonergic median raphe nucleus projection to the SCN (67).

2.1.2.3. Food/feeding

Similar to arousal stimuli, food is also known as non-photic stimulus and has an ability to reset the circadian rhythm (32). The signals of fasting or feeding lead periodic availability of macromolecules which are circulate and thus may reset the circadian clocks of the peripheral tissues (68). For instance, insulin which is released into the blood after ingestion can stimulate the expression of genes in liver, muscle or adipose tissue which are known as insulin-sensitive tissues (69). Besides, expressions of *Per1* and *Per2* might be downregulated by the high glucose concentrations in fibroblast (70).

2.1.2.4. Temperature

Temperature is also one of the non-photic synchronizers and its affect is not strong comparing with the light. However; it should be noted that even the light is the most powerful and the temperature is a weak cue for the humans, temperature might be very important synchronizer for some species such as D. melanogaster (71). Circadian rhythm is known to has an important feature called temperature compensation (32). Temperature compensation leads circadian oscillations stay resistant to the changes on temperatures and results in the period length still approximately 24 hours (72, 73). However, cells of tissues which are found outside the SCN can be synchronized by changes on temperature (74-76).

2.1.2.5. Chemical factors

Chemical factors are also counted as synchronizers of circadian rhythm. Glucocorticoid which is known as anti-inflammatory hormone and released by peripheral tissue is an example of chemical factors (77-81). Glucocorticoids act on the GREs found on promoters of core clock genes known as *Per1*, *Per2* and *E4bp4* by activating GRs (81, 82).

2.1.2.6. Mechanical stimuli and oxidative/hypoxia stress

Oxidative or hypo-toxic stimuli are thought to cause circadian clock entrainment. It was observed that in vivo, hydrogen peroxide injection caused phase shifts in peripheral clocks in kidney and liver (83). However, whether the master clock is affected by oxidative stress or not is still unclear (32).

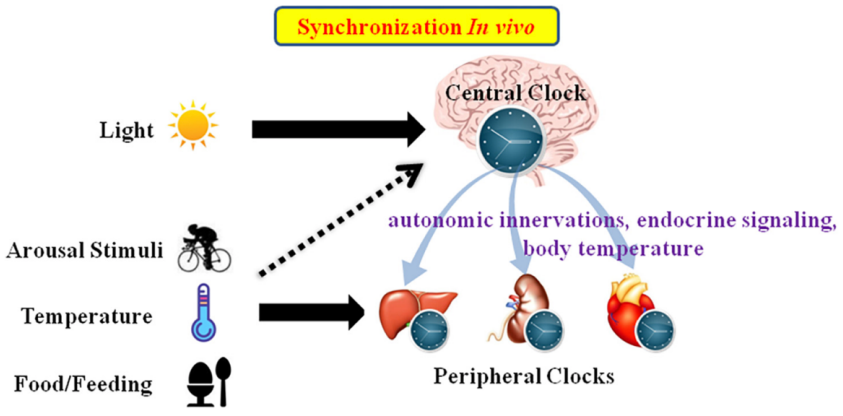


Figure 2.3. Summary of circadian rhythm synchronizers (32).

2.1.3. Relationship between circadian rhythm and metabolism

Metabolism can be explained as a whole biochemical reaction which molecules are transformed for energy generation and structural building in organisms. The main characteristics of metabolic regulation are known to be highly conserved among all form of life (51). During development, growth, tumorigenesis or aging; changes of cell fate occur and these are accompanied by wholesale rewiring of cellular metabolism (51, 84, 85).

Circadian rhythm is found at both cellular and organellar metabolism (86). Thus, circadian rhythm has the ability to regulate and control the coordination of organ functions (87). However, it is also known that circadian rhythm is not just the source of the rhythmicity on metabolic process, metabolic states or signals have also the ability to feedback to the circadian rhythm. In other words, metabolism might be counted as both output and input of circadian rhythm and its regulation (Figure 2.4). Thus, this input-output feedback mechanism between metabolism and circadian rhythm is required for the flexibility which is essential for the physiological responses of cells, tissues and whole organisms (51). Clocks found in cells can generate rhythmicity of various metabolic functions and they are independent from the systemic signals which are generated in body, but they are still very important for the synchronization of clocks found in whole-body (51). Even the most known regulations of circadian rhythm are sleep/wake and fasting/feeding cycles, it is also known that circadian clock has a various role in different metabolic events and systems such as homeostasis, behavioral and physiological processes, cardiovascular health, body temperature, glucose and lipid metabolism and endocrine hormone (18, 88). The interaction between circadian and metabolic processes is provided by the neuroanatomic and neuroendocrine levels. Thus, metabolic homeostasis is regulated by circadian clock. In other words, the anatomic connections which are found between the brain centers required for circadian rhythmicity and control for appetite and energy expenditure regulates the whole body metabolism based on circadian clock network (20, 89-92).

Besides, rhythmicity genes which are also known as clock genes are also showed that they are involved in regulation of intermediary metabolism such as mitochondrial oxidative phosphorylation, carbohydrate metabolism and transport, adipocyte differentiation, lipid biosynthesis and cholesterol synthesis and degradation (93, 94). It was also showed that when there is a mutation on the core molecular clock, the rhythmic expression of the various key metabolic genes are disrupted (87, 93, 95, 96).

Because circadian clock and its related genes are found in almost every cell; the rhythmic regulation of metabolism are found in most organs. For instance, 40% of the kidney genes are thought to be rhythmically expressed (97). In each organ, circadian clock is found for the regulation of the most prominent physiological functions of the organ. In kidney; blood flow, ion-water excretion and glomerular filtration rate were showed as the events which are modulated by circadian clock (98). Similar to kidney, rhythmicity in metabolic event of pancreas can be also observed. For instance; in pancreas insulin and glucagon excretion is controlled by circadian clock (99). In gastrointestinal tract; almost all metabolic and endocrine functions which are in close collaboration with the microbiome is also thought to be synchronized by the circadian clock (51). Besides all other organs, liver is the most well-studied organ in circadian biology. Among its many functions; metabolism of carbohydrate, lipids, amino acids, bile acids and the synthesis of detoxification enzymes, hormones and bile components are known to be regulated by circadian control of the liver (100, 101).

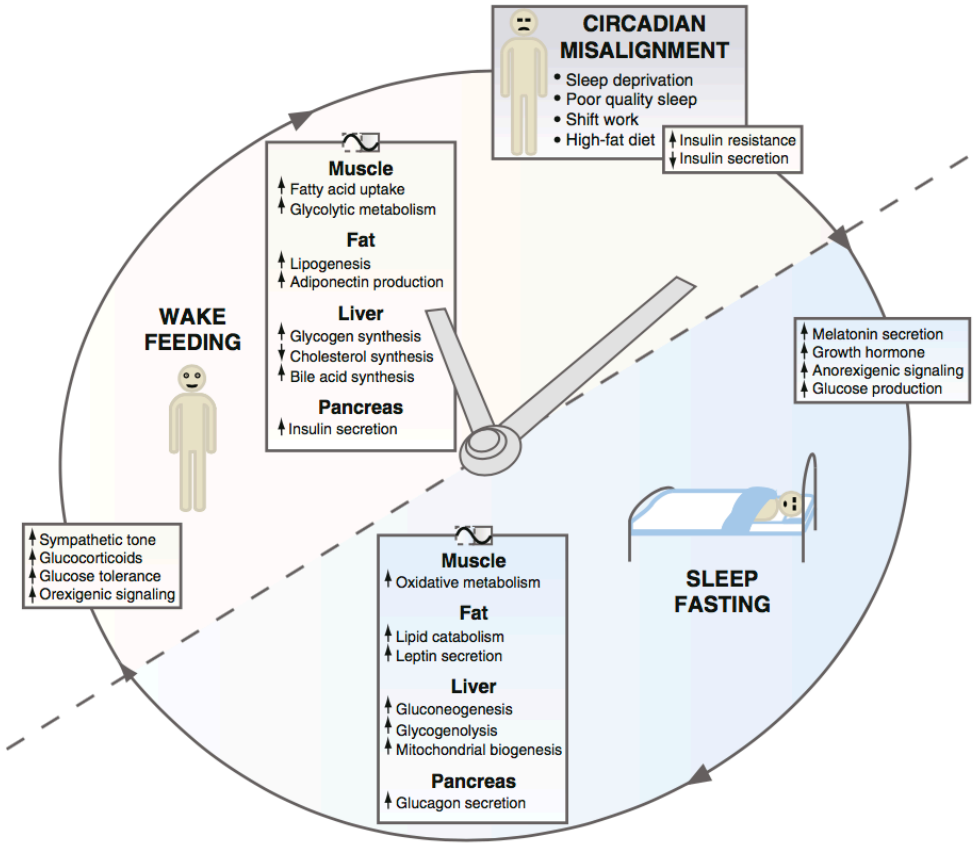


Figure 2.4. Relationship between circadian rhythm and metabolism (20).

2.1.4. Circadian rhythm related diseases

It is known that physiological functions of almost all organs are under the control of circadian clock. Thus, these physiological functions are generally affected by the external cues such as motor ability, changes of body temperatures, sleep and wakefulness, hormone secretions and cell cycle progression (28, 93). Disruptions of circadian clock by environmental or genetic defect cause dysfunction or physiological processes that occur in several organs (102). Regardless of whether the disruption is voluntary such as travel or involuntary such as advanced age, it is known that circadian rhythm disruptions have negative effect on safety, performance or productivity in organisms (21). In humans, the circadian disorders are generally caused by the misalignment between the endogenous circadian oscillators and environmental rhythm such as day-night cycle. The timing of sleep and wakefulness are synchronized and regulated by the natural conditions (day-night/summer-winter) in most animals but humans are able to override their internal biological clock cognitively. These shifts in sleep-wake cycle might cause many human physiological and mental diseases (21).

Severe imbalances in metabolic activities such as insulin and glucose metabolism were also observed in humans who experienced circadian misalignments (103, 104). Similar results were obtained in animal experiments and disorders in many tissues and organs such as diet-induced obesity, light-induced pro-inflammatory state, cardiac fibrosis and systolic dysfunction were observed due to disrupted intrinsic circadian clock (105-109). Metabolic diseases which are due to circadian clock disruptions are generally associated with defects in glucose tolerance and insulin resistance. Because these disruptions of circadian clock cause changes in glucocorticoids and melatonin levels, insulin secretion is affected (110).

Besides external timing cues, mutations in clock genes might also cause disorders and disease in both animals and human such as metabolic syndrome, hypertension and diabetes mellitus (111-113). Mutated *PER2* gene was reported as a reason of familial advanced sleep phase syndrome (114, 115). Besides, because some clock genes are in connection between the genes which are related to cell cycle such as *c-Myc*, *P53* and *P21*, clock genes are generally associated with the tumor development (116).

Disrupted circadian clocks are known to be associated with the formation and development of various diseases such as cancer (117-119), dysplasia (120-122)

cardiovascular disease (116), obesity (123), diabetes (124), sleep disorder (125), neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (126). Disorders of circadian clock are reported to be the causes of developmental dysfunctions in some organs such as brain and bone (127). Similar like organ-base effects of circadian clock disruptions, circadian clock is very important for mood-related behaviors. For instance, loss of REV-ERBα causes modulations in adult hippocampal neurogenesis and changes in mood-related behaviors (128). Depression is also very common among the population who has disrupted circadian rhythm (125). Also, it is known that circadian rhythm disruptions are associated with the emotional and psychotic attacks, and psychiatric disorders (125).

It is known that circadian rhythm is also involved in both male and female fertility and reproductive health (129-131). Thus, any effect can disrupt circadian rhythm is thought to affect reproductive health negatively and cause reproductive health related diseases (132). It is reported that environmental circadian disruptions such as rotating- or night shifting work which can be chronically or at some period during the employment can affect the reproductive function and these kind of circadian disruptions are known to be associated with the increased risk of endometriosis, increased latency to pregnancy, miscarriages and abnormal reproductive cycles in humans (133-137). It is also reported that because the environmental circadian disruptions affects the synchronization between circadian clocks in the ovary, pituitary gland, uterus, oviduct and mediobasal hypothalamus negatively, diseases like polycystic ovarian syndrome (PCOS), endometriosis or various malignant endocrine tumors which are related with the female infertility may be observed (138).

In order for the reproduction system to function properly, the hormones associated with the reproductive system must be regulated by circadian rhythm. For instance; luteinizing hormone (LH) surge, estrus cycle, and timing of insemination and fertilization are known to be controlled by the clock genes (130, 139). It is interesting to note that this interaction is not only one-way; expression of clock genes might also be affected by the hormones associated with the fertility (116, 140). Disruption of circadian clock with external stimuli, reproductive physiology can be affected and altered menstrual cycles, increased menstrual pain, altered follicular phase length, changes in the level of follicle stimulating hormone (FSH) secretion, low birth weights and greater incidence of spontaneous abortion are reported (141, 142).

2.2. Female Reproductive System

Female reproductive system is known as the well-developed system that is a series of interconnected organs (143). Female reproductive system is required for the production of eggs, support of the embryo which is developing and also for the female health. Besides the function of female reproductive system on female production, it is also known that it has an impact on functions associated with the other systems (non-reproductive systems) (143). Besides histological and anatomical literature on female reproductive system, with the improvements in molecular and genetic techniques, understanding of female reproductive system has expanded (144). With the integration of hypothalamus and pituitary, female reproductive system contains two essential components: uterus which is a main supporter for the developing fetus and the ovaries which is responsible for the production of female gametes (144).

2.2.1. Ovaries

Ovaries, the female gonads, are known as the source for the germ cells and the supplier of steroid sex hormones (145). Ovaries are found in the ovarian fossa located in the pelvis and between the uterus and pelvic sidewall. Ovaries are bound with the infundibulopelvic ligament and uteroovarian ligament to the pelvis and uterus. Their shapes are ellipsoid, and they are characterized by the presence of follicles (146). Follicles are found in the cortex of the ovaries and the medulla of the ovaries contains somatic cells (147). Cortex is referred to the outer region of the ovaries and the medulla refers to its inner region (148). Ovaries are also covered by the epithelium which is called as the ovarian surface epithelium or germinal epithelium (149). Besides germinal epithelium, ovary is also surrounded with the tunica albuginea known as the capsule of the connective tissue (148). Follicles found in ovaries are able to respond to the cyclic pituitary gonadotropin secretions and by this way, secretion of sex steroids and production of fertilizable ovum are maintained and regulated. Thus, steroidogenesis and biosynthesis of estrogen, progestin, androgens and proteins are some of the ovary functions (150). Monthly, ovary undergoes ovarian cycle which is known as the dynamic formation, growth and ovulation of ovarian follicles and also formation of corpora lutea (151-153).

2.2.2. Ovarian cycle

Ovarian cycle is known as the highly orchestrated and very complicated developmental process. During ovarian cycle, functional interactions and communication between oocyte, granulosa cells, theca cells and stromal cells are observed (154). Ovarian cycle leads the follicle development and oocyte maturation and eventually lead apoptosis or ovulation. At the end of the cycle, ovulation results whether with fertilization or not (154). Hypothalamic and pituitary functions are required for the regulation of ovarian cycle (Figure 2.5) (154). Then, ovarian cycle plays a role in regulation of oviduct, uterus and cervix functions and also it influences tissues and cells such as adipocytes and immune cells (155). Ovarian cycle is able to respond to the environmental external cues such as the length of the day (155). Ovarian cycle consists of two phases: follicular phase in which follicles grow, enlarge for the ovulation and luteal phase in which development of corpus luteum from ruptured follicle after ovulation (146).

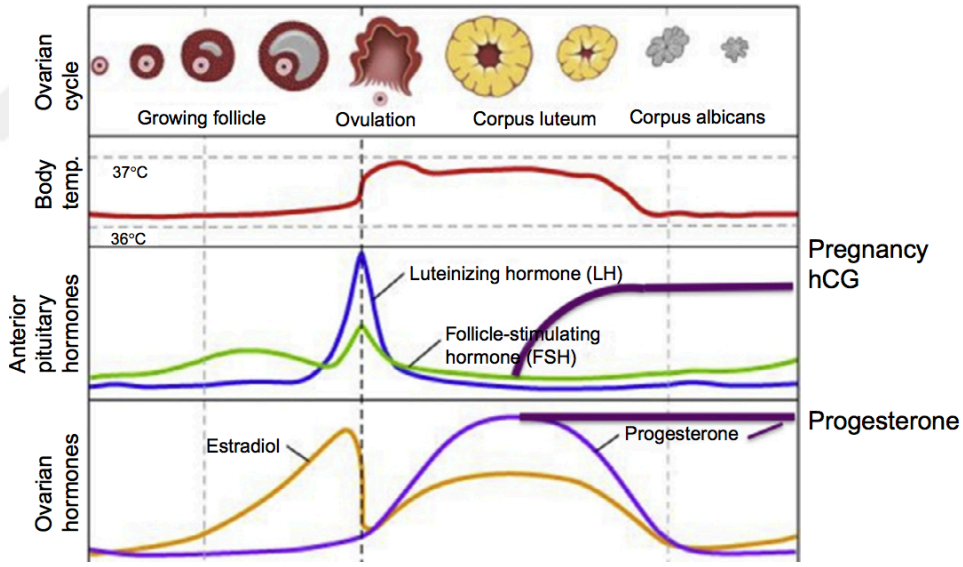


Figure 2.5. Scheme of ovarian cycle (154).

2.2.3. Ovarian folliculogenesis

Primordial follicles are characterized by the single layer of squamous somatic cells which are called as pre-granulosa cells and surrounds the oocytes (145). Oocytes found in primordial follicles are arrested in the prophase I of meiosis (156, 157). These follicles are quiescent and able to be activated to form the growing pool of follicles (Figure 2.6).

Primordial follicles are known as the ovarian reserve (158). After follicular growth, these follicles may go through either atresia or ovulation (159).

During follicular development, primordial follicles change into the primary follicles which are formed by surrounding a single layer of cuboidal shape granulosa cells and a core oocyte whose growth is also initiated (Figure 2.6) (160).

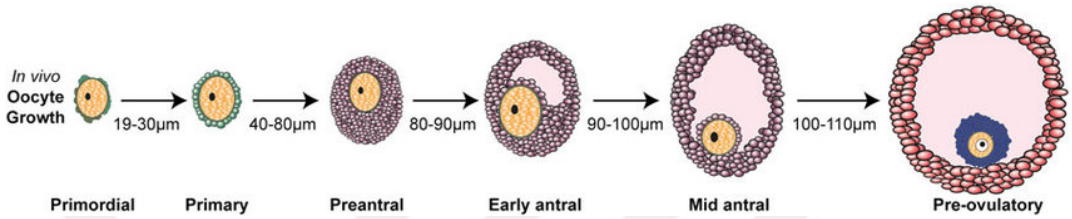


Figure 2.6. Illustration of folliculogenesis and oogenesis (161).

Primary follicles then continue to grow and form secondary follicles (Figure 2.6). Primary and secondary follicles develop in response to the Anti-Müllerian hormone which is a marker of the follicle reserve (158). Secondary follicles are characterized by the two or more layers of granulosa cells and oocyte which is in mid-growth stages. In secondary follicles, additional cell layers are also observed outer of the granulosa cells and they are called theca cells (162). Theca cells are separated from the granulosa cells with the basement membrane and encapsulate the secondary follicles. These cell layers are divided into two different layers called: theca interna and theca externa. Theca cells are required for the conversion of the estrogen which are secreted by the granulosa cells (145, 162). Theca cell layers are vascularized while the granulosa cell layers are remain as avascular (163). Oocyte found in follicle functions in regulation of the proliferation and differentiation of granulosa and theca cells (151, 164-166). Secondary follicles which are also referred as pre-antral follicles are known to be independent to the gonadotropins and regulated by the interaction between oocyte and the surrounding cells (145).

Development of follicles continues, and the antrum formation is observed in follicular structures (Figure 2.6). Antrum separates the granulosa cell layers into the mural granulosa cells (regulate steroidogenesis) and cumulus cells (found adjacent to the oocyte) (151). These antral follicles which are also known as Graafian follicle are regulated by the FSH and LH which are pituitary gonadotropins. Thus, under control of these hormones,

maturation and ovulation occur. When FSH is required for the granulosa cell proliferation and follicle survival, LH is required for the ovulation (145, 167).

At the end of the development and maturation of follicles, dominant follicle is selected for the ovulation which occurs in response to the LH surge (153). As a response to the LH surge, resumption of meiosis in the oocyte, rupture of follicle and the release of a cumulus-oocyte complex which has a fertilizable oocyte occur during ovulation. Resumption of meiosis and the continuation to the metaphase of the second meiotic division is known as the oocyte maturation. At metaphase II stage, meiotic division is arrested again. Thus, the ovulated follicle consists of metaphase II arrested oocyte (145). When the oocyte transferred from the ovary, the remaining granulosa and theca cells are reprogrammed and differentiated into luteinized structure called the corpus luteum (151, 153).

2.2.4. Relationship between female reproductive system and circadian rhythm

Similar like other systems, circadian clock is very important for the controlling and regulation mechanism of reproductive system (Figure 2.7). In mammalian reproduction, circadian rhythm is thought to influence the cycles, ovulation time, finding mate and sexual receptivity to maximize the reproductive success (168).

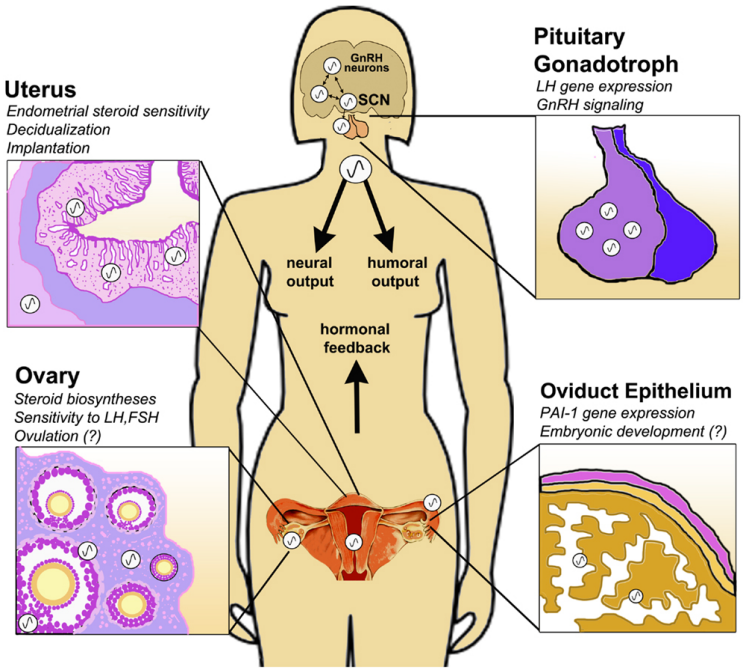


Figure 2.7. Relationship between circadian clock and female reproductive system (169).

2.2.4.1. Relationship between hypothalamo-pitutary-ovarian axis and circadian rhythm

Because the circadian rhythm is very important for the regulation of gene expression, hormone secretion, metabolism and behavior; this circadian timing system is also involved in endocrine and reproductive system and their-related organs (170-172). SCN was showed as the regulator of the hormone secretion via a complex interplay of neuroendocrine, endocrine and autonomic timing cues (173-176). Besides the brain which is the location of SCN, molecular oscillators are reported in also each endocrine tissue. It is also reported that autonomous clocks have the ability to maintain the timing of hormone synthesis and secretion (177-179).

Hypothalamo-pituitary-ovarian (HPO) axis might be an excellent example for the relationship between the endocrine system and circadian clock. For the HPO axis function, it is well-known that the coordination between the neural, neuroendocrine and endocrine oscillators is very essential (172). Synchronization between these oscillators are generated by the timing cues which are autonomic nervous cues and neuroendocrine releasing factors called hormones. Besides, circulating hormones can generate a feedback mechanism and by this way, they can modulate the phase and the amplitude of tissue sensitivity to neuroendocrine and endocrine cues (172).

Ovary has a sensitivity for the gonadotropins such as LH (Luteinizing Hormone) and FSH (Follicle stimulating Hormone) and dependent to the timing of ovary clock and timing cues which are generated from central and peripheral origins. During the daytime; photic cues are collected by SCN which is a central pacemarker (172). In SCN, these cues are transferred to the pineal and pituitary by the activity of the hormone neurons released in the basal hypothalamus and forebrain. Via autonomic nervous cues, SCN can also regulate the timing of the ovarian clock (172). FSH and LH are associated with the production of estrogen by ovarian follicles in females and the 24h rhythmicity of FSH in both during follicular and luteal phase was reported. Similar to FSH it is known that LH has also 24h circadian rhythmicity during follicular phase but not during luteal phase (180).

Ovarian steroids generate a feedback mechanism and by this way they can also modulate the expression of clock genes in other tissues such as uterus, pituitary and release of gonadotropins (172). For instance, it is known that estrogen can modulate the

expressions of *Per1* and *Per2* genes (181, 182). Besides, they can also show rhythmicity and can be involved in development and maintenance of secondary sexual characteristics (180, 183). 24h circadian rhythmicity of estrogen was reported during follicular phase but there was no evidence of the rhythmicity of estrogen during luteal phase (180). Androgens are also thought to have 24 rhythmicity with a peak in the early morning (184). Androgens are also known as the hormones which have tissue-specific effects on *Per2* expression (185). Melatonin derived from the pineal gland is known as a circadian clock hormone and a powerful free radical scavenger. It protects the oocyte from oxidative stress, especially during the ovulation (186). Melatonin rhythm which is found in both cerebrospinal fluid and blood lead strengthen the circadian message that the SCN communicates to other organs via the autonomic nervous system (92, 186, 187). In ovarian follicle, melatonin receptors are expressed, and melatonin can modulate the ovarian clock indirectly (172). Melatonin is thought to be crucial for development of fetal circadian clock, for protection of the embryo/fetus from metabolic stress and for the growth, development, senescence and function of the cells in female reproductive system (188, 189). Its 24 circadian rhythmicity with a peak in the night was reported (189). It is known that melatonin can be regulated by the dark-light and seasonal cycles and its levels are lower in women with idiopathic infertility (190).

Adrenal glucocorticoids such as corticosterone may also synchronize the molecular clock of ovary (172). They are known as synchronizers of endogenous clocks (191, 192). Their 24h circadian rhythm with a peak in morning was reported (193). Glucocorticoids are thought to be associated with the regulation and control of fetal growth and development. Glucocorticoids are also known to affect the ovarian function indirectly by altering the levels of gonadotropins and metabolic hormones or growth factors which are circulating (194).

2.2.4.2. Relationship between ovary and circadian rhythm

In the female reproductive system, the rhythmicity is observed and this rhythmicity is very tightly entrained to the light:dark cycle. The relationship between the ovarian rhythmicity and light:dark cycle is mediated by the photoperiodic cues which are originated in the SCN (35, 195). In ovary, clock function is generally associated with the steroidogenesis, folliculogenesis, cellular differentiation, responsiveness to gonadotropins

and ovulation (172). Circulating LH and FSH levels oscillate in mammals, and this oscillation occurs with the diurnal rhythm marked by significant afternoon “surges” on the day of ovulation (168, 196-198). In rodents; during pro-estrous LH surge occurs in the late afternoon/early evening (196, 199). Both in mammals and rodents; it is obvious that the afternoon LH surge is thought to be crucial for ovulation, luteinization and also formation of corpus luteum(198, 200). In other words; it can be said that the ovulation time depends on LH surge and is limited to the window on the afternoon of proestrus (176, 198, 200). Thus, to be effective; LH surge must arrive at the appropriate time of day. The ovulatory responsiveness to LH surge is thought to be persisted in constant darkness and it is suggested as timing system (201). This shows us that the ovary has a circadian oscillator which shows rhythmic expression of key players in the ovulatory response pathway (172).

Besides the timing of ovary which is dependent to rhythmic sensitivity of the ovary to gonadotropins; core clock gene expressions were also reported in the ovary and in isolated granulosa cells in both mice and rats (8, 202-207). Similar to granulosa cells, expressions of clock genes are also observed in oocytes and preimplantation embryos from mice, rabbits and cattle (208-211). These core clock genes which are found in the ovary is thought to be associated with the follicular development (8, 203). It is reported that knockdown of *Clock* expression in the ovary caused reduction in litter size and oocyte release (212). Besides; cyclic expressions of *Bmal1* and *Per2* mRNA are known to be induced by gonadotropin exposure in the ovaries of hypophysectomized prepubertal rats (203). Also, the diurnal rhythms of *Per1* and *Per2* expressions were also reported that persistent across the 4- to 5-d estrus cycle in rats (8). In large preantral follicles, small antral follicles, Graafian follicles and corpora lutea; expressions of clock genes were also reported (172). Thus, ovary can regulate the ovulation time by modulating the gonadotropin receptor expressions and the timing of steroid and peptide hormone secretion (6).

2.2.4.3. Relationship between ovarian cells and circadian rhythm

The rhythms of clock gene expression are thought to be limited to the mature granulosa cells and luteal cells (206, 213). Actually, it is reported that there is no expression of clock genes in primordial and pre-antral follicles but when follicle reach the late antral or Graafian stage, clock genes start to be expressed in both granulosa and theca

cells (8, 203, 214). It is reported that cultured granulosa cells have cell-autonomous persistence of the clock (202). Also, it is demonstrated that the genes which are found in granulosa cells and associated with the follicular development such as LH receptor, prostaglandin synthetic enzymes, steroidogenic enzymes, *Cyp11a1*, aromatase, *Connexin-43* are also clock controlled genes (205, 206, 215-217). It is also reported that the gene expressions in the granulosa cells of preovulatory follicle are changed by the LH surge (218, 219). It is also known that for the successful rupture of follicles which dependent to the prostanoid signaling is regulated by the transcriptional control of circadian clock because the regulation of the expression of cyclo-oygenase-2 (*Cox2*), rate-limiting enzyme for prostaglandin synthesis, by BMAL1/CLOCK complex was reported (220).

Besides, similar to COX2 regulation, it has been reported that LH receptor gene expression is regulated by BMAL1/CLOCK directly (216). Similar to *Bmal1* and *Clock* genes, *Per2* expression is also very important for the function of granulosa cells. It has been reported that when the intercellular communication was disrupted by blockers of the gap junctions reduced the amplitude and lengthened the period of the *Per2* expression in rat granulosa cells (205).

Even the rhythmic expressions of clock genes are reported in the ovarian theca cells were reported, clock-control program of cell-type-specific gene expression in theca and stromal cells is still unclear because the isolation of purified population of these cells is very difficult (8, 203, 214). Even so, conditional deletion of *Bmal1* in theca cells but not in granulosa cells caused severe attenuation of the daily rhythm of ovarian sensitivity to LH. Thus, the clock in the theca cells can be counted as one of the regulators of the timing of ovulation (221).

2.2.4.4. Relationship between oocyte and circadian rhythm

The expressions of clock genes were reported in mouse, rabbit and bovine oocytes and in mice; it is reported that the mRNA of clock gene fluctuates with the function of developmental stage (208-211). These rhythmic oscillations are thought to be a regulator of normal oocyte maturation (172). It was reported that the knockdown of *Cry1* mRNA expression in oocytes caused the slight inhibition of meiosis in murine oocytes (208). This situation indicates that *Cry1* mRNA expression is very important for the oocyte maturation. Similar to CRY, BMAL is also thought to be effective in oocyte maturation.

Knockout of *Bmal1* in steroidogenic cells caused the failure of implantation due to abnormal progesterone secretion. This suggest that ovarian clock plays a role in female fertility (222).

2.2.5. Relationship between female reproduction diseases and circadian rhythm

Research on genetic models of clock genes and female fertility showed the importance of circadian rhythm on fertility and the relationship between the female reproduction disease and circadian rhythm (Figure 2.8). Irregular or acyclic estrus cycles and decreased reproductive rates were observed in mice which are *Per1* and *Per2* knockout (223, 224). Besides, *Per1* and *Per2* double knockout mice showed premature depletion of the ovarian follicular reserve which is related to the decreased reproductive capacity and lead to premature ovarian insufficiency (225). Thus; disrupted *Per1* and *Per2* genes are thought to be related with the decrease of ovarian follicles in especially aged mice (225).

It is also reported that the *Clock* mutant mice have higher proportions of irregular estrus cycles even normal timing of vaginal opening observed (226-228). Another study with the transgenic mouse model of *Clock* gene which can generate BMAL1/CLOCK dimer but cannot regulate PER and CRY, showed loss of circadian rhythmicity and high rate of pregnancy failure (226, 229).

Similar to *Clock* mutant mice, *Bmal1* null mice also showed irregular estrus cycles (230). Besides irregular estrus cycles, *Bmal1* knockout mice showed delayed puberty and smaller ovaries and uterus (227, 230, 231). It is also known that absence of *Bmal1* is associated with the impaired progesterone levels and implantation failures (222, 230). It is also noted that absence of *Bmal1* can be associated with the reduced estrogen levels in granulose cells (232).

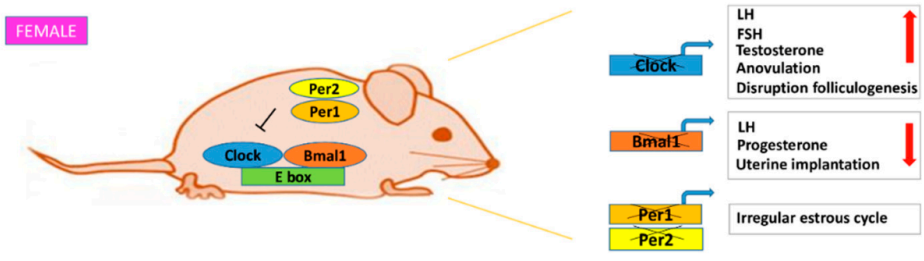


Figure 2.8. Disrupted clock genes and their effects on female reproductive system (129).

Disruption of circadian clock is also thought to be associated with the accelerated reproductive aging. It is reported that more than 80% of the *Per1* and *Per 2* mutants had lower levels of successful parturition and the numbers of pups even they became pregnant. Also, it is reported that *Per1* and *Per2* deficiency caused advanced aging (223).

Besides clock genes, hormones that have 24h rhythmicity can also affect the reproductive health and can be related with the reproductive diseases. For instance, environmental circadian disruptions might affect the ovulation through disrupts the melatonin production and secretion (186). In stress-induced women, it is known that melatonin is suppressed, and the circadian rhythm is altered. This situation affects the fertility in women and development of fetus. It is also known that in this situation, the risk of miscarriage, premature birth and low birth weight might be observed (233). It is also reported that the levels of melatonin might affect the in vitro fertilization outcomes and embryo quality (234). High level of melatonin is thought to cause infertility (235). Like melatonin, glucocorticoids might cause some diseases related with the female reproductive system. Glucocorticoids are thought to lead the disruption of ovarian cyclicity (194), and inhibit the synthesis or release of LH and FSH (236). Treatments with glucocorticoids might cause alterations in steroidogenesis and histological impairment (237). Besides, reduced total number of germ cells and ovarian volume were also observed (238). Glucocorticoids can also induce apoptosis of oocytes (239). Androgens might also affect female reproductive system. For instance, high level production of androgens is thought to be associated with the female hyperandrogenemia which is related with the PCOS by affecting *Clock* gene in ovarian rat follicles (240). Circadian rhythm and its regulators are also thought to have effects on pregnancy. For instance, there are some evidence that circadian rhythms might contribute to birth timing and disruptions of circadian rhythm might contribute to preterm birth (241). For instance, pregnant mice which are lack of clock gene failed to enter labor or showed prolonged labor and non-productive contractions (228). Disrupted circadian rhythm is also thought to cause increase the risk of hypertensive pregnancy complications due blood pressure reflects endogenous internal rhythms (242, 243).

2.3. Mammalian Target Of Rapamycin (mTOR) Pathway

mTOR is known as the target of a molecule called as rapamycin which is very important because of its various antiproliferative features (244). In 90s, TOR1 and TOR2 were demonstrated as regulators of the toxic effects of rapamycin in budding yeast with the genetic screening techniques (245, 246). Then, in mammals, mTOR was also purified and discovered. Thus, it became the physical target of the rapamycin (247-249). mTOR is known as atypical serine/threonine protein kinase and it is a member of phosphoinositide 3-kinase (PI3K)-related kinase family. mTOR is also evolutionarily conserved (10). mTOR communicate generally with the other various proteins and it generates two different complexes. These complexes are known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These complexes are differed from each other by their sensitivity to the rapamycin and their upstream/downstream factors (244). Both mTORC1 and mTORC2 is very large complexes. MTORC1 consists of 6 protein components while mTORC2 has 7. They share mammalian lethal with sec-13 protein 8 which is known as mLST8 or GβL, DEP domain containing mTOR-interacting protein (DEPTOR), Tti1/Tel2 complex and the catalytic mTOR subunit (250-253). However, regulatory-associated protein of mammalian target of rapamycin (raptor) and proline-rich Akt substrate 40kDA (PRAS40) are found only in mTORC1 which is a sensitive one to rapamycin (254-258). On the other hand, rapamycin insensitive companion of mTOR (Rictor) are found in mTORC2 (250, 259). Thus, rapamycin directly interacts with the mTOR in mTORC1 and inhibits it, but rapamycin cannot interact with the mTORC2 and affect it (244). These two different complexes are also regulated with different growth factors and nutrients. When mTORC1 is more nutrient sensitive multiprotein complex, mTORC2 is growth-factor sensitive and nutrient insensitive (250, 254, 255, 259).

mTOR is known to control cell growth, autophagy, metabolism, mRNA translation and ribosome biogenesis (260-262). mTOR controls also cell proliferation, cell maturation and maintains cell survival (10). It is also known that mTOR is one of the key modulators of aging and age-related diseases (263). Inputs regulating mTORC1 are known as growth factors, amino acids, stress, energy status and oxygen (244). These inputs might activate mTORC1 and mTORC1 can stimulate some metabolic events such as the protein synthesis, lipogenesis and energy metabolism (244, 264, 265). On the other hand, mTORC1 has an ability to inhibit autophagy and lysosome biogenesis (244, 266). Growth

factors might also activate mTORC2 and mTORC2 controls the cytoskeletal organization and cell survival also some metabolic activities (250, 259).

2.3.1. Molecular mechanism of mTORC1

Comparing to mTORC2, mTORC1 is more well-understood complex of the mTOR signaling. There are various upstream and downstream regulators of mTORC1 (Figure 2.9) (244). The inputs of mTORC1 can be classified simply as at least five major cellular and extracellular factors: stress, growth factors, oxygen, energy and amino acids. Thus, mTORC1 can regulate lipid or protein synthesis and autophagy (244). As most major upstream factors of mTORC1, tuberous sclerosis 1 (TSC1) and TSC2 are known. These factors are function as a GTPase-activating protein for the Ras homolog enriched in brain (Rheb) GTPase. Rheb which is bound with GTP, communicates with mTORC1 and stimulates its activity. TSC1/2 inactivate Rheb and regulates mTORC1 negatively (267, 268). TSC1/2 can regulate many upstream signals of mTORC1 such as insulin and insulin-like growth factor 1 (IGF1) (244). TSC1/2 can be phosphorylated and inactivated by protein kinase B (Akt/PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2) and ribosomal S6 kinase (RSK1). Thus, they can activate mTORC1 (269-273). Independent to TSC1/2, Akt may also affect the mTORC1 and cause dissociation from raptor of PRAS40 which is a mTOR inhibitor (256-258).

Besides, tumor necrosis factor-α (TNFα) may activate mTORC1 by inhibiting TSC1/2 (274). Low energy or oxygen levels and DNA damage also affect mTOR pathway activity. Low energy causes phosphorylation of TSC1/2 by monophosphate-activated protein kinase (AMPK) (267). AMPK may also interact with mTORC1 directly. It phosphorylates raptor and cause inhibition of mTORC1 (275). DNA damage stimulates Tsc2 expression and causes downregulation of PI3K-mTORC1 axis (276, 277). mTORC1 can also be activated by amino acids (278, 279).

As a downstream factors or outputs, mTORC1 controls protein synthesis by phosphorylating eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1) (280). mTORC1 communicates also with sterol regulatory element binding protein 1/2 (SREBP1/2) and through this protein, it can regulate fatty acids and cholesterol synthesis (281-283). Hypoxia inducible factor 1α (HIFα) is also known as the downstream factor of mTORC1. mTORC1, through HIFα, increases

glycolytic flux (281, 284, 285). mTORC1 causes inhibition of autophagy by suppressing the ULK1/Atg13/FIP200 which is an autophagy gene. Thus, stimulates the cell growth (266, 286, 287). Besides autophagy, biogenesis of lysosomes is also negatively regulated by mTORC1. Through transcription factor EB, mTORC1 inhibits the lysosome biogenesis (288).

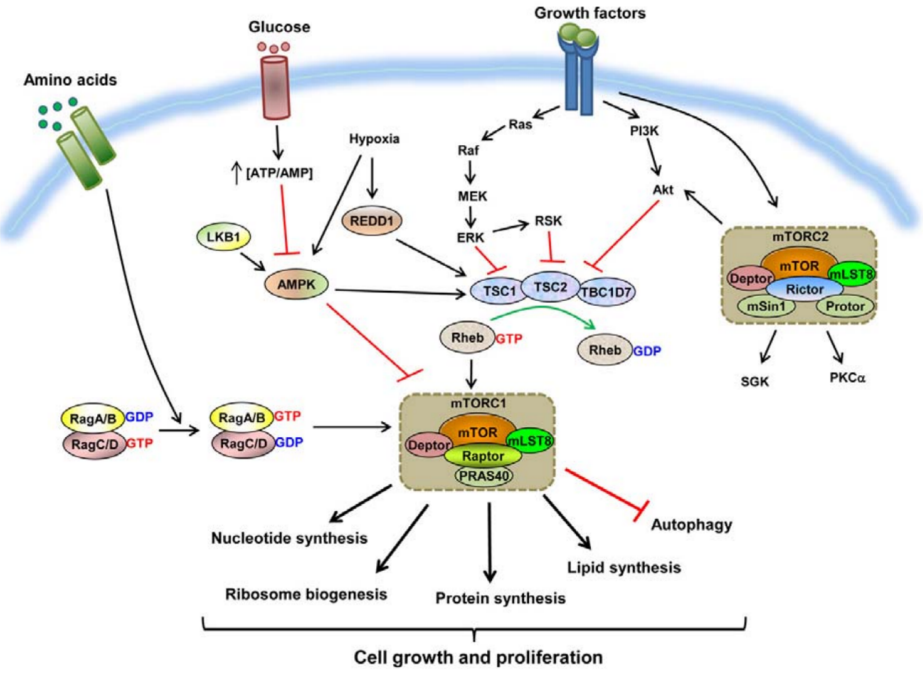


Figure 2.9. mTOR signaling pathway (289).

2.3.2. Relationship between mTOR and circadian rhythm

It is known that circadian rhythm regulates various cellular and metabolic events in tissues and organisms (86). These events can be associated with the homeostasis, glucose or lipid metabolism (18, 88). Control of appetite and energy metabolism is also provided by the circadian clock (20, 90-92). Circadian clock regulates also adipocyte differentiation, cholesterol synthesis and degradation (28).

Also, mTOR signaling pathway has different roles in similar cellular and metabolic events such as the protein synthesis, lipogenesis and energy metabolism (244, 264, 265). Thus, it would be not surprising that these two important regulators can be associated with each other.

2.3.2.1. mTOR regulation on the circadian rhythm

The multifaced role of mTOR in the circadian clock was demonstrated (50). mTOR has sensitivity to various environmental inputs such as nutrients and growth factors and this sensitivity helps mTOR to regulate cell processes such as growth, metabolism, differentiation and autophagy (290). mTOR signaling is thought to be a part of the photic entrainment pathway in SCN (50). mTOR is also known as that it can regulate autonomous clock characteristics in a variety of circadian oscillators. It is also demonstrated that mTOR has a regulative effect on SCN neurons (50).

Light at night is thought to activates mTORC1 and by this way, S6K1 is activated. Activated S6K1 phosphorylates S6 and this is associated with the translation of various mRNAs. Thus, the translational regulation can be organized through light and mTOR (291). This light-induced activation of mTORC1 is known as the important regulator of the photic entrainment of the SCN clock (292). Besides, the effect of the rapamycin, which is a mTOR inhibitor, on behavioral phase shift was demonstrated by its inhibition of light induced PER1 and PER2 proteins in SCN (50).

The relationship between the SCN synchrony and mTORC1/4E-BP1 pathway was also demonstrated. It is known that rhythmic mTORC1 signaling phosphorylates and inhibits 4E-BP1 and by this way, mRNA translation of Vip is promoted (293-295). Thus, the level of VIP which regulates the synchrony of SCN cells and the clock gene oscillation in SCN increases (50).

It is reported that downregulation of PI3K and mTOR cause alterations in circadian period (295). Inhibition of mTOR leads increase in period and decreases the amplitude in hepatocytes and adipocytes (295). For instance, constitutive activation of mTOR in Tsc-/- fibroblasts causes alteration in clock gene oscillations and elevation in the levels of CRY1, BMAL1 and CLOCK (295, 296). Heterozygous mTOR knout mice also showed long circadian periods (294).

2.3.2.2. Circadian rhythm regulation on mTOR

It is demonstrated that mTORC1 activities show robust circadian oscillations in the SCN under constant conditions (294, 297). For instance, in brain, it is reported that mTORC1 activities has daily oscillations in hippocampus and frontal cortex which are the important regions for circadian rhythms, feeding, learning, memory and emotions (298-

300). Also, mTOR rhythmicity was observed in the liver, cardiac and skeletal muscles and adipocytes (298, 301-304). The role of mTOR signaling as an output pathway which links circadian rhythm to mRNA translation was also reported. They found that the temporal translation of mRNAs which are involved in ribosome biogenesis is affected by the circadian clock (302). The transcriptional feedback loop in the circadian clock is thought to be coupled to a translational regulatory loop which is mediated by the mTOR pathway (304). It is demonstrated that BMAL1 regulates as a translation factor and is associated with the protein synthesis which is regulated also by mTOR. It is reported that BMAL1 is controlled by the rhythmic phosphorylation at Ser42 by mTORC1/SbK1 pathway which is very important for the protein synthesis. Thus, it is thought that mTORC1/S6K1 pathway lead the communication between circadian clock and rhythmic translation via BMAL1 (304).

2.3.3. Relationship between mTOR and female reproductive system

It is demonstrated that mTOR regulation is very important for folliculogenesis (14), oocyte meiotic maturation (305), ovarian somatic cell proliferation and steroidogenesis (306), ovarian aging (15) and embryonic development (15) (Figure 2.10).

It was reported that when mice are lack in the *Tsc2* gene in their oocyte, activated pool of primordial follicles were observed because of the increase in mTOR activity in oocytes. Thus, this situation caused depletion of follicles in early adulthood and premature ovarian failure features. These results suggested that suppressed mTOR activity which is mediated by TSC complexes is important for dormancy of primordial follicles and preservation of the follicular pool (307). It was also demonstrated that conditional knock out of mTOR in primordial or growing oocytes led infertility and influenced oocyte quality, granulosa cell fate besides follicular development. In primordial follicles, knock out of mTOR caused progressive degeneration of oocytes and loss of granulosa cell identity. mTOR deletion also caused DNA damage in oocytes and alteration in transcriptomes thus affected the oocyte quality negatively (305). When TSC1 was homozygous deleted in reproductive tract somatic tissues in mice, complete infertility was reported because of the pleiotropic effects on follicle recruitment (308). Besides oocyte related role of mTOR in folliculogenesis, role of mTOR in oocyte maturation was also demonstrated. It was reported that rapamycin causes inhibition of spindle migration and

asymmetric division in mouse. Thus, it can be said that oocyte maturation is regulated by the mTOR-mediated signaling pathways (309). mTOR which is associated with the eIF4F pathway is also thought to be important for the translation of various transcripts which have roles in normal spindle assembly, chromosome alignment and segregation (310). When rapamycin which is an inhibitor of mTOR was injected intraperitoneally into the rats, it was observed that the ovarian lifespan of rats lengthened due to inhibition of transition of primordial follicles to the developing follicles and preservation of the follicle pool. The number of primordial follicles in the rapamycin-treated group was also observed as twice the control group (15). The effect of mTOR found in granulosa cells in ovulation and corpora lutea was also reported. Disruption of *Tsc1* in mice ovarian granulosa cells is thought to promote ovulation and accumulation of corpora lutea in mice through mTOR signaling pathway (311).

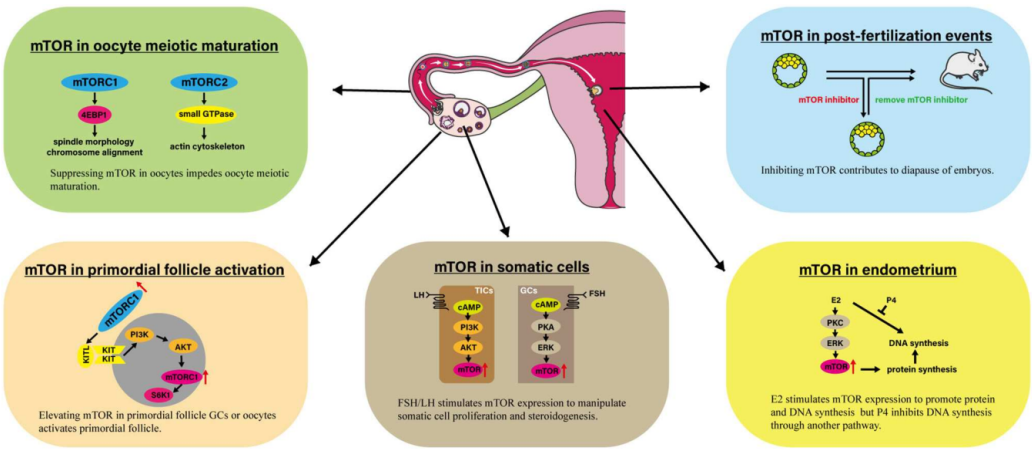


Figure 2.10. Relationship between mTOR and female reproductive system (312).

mTOR regulation on granulosa cell proliferation was also reported. Inhibition of mTOR with rapamycin was associated with the reduced FSH-mediated increase in cyclin D2 mRNA expression. Also, the role of mTOR in FSH-mediated increase in granulosa cell proliferation was also reported in rats (313). Besides mice and rats, the role of mTORC1 signaling was observed also on human granulosa lutein cells. It is reported that human chorionic gonadotropin treatment caused increase in the expression of downstream factors of mTOR signaling and also cholesterol side chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase type 1 and steroidogenic acute regulatory protein messenger mRNAs.

These increases were reported to be inhibited by rapamycin pretreatment. These results show the role of mTOR in steroidogenesis in human granulosa lutein cells (314). The role of mTOR on the communication between the somatic cells, microenvironment and germ cells for follicular activation and proper reproductive lifespan in mammals was also demonstrated. It is reported that microenvironment which surrounds the primordial follicles may activate the mTORC1-KITL signaling in granulosa cells of primordial follicles and lead the awakening of dormant oocytes for the follicular activation in mice (315). LH/hCG treatment was demonstrated to increase the expression of downstream factors of mTORC1, and mTORC1 inhibitors such as rapamycin can block the LH/hCG-regulated stimulation of the steroidogenic enzyme mRNAs. It is also demonstrated that knockdown of mTOR caused the increase in the expression of steroidogenic enzymes. Taken together, it is reported that the role of mTORC1 was demonstrated in androgen biosynthesis by theca-interstitial cells (315).

2.3.4. Relationship between mTOR and female reproduction diseases

mTOR is known as an inhibitor of apoptosis and promoter of cell cycle progression (10). Besides, it is also known that it has various roles in cell survival, proliferation and angiogenesis thus in invasion and metastasis of tumor cells (316-318). The roles of mTOR in diseases which are related with the female reproduction such as ovarian neoplasm, polycystic ovary syndrome, premature ovarian failure and ovarian aging were reported (10).

It is reported that TSC/mTOR signaling is very important for the ovarian function such as quiescence, activation and survival of primordial follicles, granulosa cell proliferation, differentiation and meiotic maturation of oocytes (13). Altered follicular development and anomalies of ovulation were showed in ovaries with deregulated mTOR signaling pathway (319-321). It is reported that PI3K/AKT/mTOR signaling is involved in increased invasion of ovarian cancer cells (267, 322). It is also demonstrated that activated mTOR signaling is found in about half of all high grade serous ovarian cancer patients (323, 324). mTOR is thought to regulate mRNA translation and protein synthesis which is involved in tumor cell growth, angiogenesis and survival (325).

The relationship between mTOR and PCOS was also reported. Insulin resistance which is the one of the characteristics of PCOS is thought to be associated with the

overexpression of mTOR signaling (10, 326). In DHEA-induced PCOS mouse model, it is reported that the expression of mTOR and phsopho-mTOR is high (327). Another study showed that expression of mTOR protein decreased in luteal granulosa cells of PCOS patients (328). Also, rapamycin which is a mTOR inhibitor, is thought to be used to eliminate metabolic syndrome with PCOS because it cause enhanced insulin sensitivity (329).

Premature ovarian failure is known as decreased follicle number in ovary before expected age of menopause (330). It is suggested that inhibition of mTORC1 plays role in preservation of ovarian function and fertility (331). Similarly, mTOR inhibitor, rapamycin, is suggested to prevent primordial follicle activation via PI3K/AKT/mTOR signaling pathway. Therefore, it is reported that mTOR inhibition can be used as a treatment for protection of ovarian reserve and prevention of POF which is characterized by diminished primordial follicle pool and uncontrolled primordial follicle activation (332). Similar to POF results, research on relationship between mTOR and ovarian aging suggested that inhibition of mTOR signaling by rapamycin may lead controlled reservation of follicle pool and prolongs the ovarian lifespan in rats and mice (15, 333).

2.3.4.1. Relationship between mTOR and ovarian/oocyte aging

It is known that female age is very important determining factor for the female reproduction (334). During normal procedure; follicular loss occurs because of the ovulation but the major loss of follicles is occurred because of the atresia before ovulation (335). Increasing chronological age, female reproductivity which refers to the production of offspring decreases (336). The reproductive aging is associated with the decrease in quantity and the quality of the oocyte located in the follicles which are found in the cortex of the ovary (337). Thus, physiological aging is generally resulted with the primordial follicle loss (338). It is known that increased mTOR signaling in granulosa cells of primordial follicle or oocytes causes activation of primordial follicle (312). Thus, primordial follicle reserve might decrease rapidly because of the elevated activation of primordial follicle. It is also reported that inhibition of mTOR signaling lead longer ovarian lifespan. For instance, increase in number of primordial follicles was observed in rapamycin treated rats compared to the control rats (15, 339). Another study which showed the protective effect of rapamycin on primordial follicle reserve demonstrated that 2-weeks

regimen of rapamycin led the sufficient extension of ovarian lifespan independent from the age at treatment initiation (340).

The loss of oocyte quality is thought to be caused by the increase in meiotic nondisjunction which leads aneuploidy in the early embryo at higher female ages (341-344).

Decrease in both number and quality of follicles is observed during the third and fourth decade of life and the process behind the ovarian aging remains unnoticed (336). Conditional knockout of mTOR in primordial follicle and growing oocytes is thought to be associated with the infertility. This knockout affected the oocyte quality, granulosa cell fate and follicular development. Knockout of mTOR in primordial oocytes showed degeneration of oocytes and defective follicular development (305).

Ovarian aging has some clinical signs. Shortening of the menstrual cycle length can be counted as the important clinical sign of the ovarian aging (345). During ovarian aging, irregular menstrual cycles and reduced follicle numbers are observed, and they can be one of the signs of ovarian aging. Generally, follicles have insufficient availability and this situation results to lengthened cycles or missed periods. This stage is thought to be menopausal transition (346). Similar to loss in quantity of follicles and oocytes, some endocrine abnormalities such as changes in Inhibin B and AMH levels are also observed during ovarian aging (336). AMH is secreted by granulosa cells of antral and preantral follicles and it can be the marker for the size of the primordial follicle pool (347, 348). Because the number of primordial follicles is associated with the number of antral follicles at all ages, decline in primordial follicle number is generally observed with the decline in size of antral follicles (349). It is speculated that elevated mTOR signaling results increased follicular growth but many of these are thought to be atretic due to insufficient mTOR in large antral follicles (308).

Oocyte quality is also affected because of the ovarian aging. It is known that fresh matured oocytes are interact with the first polar body and the thick zona pellucida which formed by glycoproteins (350). Organelles, microfilaments and MII spindles which are found in fresh oocyte are regularly distributed (351-353). However, during oocyte aging, morphological alterations start to be observed in polar body, zona pellucida, cytoskeleton, mitochondria, spindle organization and cortical granules (350). Oocyte quality is decreased because of the decreased quality of oocyte cytoplasm followed by the increased genome

abnormalities (354, 355). Age-related changes in the meiotic spindle formation and the chromosome alignment result in oocyte embryo aneuploidy. Normally, spindles are oriented vertically to the oocyte membrane and they are associated with each pole of oocyte. However, in aged oocyte, spindles became elongated and smaller (351, 356-359). mTOR signaling is also reported during oocyte meiotic maturation. mTOR is reported in the cytoplasm at the GV stage and on the spindle during MI-MII stages in mice.

The localization of mTOR is thought to change with time (360). From germinal vesicle (GV) to metaphase I (MI), mTOR mRNA was reported to be expressed and increased during metaphase II (MII) (309). Chromosomes are also affected by the oocyte aging. When symmetrically arranged chromosomes are observed in fresh oocytes, chromosomes start to migrate centripetal and decondensed in aged oocytes (351, 361-365). Also, in aged ovaries, mitochondrial DNA of oocytes become instable and mitochondrial abnormalities are observed (355). When the mitochondria are intact in fresh oocytes, mitochondria matrix start to swell because of decreased membrane potential in aged oocytes (366). It is reported that mTOR activation by GAS6 depletion, MII oocytes exhibited mitochondrial accumulation and aggregation due to mitophagy inhibition (367).

Zona pellucida hardening is one of the other alterations which is observed in oocyte with age. Granulofibrillar zona pellucida which is interconnected with pores is observed in fresh oocytes but in aged oocytes, zona pellucida becomes harden and get cobblestone appearance (368-371). Affected perivitelline space is also associated with the oocyte aging. When perivitelline space is small in fresh oocytes, oocyte aging cause perivitelline space to become large (371). In fresh oocytes, cortical granules are found in a line beneath the oocyte membrane and they are also altered with the age. Cortical granules undergo migration and partial exocytosis can be observed in aged oocyte (369, 370, 372, 373). Rapamycin treatment was reported to disrupt the formation of the cortical granule-free domain (309). With age, telomere shortening, and dysfunctions cause also decrease in oocyte quality (374). mTOR is known as one of the regulators of telomerase activity at both translational and post-translational level. Rapamycin which inhibits the mTOR was reported to decrease telomerase activity (375). Besides, reactive oxygen species (ROS) damages are often in oocytes whose quality decreased because of the age and ROS levels can be used as a marker for aged oocytes (334).

In aged oocytes, an increase in the amount of the ROS is generally observed (350). All these alterations which occur because of the oocyte aging and affect the oocyte quality

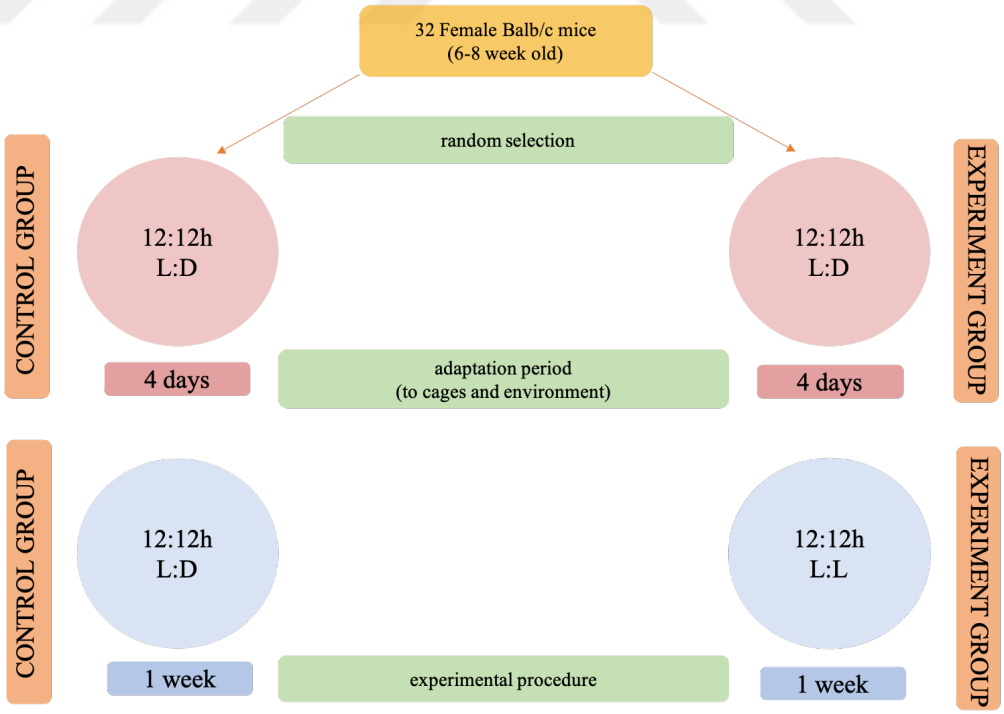
influence the fertility. It is known that oocyte aging is associated with the decreased fertilization rates (376, 377). Thus, because of these problems related with the oocyte aging, assisted reproductive techniques may be needed (350).

3. MATERIALS AND METHODS

3.1. Animals and Experiment Design

A total of 32 female 6-8-week-old Balb/c mice which enter estrous cycle were used in the study (Table 1). Animals have not been mated and used in any study before. Mice were fed with normal food and water. Food and water were available ad libitum. The temperature was maintained at 21-23°C. Mice were randomly assigned to one of two groups as 12:12h L:D and 12:12h L:L (Table 1) Before the experiment started, the 12:12h L:L (experimental group) mice spent 4 days in the experiment room with normal lightening conditions so that the mice could get used to the ambient conditions. During the experiment, 12:12h L:D (control group) was housed in a 12:12h light:dark cycle and 12:12h L:L (experiment group) was housed in a constant light conditions 12:12h light:light for 1 week (Table 1).

Table 3.1. Experimental design of the study



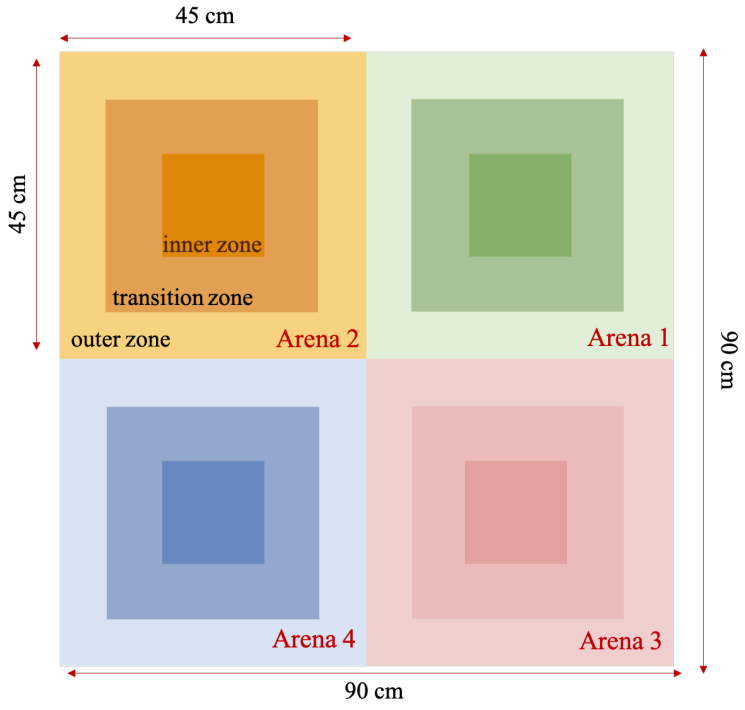
3.2. Body Weight and Food Intake

All animals were weighed at the arrival and at the end of the experiment. Food ingestion for a 12h cycle was monitored at 7.00 am and 19.00 pm for each day during experiment (1 week) by weighing the initial and the remaining food in the bin for a 12 cycle.

3.3. Open Field Test

Animals were tested in the open field test (OFT) after 1 week-experiment. OFT was performed using a black acrylic square arena (90 cm wide x 90 cm long, divided in 4 equal squares), illuminated with 100lux bulb located above the field (Table 2). The camera was also located above the field. The room temperature was 21°C. All mice were introduced in the OFT facing to square wall, by leaving observer out of view of mice. All mice were left in the OFT for 10 min. After each test, the field was cleaned with a clean cloth followed by 96% ethanol solution. OFT was performed for both groups at the same day, during the light time period of 12:12h L:D group. From the videotape recording, the following analysis were done: the number of fecal boli, the time spent in inner square, velocity and total distance of mice.

Table 3.2. The open field test set-up



3.4. Vaginal Smear

Vaginal smear was performed for monitoring estrous cycles after OFT right before cervical dislocation of mice. During vaginal smear application; Pasteur pipette was used to wash the vagina of the animal physiologically with saline then spread it on a clean slide. After covering the slide with the coverslip, smear examples were observed under light microscope. The vaginal smears which were characterized by the predominance of nucleated vaginal epithelial cells and very few leukocytes were counted as proestrus (378). When the vaginal smears characterized by large, nonnucleated cornified squamous epithelial cells were counted as estrus, the smears contained a mix of cell types were counted as metestrus (378). Diestrus was defined when the vaginal smear examples contained leukocytes predominantly (378).

3.5. Tissue Processing and Morphological Analysis With PAS Staining

Animals were dissected by cervical dislocations. Right ovaries from mice were fixed immediately in Neutral Formalin solution (Formalin, Buffered, 10% (Phosphate Buffer/Certified), Fisher Scientific, SF100-4) for 12h. Following fixation, ovaries were washed for 3 times for 10 min. and then dehydrated in a graded ethanol series from water. After dehydration step, ovaries were cleared with xylene and then embedded in paraffin with paraffin embedding station (Leica, EG 1160). Paraffin-embedded ovaries were cut into 5 μm histological sections by microtome (Leica, RM 2245) and serial sections were mounted on poly-L-Lysine glass slides. For ovarian morphology, some sections were chosen for PAS staining. These sections were incubated for 40 min at 60°C to remove paraffin and for 2 times for 20 min left in xylene for complete removal of paraffin. Then sections were rehydrated by decreasing alcohol series for 10 min for each (100%, 96%, 90%, 80% and 70%). After rehydration, the sections were stained with Periodic Acid Schiff Kit (Periodic Acid Schiff, Bio Optica, 04-130802). After washing the sections with water, they were passed through increasing alcohol series and cleared with xylene. Finally, the sections were covered with coverslip using xylene-based mounting medium (Bio Mount HM, Bio-Optica, 05-BMHM500). Examination was done under the light microscope (Leica, DM 500) and images of slides were taken under microscope (Leica, CTR 6000) with LASV4.1 computer program.

3.6. Hematoxylin and Eosin Staining

After sections were evaluated morphologically, follicle counting was performed on serial sections. Sections other than PAS-stained sections were stained with hematoxylin and eosin for follicle counting. These sections were incubated for 40 min at 60°C to remove paraffin and for 2 times for 20 min left in xylene for complete removal of paraffin. Then sections were rehydrated by decreasing alcohol series for 10 min for each (100%, 96%, 90%, 80% and 70%). After rehydration, sections were washed with distilled water and were stained with hematoxylin (Gill's hematoxylin No.3, Bio-Optica, 05-06015/L) for 2 min. Then, the sections were washed in running tap water. Eosin (Eosin Y Alcoholic Solution, Bio-Optica, 05-10003/L) staining was performed for 1 min. Sections were washed with distilled water for 2 min. Sections were dehydrated with increasing alcohol series and cleared with xylene for 40 min. Finally, the sections were covered with coverslip using xylene-based mounting medium (Bio Mount HM, Bio-Optica, 05-BMHM500).

3.6.1 Follicle counting

Follicle counting was done under the light microscope (Leica, DM 500). Follicles were classified and counted to assess the effect of the disrupted circadian clock on ovarian follicles. Only those follicles in which the nucleus of the oocyte was clearly visible were scored. A follicle was counted as primordial when it contained an intact oocyte surrounded by a single layer of flattened squamous follicular cells (379). A primary follicle was defined as an enlarged oocyte surrounded by a single layer of cuboidal granulosa cells. Oocytes with two or more layers of granulosa cells but no visible space between granulosa cells were identified as pre-antral follicles. Antral follicles were scored when containing several layers of granulosa cells, and oocyte with a clear nucleus, an antrum and a theca layer (379). Atretic follicles were counted only when a degenerating oocyte and pyknotic granulosa cells observed (379). Corpus luteum and apoptotic cells were also recorded. Data was presented as a number of follicles per developmental stage.

3.7. Immunofluorescence Staining

5 μm histological sections cut by microtome (Leica, RM 2245) and mounted on poly-L-Lysine glass slides were deparaffinized in the incubator (60°C) for 30 min. To be able to remove paraffin from the sections, they were cleared with xylene for 2 times for 20

min. Sections were rehydrated by decreasing alcohol series (100%, 90%, 80% and 70%) for 10 min in order to remove xylene. Sections were washed by distilled water for 5 min then by PBS (Phosphate Buffer Saline, pH:7.2-7.4) for 5 min. For antigen retrieval, sections were heated in microwave (around 600 Watts) in Citrate buffer (10mM, pH 6.0) prepared by dissolving sodium citrate tribasic dihydrate (Sodium Citrate Tribasic Dihydrate, Sigma Aldrich, 6132-04-3) in distilled water, followed by about 5 min low watt (300 Watt). After the sections were heated in microwave, they were left in room temperature to cool down and this process was repeated two times more. Then sections were cool down at the room temperature for 20 min. Then a circle was drawn around the tissues on the slides with a hydrophobic barrier pen (Liquid Blocker, Super Pap Pen) and slides were washed with PBS for 3 times 5 min. 5% Normal Goat Serum (prepared in 0.1 %Triton-X- PBS) (Normal Goat Serum, Chemicon International, S26-100mL) was used for blocking for 1h at room temperature. After removal of blocking solution, sections were not washed, and they were incubated with the primary antibodies; NTY in 1:100 (Nitrotyrosine Polyclonal Antibody, Invitrogen, A-21285) and ZP3 in 1:50 (ZP3 Polyclonal Antibody, Proteintech, 21279-1-AP) which were prepared in blocking solution at +4°C for overnight. After the incubation with primary antibodies, sections were washed with PBS-T (1000µl Tween in 1L PBS) for 3 times for 5 min and then incubated with secondary antibody in 1:250 (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody Alexa Fluor 488, Therma Fisher, A-11008) for 1,5h at room temperature. Sections were washed with PBS-T and mounted with DAPI (Fluoroshield with DAPI, Sigma-Aldrich, F6057) for nucleus staining. The examination was performed by using confocal microscope (Zeiss, LSM780).

3.8. Immunohistochemistry

5 µm histological sections cut by microtome and mounted on poly-L-Lysine glass slides (Poly Lysine, Thermo Fisher Scientific, 165014) were deparaffinized in the incubator (60°C) for 1 hour. To be able to remove paraffin from the sections, they were cleared with xylene for 2 times for 20 min. Sections were rehydrated by decreasing alcohol series (100%, 90%, 80% and 70%) in order to remove xylene. Sections were washed by distilled water for 5 min then by PBS (Phosphate Buffer Saline, pH:7.2-7.4) for 5 min. For antigen retrieval, sections were heated in microwave (around 600 Watts) in Citrate buffer

(10mM, pH 6.0) prepared by dissolving sodium citrate tribasic dihydrate (Sodium Citrate Tribasic Dihydrate, Sigma Aldrich, 6132-04-3) in distilled water, followed by about 5 min low watt (300 Watt). After the sections were heated in microwave, they were left in room temperature to cool down and this process was repeated two times more. Then sections were cool down at the room temperature for 20 min. Then a circle was drawn around the tissues on the slides with a hydrophobic barrier pen and slides were washed with PBS for 3 times 5 min. For blocking endogenous peroxidase enzymes, sections were incubated with 3% hydrogen peroxide (prepared in methanol) for 10 min. Sections were washed with distilled water for 2 times for 5 min, then with Tween-20 PBS for 2 times for 5 min. 5% Normal Goat Serum (prepared in 0.1 % Triton-X-PBS) was used for blocking and the sections were incubated with Normal Goat Serum for 1h at room temperature. After removal of blocking solution, sections were not washed, and they were incubated with the primary antibodies Nitrotyrosine Polyclonal Antibody in 1:50 (Nitrotyrosine Polyclonal Antibody, Thermo Fisher Scientific, A-21285) which were prepared in blocking solution at +4°C for overnight. After the incubation with primary antibodies, sections were washed with PBS-T (1000µl Tween in 1L PBS) for 3 times for 5 min and then incubated with biotinylated secondary antibody for 1,5h at room temperature. Sections were washed with PBS-T for 3 times for 5 min and then incubated with streptavidin for 1,5h at room incubated at room temperature. After slides were washed with PPS-T for 3 times for 5 min, slides were incubated with the mix of DAB and DAB substrate 1:20 (UltraVision Detection System Large Volume DAB Substrate System (RTU), Thermo Fisher Scientific HD16378). Sections were washed with PBS-T for 3 times for 5 minutes. Counterstaining was performed with hematoxylin (Gill's hematoxylin No.3, Bio-Optica, 05-06015/L) and counterstaining slides were washed with tap water. Sections were dehydrated by increasing alcohol series (70%, 80%, 90% and 100%). Then they were cleared with xylene for 2 times for 20 min. The sections were covered with coverslip using xylene-based mounting medium (Bio Mount HM, Bio-Optica, 05-BMHM500) and examination was performed by using light microscope.

3.9. Western Blot

Left ovaries of all mice were used for protein isolation. After cervical dislocation, tissues were stored -80°C. Western Blot analysis were used to determine protein levels of P-mTOR, mTOR and PER2 in ovaries of 12:12h L:D and 12:12h L:L group.

3.9.1. Protein isolation and tissue homogenization

470µL RIPA lysis buffer, 10µL sodium ortho-vanadate, 10µL protein inhibitor cocktail (PIC), and 10µL phenylmethylsulphonyl fluoride (PMSF) (RIPA Lysis Buffer System, SantaCruz Biotechnology, sc-24948A) were added and total 500µL mixture was prepared for each ovary. For tissue homogenization; 200µL out of total mixture (500µL) and zirconium oxide beads (0.9-2.0mm Stainless Steel Beads, Next Advance) were added to ovary tissues and homogenization was performed by homogenizer (Bullet Blender Storm 24, Next Advance). After homogenization, the supernatant was transferred to another Eppendorf and the remained 300µL of mixture was added to the Eppendorf. The supernatant was centrifuged at 14800 rpm for 15 min. After centrifugation, supernatant was taken to a clean Eppendorf without touching the pellet and oil portions. Centrifugation process was repeated. The prepared samples were kept at -20°C overnight. After the qubit measurements were done, the master mix was prepared.

3.9.2. Sample preparation

Master mix was prepared by adding 5µL LDS sample buffer (NuPAGE LDS Sample Buffer (4X), Invitrogen, NP0007) and 2µL reducing agent (NuPAGE Sample Reducing Agent (10X), Invitrogen (10X), NP0009) for each ovary. 7µL of master mix and 13µL sample were added to the PCR tubes. This total 20µL mixture was vortexed and then centrifuged. Then; for protein denaturation, the tubes were placed to the Thermal Cycler (95°C) for 5 min.

3.9.3. Gel loading

Tape on the bottom of the gel was removed and the gel was washed with distilled water. Gel was placed on the tank; the tank was locked. Inner chamber was filled up to upper electrode with fresh running buffer. Running buffer was prepared with 950ml water

and 50ml running buffer (NuPAGE MES SDS Running Buffer (20X), Novex, NP0002). The wells were washed with running buffer before the loading of samples. 18μL samples and 8μL ladder were loaded to the wells. Gel was run first at 120 Volt then at 80 Watt for around 2,5h.

3.9.4. Transfer

After gel running process, gel was removed from the tank. The cassette was washed with distilled water. Running buffer was removed from the tank. Nitrocellulose membrane and filter papers were placed into transfer buffer for 20 min for activation. Transfer buffer was prepared with 3gr Tris (Tris(hydroxymethyl)aminomethane, Thermo Fisher Scientific, 17926), 14.3gr glycine (Glycin für die Molekularbiologie, BioFroxx, 56-40-6) and 200ml methanol and completed with distilled water, with a total volume of 1000ml. Solution was stored at +4°C to cool it down. Sandwich structure was formed bottom to the top as filter paper x2/membrane/gel/filter paper x2. The sandwich was placed into the tank and the tank was filled with transfer buffer. Wet transfer was performed at 15V, +4°C overnight.

3.9.5. Blocking

After transfer, membrane was removed from the tank and washed with TBS-T before proceeding. Blocking solution was prepared with 10ml TBS-T and 0,5 gr milk powder. Membrane was blocked in 5% milk power solution at room temperature for 1 hour.

3.9.6. Primary antibody incubation

After blocking solution was discarded, membrane was incubated with approximately 10 ml of primary antibody on the shaker at +4°C overnight. Primary antibody was diluted with either 5% blocking solution or 5% Bovine Serum Albumin (prepared in TBS-T). mTOR (mTOR (7C10) Rabbit mAb, Cell Signaling, #2983) in 1:1000 and p-mTOR (Phospho-mTOR (Ser2448) (D9C2) Rabbit mAb, Cell Signaling, #5536) in 1:1000 were prepared in 5% BSA. PER2 (PER2 Polyclonal Antibody, abcam, #ab180655) in 1:500, Caspase-3 (Caspase-3 (D3R6Y) Rabbit mAb, Cell Signaling, #14220) in 1:1000 and β-actin (β-actin (H10D10) Mouse mAb, Cell Signaling, #3700) in

1:1000 were prepared in 5% milk powder. After primary antibody incubation, membrane was washed with TBS-T for 3 times for 5 min at room temperature. β-actin was used as an internal control.

3.9.7. Secondary antibody incubation

Primary antibody solution was collected for future use. For secondary antibody incubation, membrane was incubated with approximately 10 ml secondary antibody on the shaker at room temperature for 2h. Anti-rabbit secondary antibody (Anti-Rabbit IgG, HRP-linked Antibody, Cell Signaling, #7074) in 1:1000 was prepared in 5% milk powder and used for mTOR, p-mTOR and PER2 antibody. Anti-mouse secondary antibody (ECL Mouse ıgG, HRP-linked whole Ab, Amersham, #NA931V) in 1:2000 was prepared in 5% milk powder and used for β-actin. After incubation, membrane was washed with TBS-T for 6 times for 5 min. at room temperature. The membrane was placed in TBS until ECL application.

3.9.8 Chemiluminescent setection and analysis

Mixture of ECL substrate and ECL (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific, 32106) in 1:1 was used for one membrane. ECL mixture was added on top of the membrane drop by drop and ChemiDoc^{TM} MP Imaging System (170-8280) was use for gel imaging. ImageLab software was used for further analyses.

3.10. Statistical Analysis

Test were analyzed with t-test (Student's test).. Statistical calculations were performed using GraphPad Prism version 7.0.

4. RESULTS

4.1. Food Intake and Body Weight Change Results

In order to check whether the experimental setup we designed caused a disruption in the circadian rhythm of the animals or not, we set food intake and body weight change analysis as the criterions.

Food ingestions of animals was monitored at 07.00 am and 19.00 pm for each day during experiment by weighing the initial and the remaining food in the bin. We demonstrated that the total food intake of 12:12h L:L group for one week is significantly higher than the 12:12h L:D group (Figure 4.1A). In order to show whether there is any disruption in the food intake cycles of the mice that are more active at night and less active during the day in normal cycle, we monitored both daytime (07.00 am-19.00 pm) food intake and nighttime (19.00 pm-07.00 am) food intake. We observed that daytime food intake of 12:12h L:L group is significantly higher than 12:12h L:D group when there is no significantly difference in nighttime food intake of them (Figure 4.1B and 4.1C). We showed that housing in a constant light for one week caused a change in the food intake cycles of the mice because of the disrupted circadian rhythm.

In addition to food intake analysis, all animals were weighed at the arrival and at the end of the experiment to analyze the body weight change of animals. At the end of the one week experiment, we showed that mice in 12:12h L:L group gained significantly less weight than those in 12:12h L:D group (Figure 4.1D). We also demonstrated that the negative correlation between body weight change and food intake. Mice in 12:12h L:L group gained less weight although they ate more than 12:12h L:D group. We resulted that housing in constant light for one week caused a negative correlation between body weight change and food intake of the mice because of the disrupted circadian rhythm.

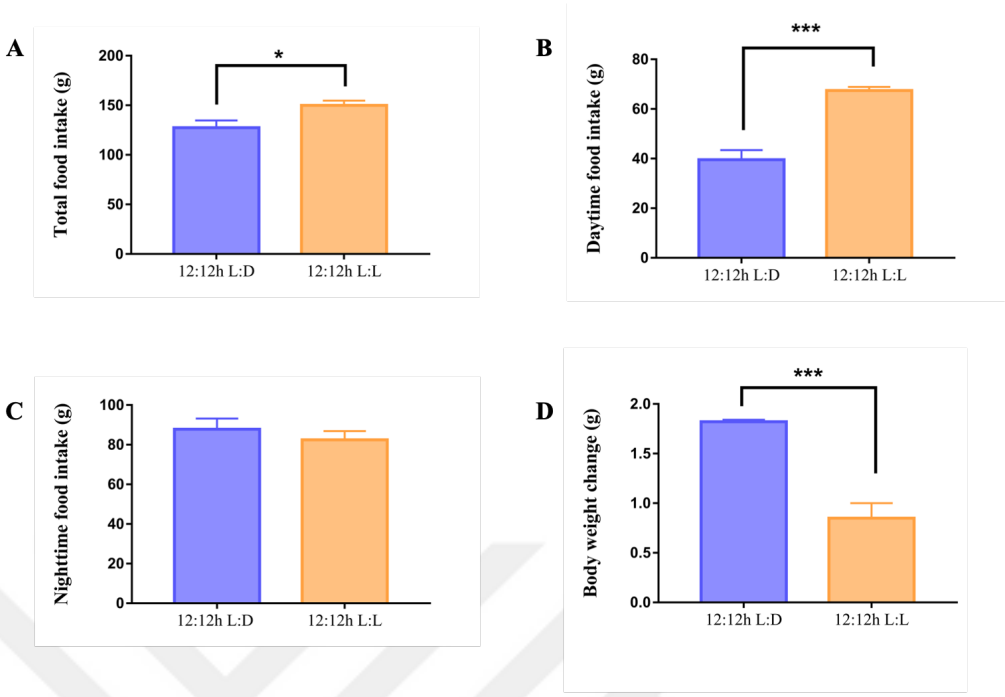


Figure 4.1. Food intake and body weight change analysis. Total food intake analyses for one week of both 12:12h L:L group and 12:12h L:D group is shown (A). 12:12h L:L group consumed significantly more food than 12:12h L:D group during one-week experiment. Daytime and nighttime food intake analyses for one week of both 12:12h L:D and 12:12h L:L are shown (B-C). Daytime food intake of 12:12h L:L group is significantly higher than 12:12h L:D group. One-week body weight changes of mice in 12:12h L:L and 12:12h L:D groups (D). Mice in 12:12h L:L group gained significantly less weight than those in 12:12h L:D group (n:15) ($p<0.05$).

4.2. Open Field Test (OFT) Results

After 1-week experiment, open field test was performed on mice of both 12:12h L:D and 12:12h L:L groups in order to monitor the possible behavioral changes due to constant light housing. After OFT was performed, to analyze locomotor and anxiety-like behavior in mice, following analyses were done: the number of fecal boli, total distance, velocity, time spent in inner zone, in transition zone and in outer zone (Figure 4.2).

Mice were allowed to complete the 10 min test in the OFT and removed from the maze. After mice were removed, the number of fecal boli deposits were manually counted. We monitored that the number of fecal boli of 12:12h L:L group is significantly higher

than those in 12:12h L:D group (Figure 4.2A). This increased number of fecal boli might indicate increased anxiety and emotionality of mice in 12:12h L:L group.

Total distance travelled by mice in 12:12h L:L group during 10-minute experiment and the velocity of them were found as higher than those in 12:12h L:D group. However, no significant difference was found in these analyses (Figure 4.2B and 4.2C).

Similarly, when the 12:12h L:D and 12:12h L:L groups were compared, no significant difference was found between the time spent in inner, transition and outer zone (Figure 4.2D). Although fecal boli analysis showed that the mice in 12:12h L:L group had a tendency to anxiety, other OFT analyses showed that there was no significant difference between animals' locomotor activities and emotionality.

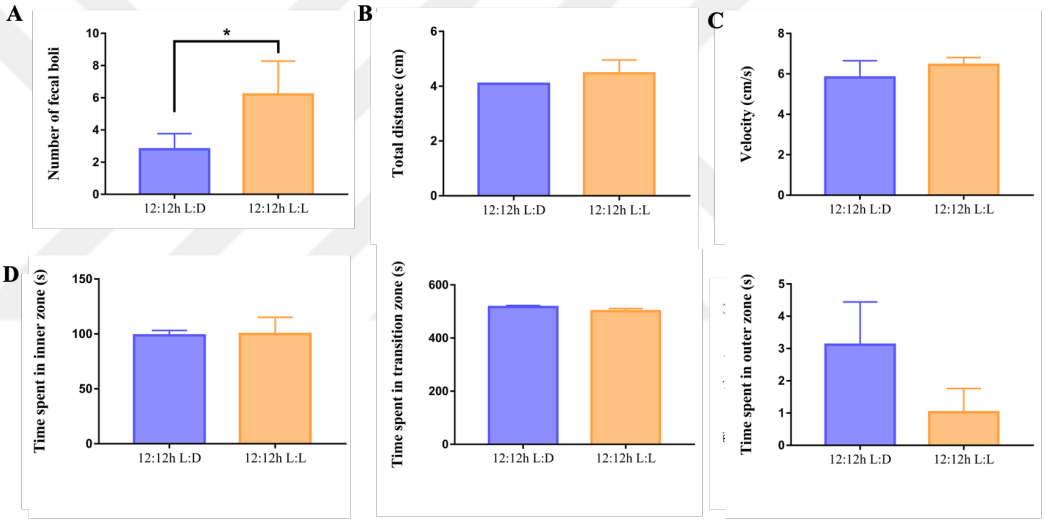


Figure 4.2. Open field test results. Total number of fecal boli of mice of both 12:12h L:D and 12:12h L:L groups is shown (A). The number of fecal boli of 12:12h L:L group is significantly higher than the those in 12:12h L:D group during 10-minute OFT (A). Total distance travelled by mice of both 12:12h L:D and 12:12h L:L groups is shown (B). No significant difference was found. Velocity of mice during 10-minute OFT is shown (C). No significant difference was found. Time spent in inner, transition and outer zone is shown (D). No significant difference was found (n: 8) ($p<0.05$).

4.3. Vaginal Smear Results

After OFT, vaginal smear was performed for monitoring estrous cycles of mice in both 12:12h L:D and 12:12h L:L groups (Figure 4.3). After the vagina of mice were washed with saline, the unstained smear samples were observed under light microscope.

Three main cell types are observed in vaginal smear samples of mice: nucleated epithelial cells, cornified squamous epithelial cells, and leukocytes. The stages of the estrus cycle were classified into 4 phases as diestrus, proestrus, estrus and metestrus according to the cell types which were observed on smear samples.

The smear samples consist of predominantly nucleated epithelial cells; it is called as pro-estrus. The smear samples which consist of leukocytes, cornified and nucleated epithelial cells were called metestrus. Estrus which is characterized by enucleated cornified cells and diestrus characterized by leukocytes were not observed among both 12:12h L:D and 12:12h L:L groups. While it was observed that all of the mice in the 12:12h L:L group were in the pro-estrus cycle (Figure 4.3C and 4.3D), we resulted that some of the mice in 12:12h L:D group were in the pro-estrus and the other part was in the metestrus (Figure 4.3A and 4.3B). Although the same methods were followed during the smear collection, cell numbers in smear samples from the mice in 12:12h L:L group were lower than those in 12:12h L:D group.

Since the smear results obtained at the end of the experiment did not give an idea whether the phases of estrus cycle were shortened/lengthened or skipped, we did not establish a connection between estrus cycle and circadian rhythm disruption caused constant light.

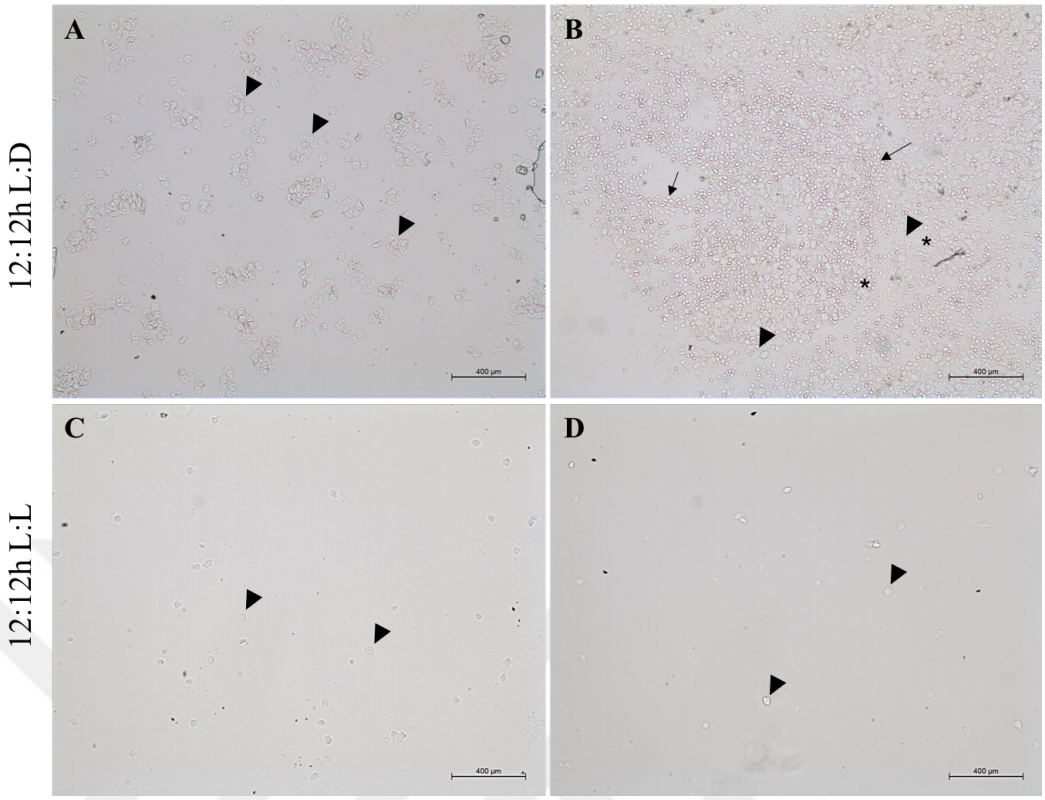


Figure 4.3. Vaginal smear results. Unstained vaginal smear samples of mice in 12:12h L:D group are shown (A, B). In 12:12h L:D group, some mice were in proestrus (A) and some of them were in metestrus (B). Arrowheads indicate nucleated epithelial cells. Arrows indicate leukocytes and asterix (*) indicate cornified epithelial cells. All mice in 12:12h L:L were in proestrus according to vaginal smear analyses (n:10) (C, D).

4.4. Morphology and Histological Analysis

Morphological analysis of ovaries from mice in 12:12h L:D and 12:12h L:L groups were identified with Periodic Acid Shift (PAS) and Hematoxylin and Eosin (H&E) staining. Sizes of ovaries from 12:12h L:L group were similar to those in 12:12h L:D group. Although there is no significant difference between the sizes of ovaries, it was obvious that the ovaries from 12:12h L:L group was surrounded with a large amount of lipid tissue comparing to 12:12h L:D group. Stroma of the ovaries from 12:12h L:L group was disrupted. We observed that the fibrous structure of the stroma of the ovaries from 12:12h L:L group was deteriorated and became discontinuous (Figure 4.4). We have observed all stage-developing follicles and corpus luteum throughout the ovaries from 12:12h L:L group like those in 12:12h L:D group (Figure 4.5, 4.6 and 4.7). We noted that the ovaries from 12:12h L:L group contain quite few comparing to control group ($p<0.05$)

(Figure 4.5). We also observed corpus luteum structures and atretic follicles in the ovaries from 12:12h L:L group. We demonstrated that ovulation took place in the ovaries from 12:12h L:L group due the existence of corpus luteum (Figure 4.7A, 4.7A'). More atretic follicles were noted in the ovaries from 12:12h L:L group (Figure 4.7B, 4.7B'). Although we demonstrated the follicles at every stage of development in the ovaries of 12:12h L:L group, we observed that the structures of follicles are impaired (Figure 4.8). The granulosa cells were not in close cell-cell contact with each other. Follicles did not generally display a spherical-shape appearance (Figure 4.8). Besides unhealthy follicles, the atretic follicles were also more depressive than we observed in 12:12h L:D group (Figure 4.8). We also observed more apoptotic and luteinized cells in the ovaries from 12:12h L:L group comparing to the 12:12h L:D group (Figure 4.9).

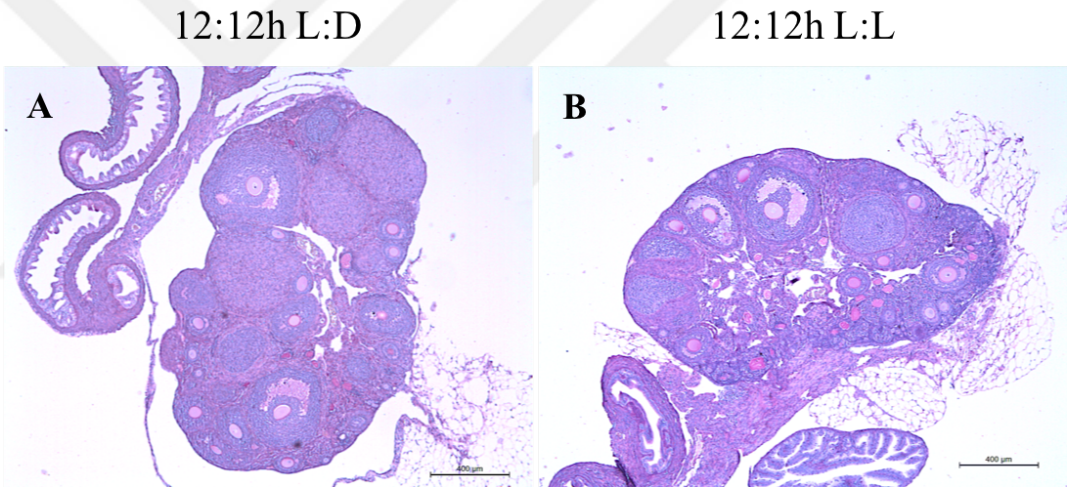


Figure 4.4. Ovaries of 12:12h L:D and 12:12h L:L groups. The structure of cortex and medulla of the ovaries from both 12:12h L:D and 12:12h L:L groups are shown (A, B). Ovaries stained with Periodic Acid Shift staining.

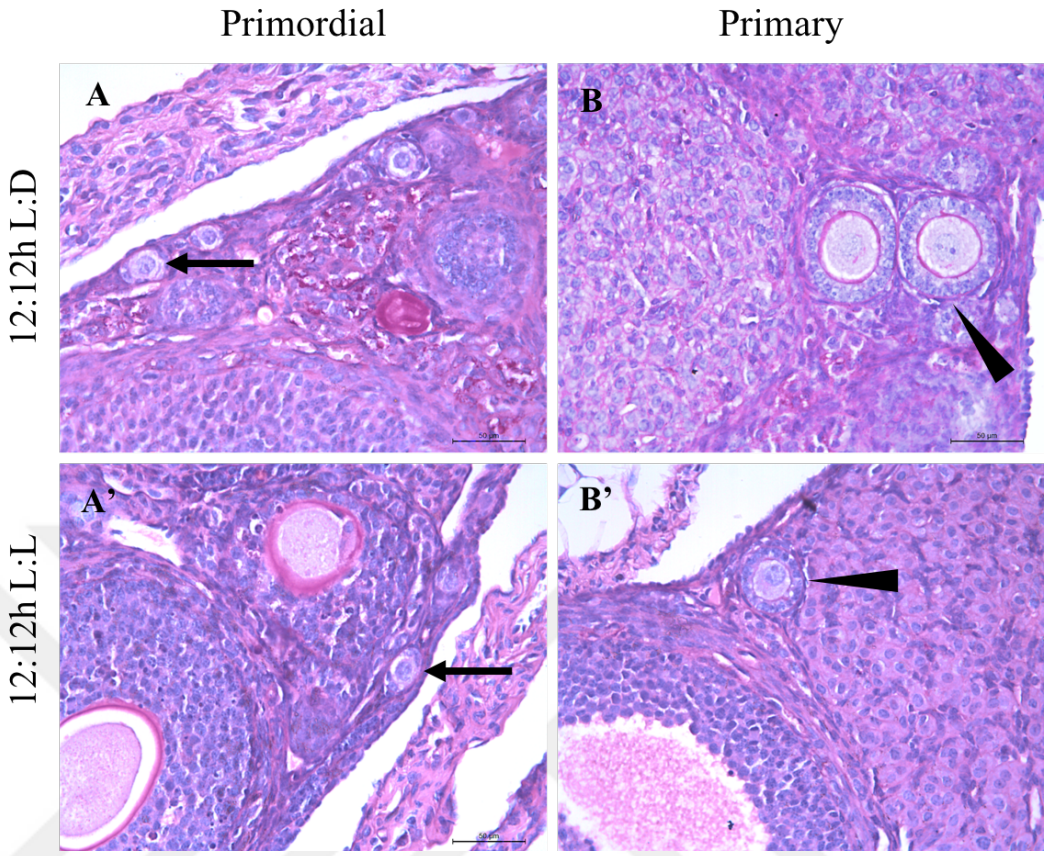


Figure 4.5. Primordial and primary follicles of 12:12h L:D and 12:12h L:L groups. PAS stained primordial follicles and primary follicles are shown here. Primordial follicle of 12:12h L:D group is indicated with black arrow (A). Primordial follicle of 12:12h L:L group which is indicated with black arrow (A'). Primary follicle of 12:12h L:D group is indicated with black arrow head (B). Primary follicle of 12:12h L:L group is indicated with black arrow head (B').

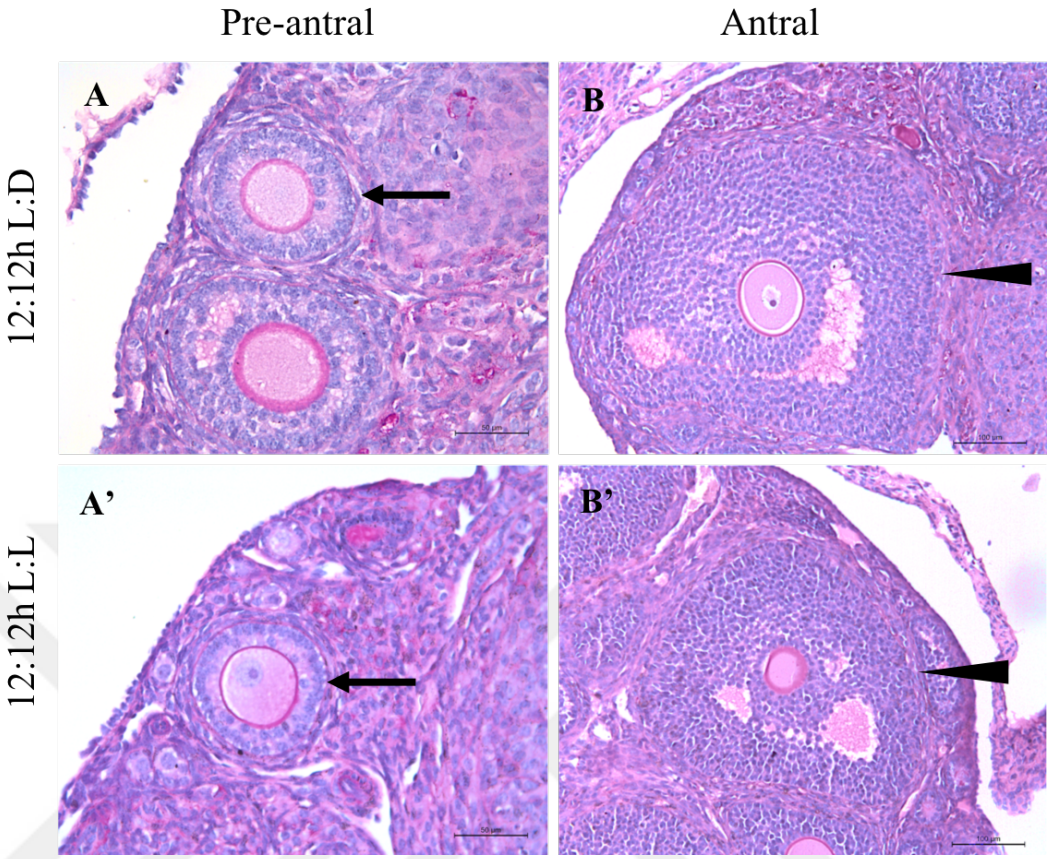


Figure 4.6. Pre-antral and antral follicles of 12:12h L:D and 12:12h L:L groups. PAS stained pre-antral follicles and antral follicles are shown here. Pre-antral follicle of 12:12h L:D group is indicated with black arrow (A). Pre-antral follicle of 12:12h L:L group which is indicated with black arrow (A'). Antral follicle of 12:12h L:D group is indicated with black arrow head (B). Antral follicle of 12:12h L:L group is indicated with black arrow head (B').

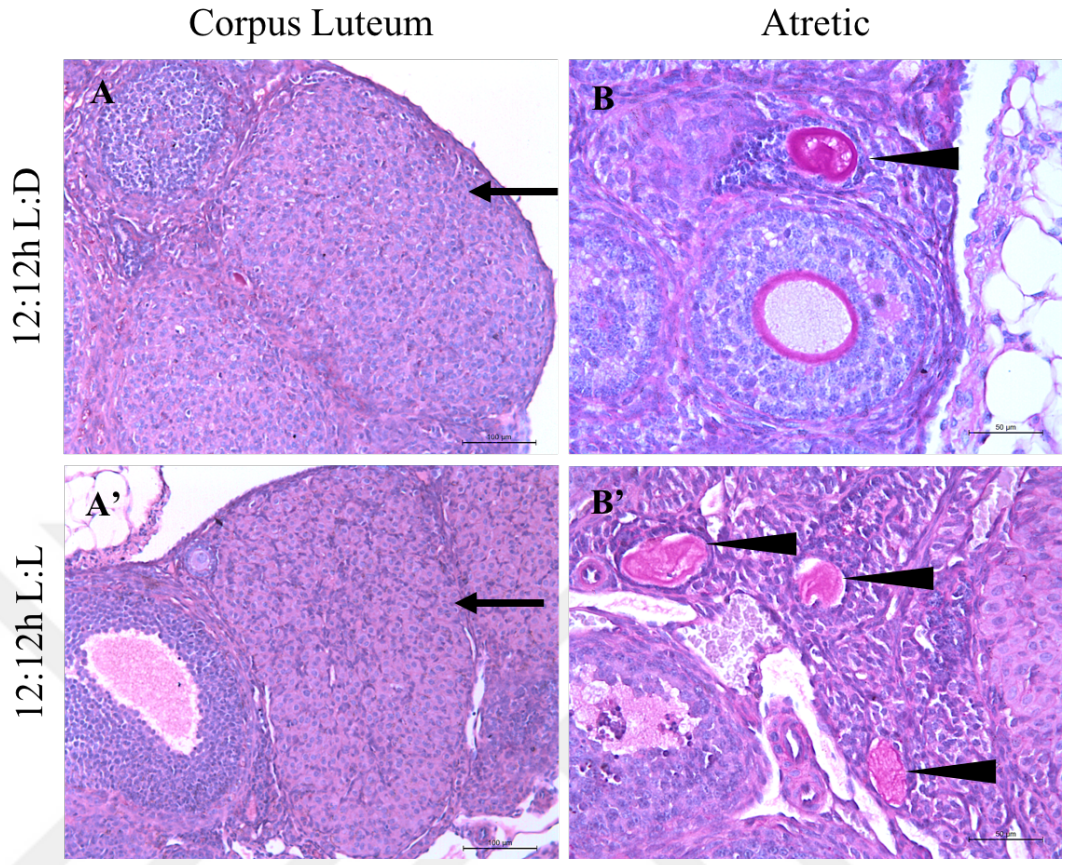


Figure 4.7. Corpus luteum and atretic follicles of 12:12h L:D and 12:12h L:L groups. PAS stained corpus luteum and atretic follicles are shown here. Corpus luteum of 12:12h L:D group is indicated with black arrow (A). Corpus luteum of 12:12h L:L group which is indicated with black arrow (A'). Atretic follicle of 12:12h L:D group is indicated with black arrow head (B). Atretic follicle of 12:12h L:L group is indicated with black arrow head (B').

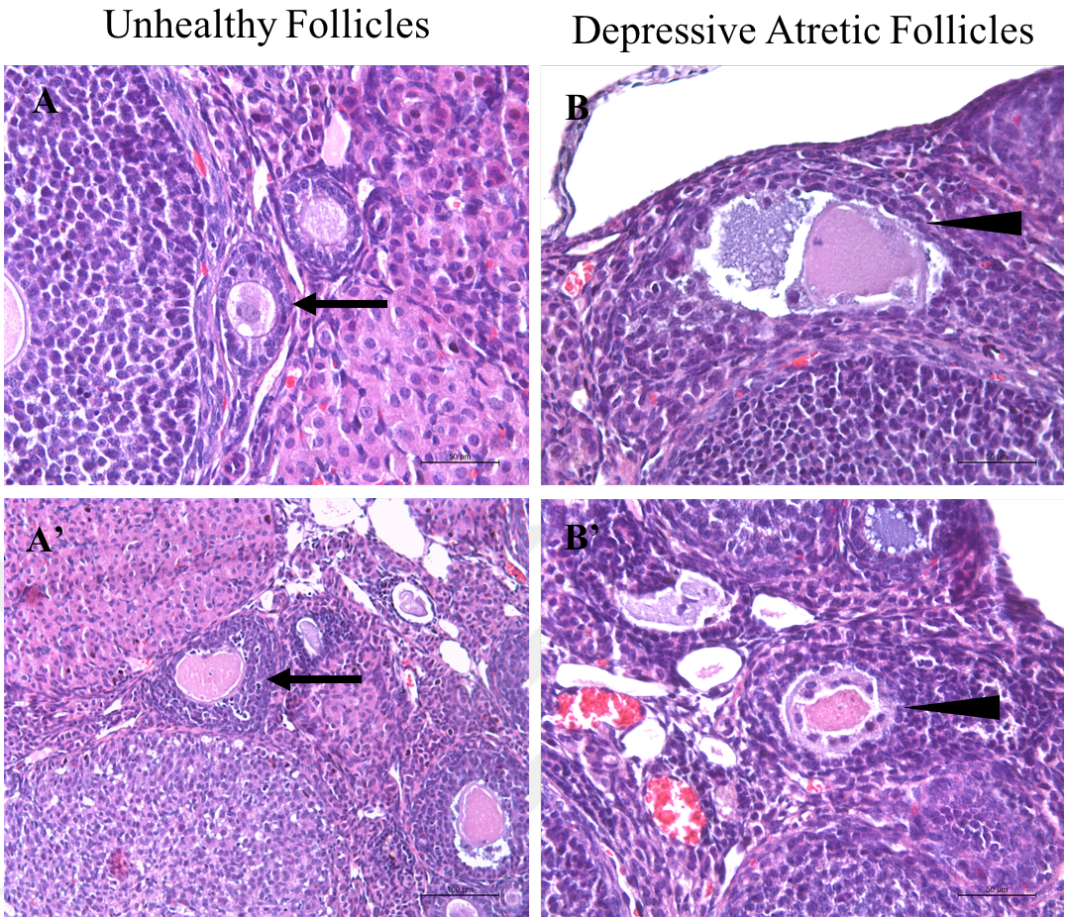


Figure 4.8. Unhealthy and depressive atretic follicles in 12:12h L:L group. Hematoxylin and eosin stained follicles of 12:12h L:L group are shown here. Unhealthy follicles found in 12:12h L:L group are indicated with black arrows (A, A'). Depressive atretic follicles found in 12:12h L:L group are indicated with black arrow heads (B, B').

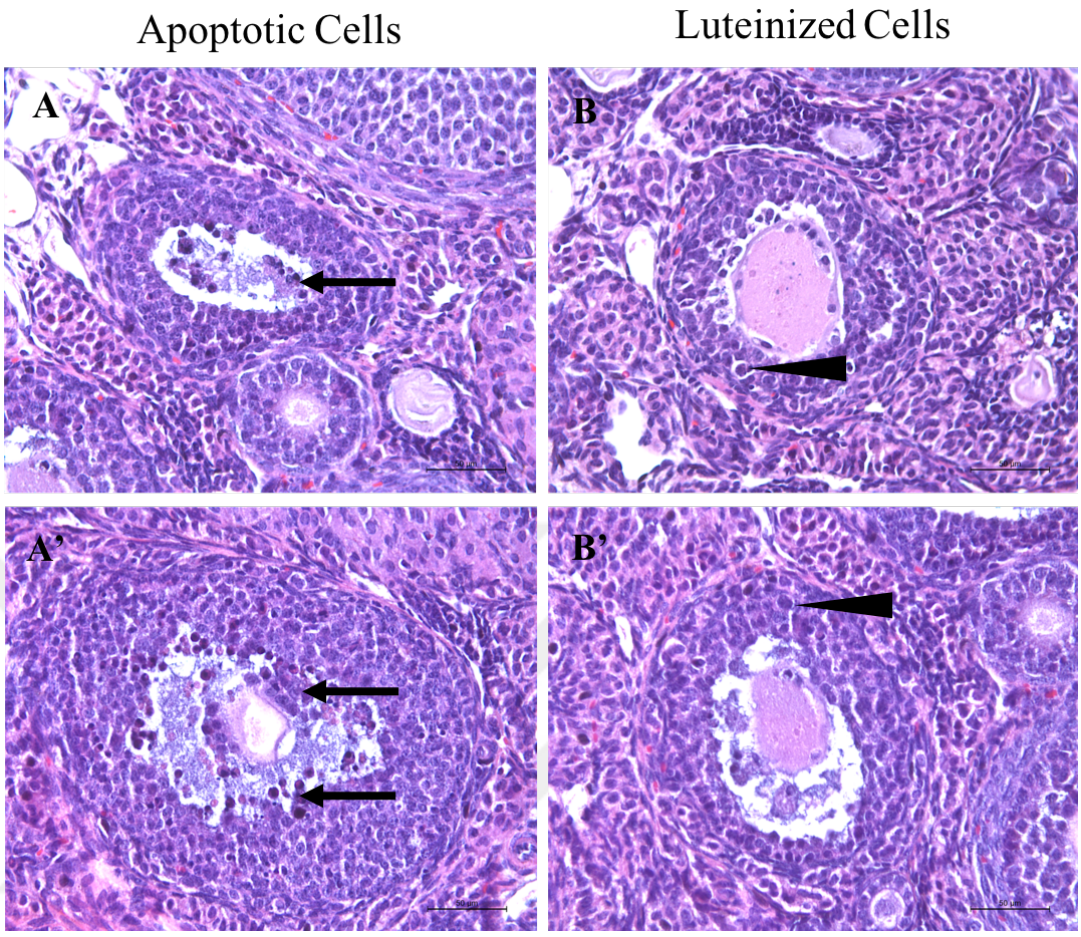


Figure 4.9. Apoptotic and luteinized cells in 12:12h L:L group. Hematoxylin and eosin stained follicles of 12:12h L:L group are shown here. Apoptotic cell clusters found in 12:12h L:L group are indicated with black arrow (A, A'). Luteinized cells found in 12:12h L:L group are indicated with black arrow heads (B, B').

4.5. Follicle Counting

For follicle counting, paraffin-embedded ovaries were cut into 5µm serial sections by microtome. Serial sections were stained with hematoxylin and eosin and examination was done under the light microscope. Follicles were classified and counted to assess the disrupted circadian clock on ovarian follicles. Only those follicles in which the nucleus of the oocyte was clearly visible were scored and then the total number of follicles obtained was divided by the number of sections counted. Thus, the proportion of follicles at different developmental stages in the ovaries of both the 12:12h L:D and 12:12h L:L group was obtained. Graphs were created to make comparisons between groups. Primordial follicles were recognized by a single layer of flattened (squamous) follicular cells

surrounding oocyte. Primary follicles were characterized by a single layer of cuboidal granulosa cells which surround the enlarged oocyte. Pre-antral follicles identified by many layers of granulosa cells consisting with small a few antral spaces between them. Antral follicles were scored when several layer granulosa cell, antrum and theca layer were obvious. Follicles consisting of degenerating oocyte and pyknotic granulosa cells were scored as atretic follicles.

As a result of the evaluations, we found that the number of primordial follicles in the ovaries of the experiment group is significantly lower compared to those of the ovaries of the control group (Figure 4.10A). We started to observe zona pellucida structures right before late primary follicles (Figure 4.10).

Thus, we interpreted that the primordial follicle pool which is also known as the ovarian reserve was reduced in the ovaries of mice in 12:12h L:L group. It should be noted that lower ovarian reserve might be related to ovarian aging. Therefore, it was thought that mice of 12:12h L:L group tend to ovarian aging and ovarian aging might be triggered.

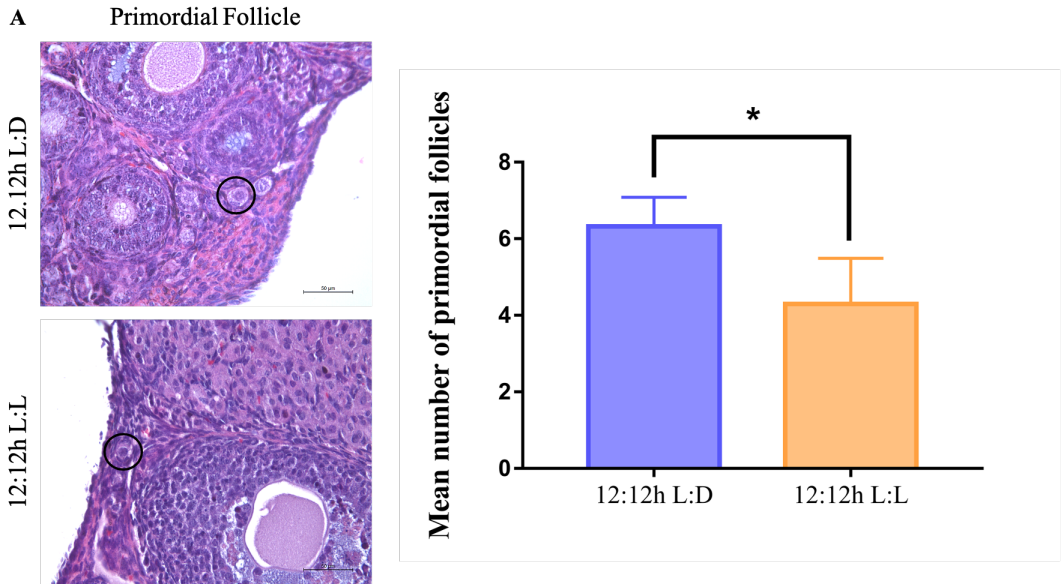
Number of primary follicles were lower in ovaries of mice from 12:12h L:L group compared those of 12:12h L:D group. We reported no significant difference between groups based on primary follicle numbers (Figure 4.10B). Like primary follicles, even the number of antral follicles were higher in ovaries from 12:12h L:L group compared to ovaries from 12:12h L:D group, there were no significant difference between the groups (Figure 4.10D).

However, we demonstrated that the number of pre-antral follicles in the ovaries of the 12:12h L:L group is significantly higher compared to those of the ovaries of 12:12h L:D group (Figure 4.10C). This result showed us that primordial follicle activation was higher and faster in the ovaries of 12:12h L:L group. Therefore, this result can be interpreted as follows; primordial follicles in the ovaries of mice whose circadian rhythm was disrupted by constant light for 1 week had undergone a rapid activation of primordial follicle without follow healthy developmental timing. Thus, this situation may have caused a rapid reduction of the primordial follicle pool. It should be noted here that it is known that excessive primordial follicle activation might lead development of premature ovarian insufficiency which is known a condition characterized by menopause before age 40 (380). It should also be noted that menopause is known as the final step of ovarian aging (336).Combining the results obtained with the literature, we have shown that the disruption

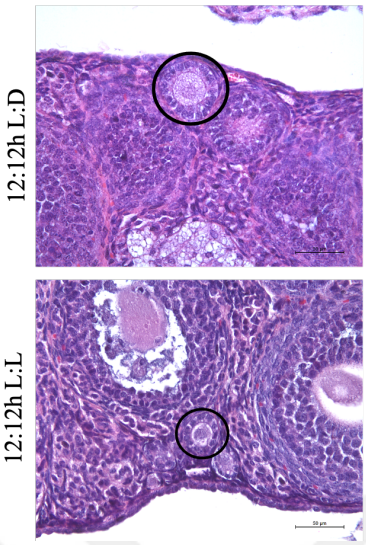
of the circadian rhythm with light caused decreased primordial follicle number and increased pre-antral/antral follicle number associated with the ovarian aging. When the ovaries of 12:12h L:D and 12:12h L:L group were compared, we did not see a significant difference between the number of corpus luteum (Figure 4.10E).

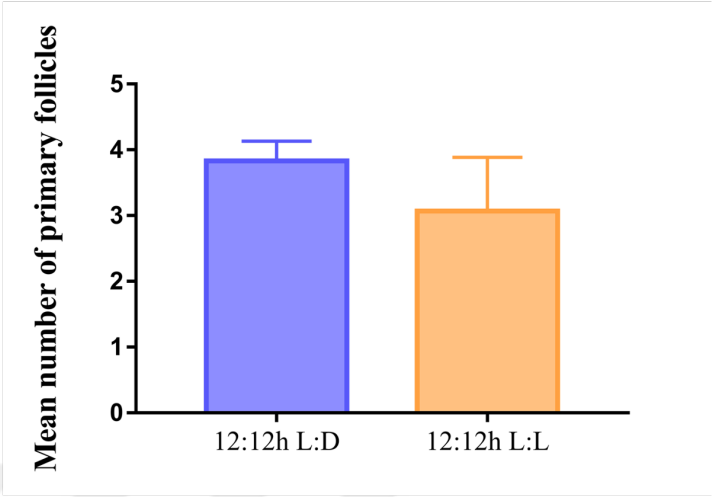
However, a significant difference was noted between atretic follicle numbers in ovaries of 12:12h L:D and 12:12h L:L group (Figure 4.10F). In addition to the high number of atretic follicles in ovaries from 12:12h L:L group, we have seen that pre-antral and pre-ovulatory follicles also go to atresia in mice from 12:12h L:L group. However, it is known that pre-antral and pre-ovulatory follicles rarely undergo atresia (334, 335). We evaluated these results which we observed in 12:12h L:L group, that the disruption of the circadian rhythm accelerates the aging of the ovary and impairs development of healthy follicles. Besides, we observed significant decrease in 12:12h L:L groups according to their total follicle numbers (Figure 4.10G).

Follicle counting results showed us that disruption of circadian rhythm with constant light for 1 week caused consequences which might be associated with the ovarian aging and negative effects on ovarian health because it is known that follicular reserve declines at an exponential rate that gradually changes throughout life leading to accelerated rate of atresia during years preceding menopause (381-384).

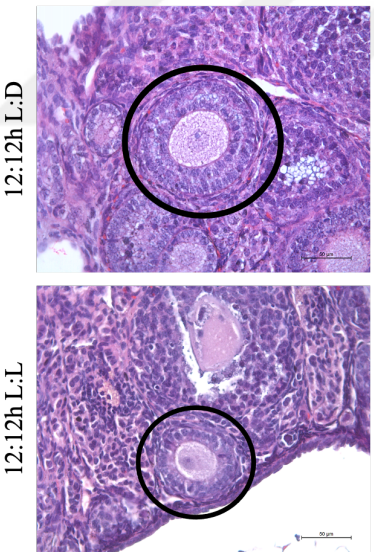


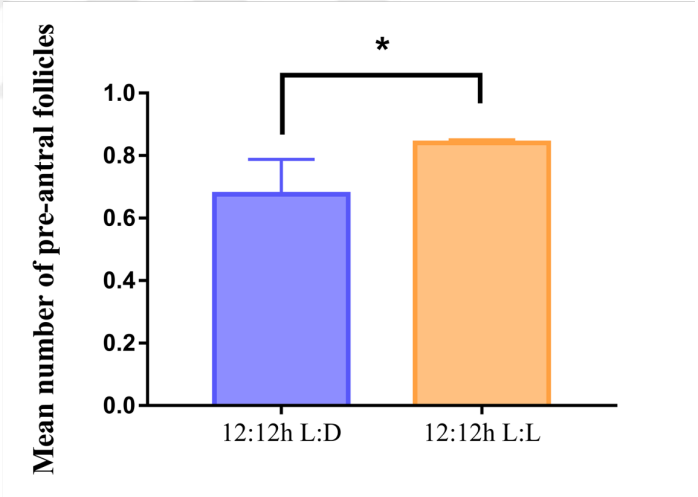
B Primary Follicle

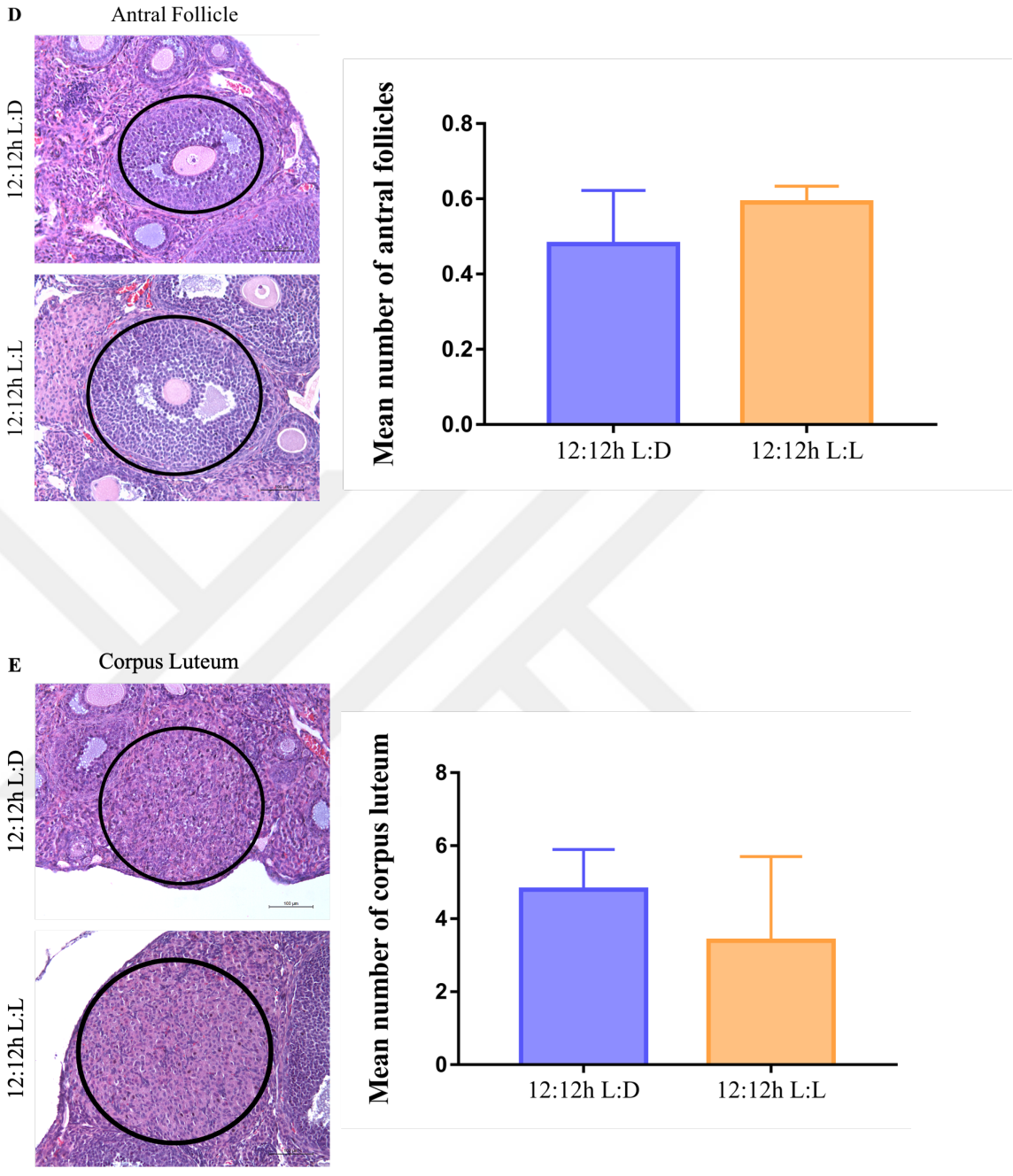




C Pre-antral Follicle






D
Antral Follicle
12:12h L:D
12:12h L:L
Mean number of antral follicles
0.8
0.6
0.4
0.2
0.0
12:12h L:D
12:12h L:L
E
Corpus Luteum
12:12h L:D
12:12h L:L
Mean number of corpus luteum
8
6
4
2
0
12:12h L:D
12:12h L:L

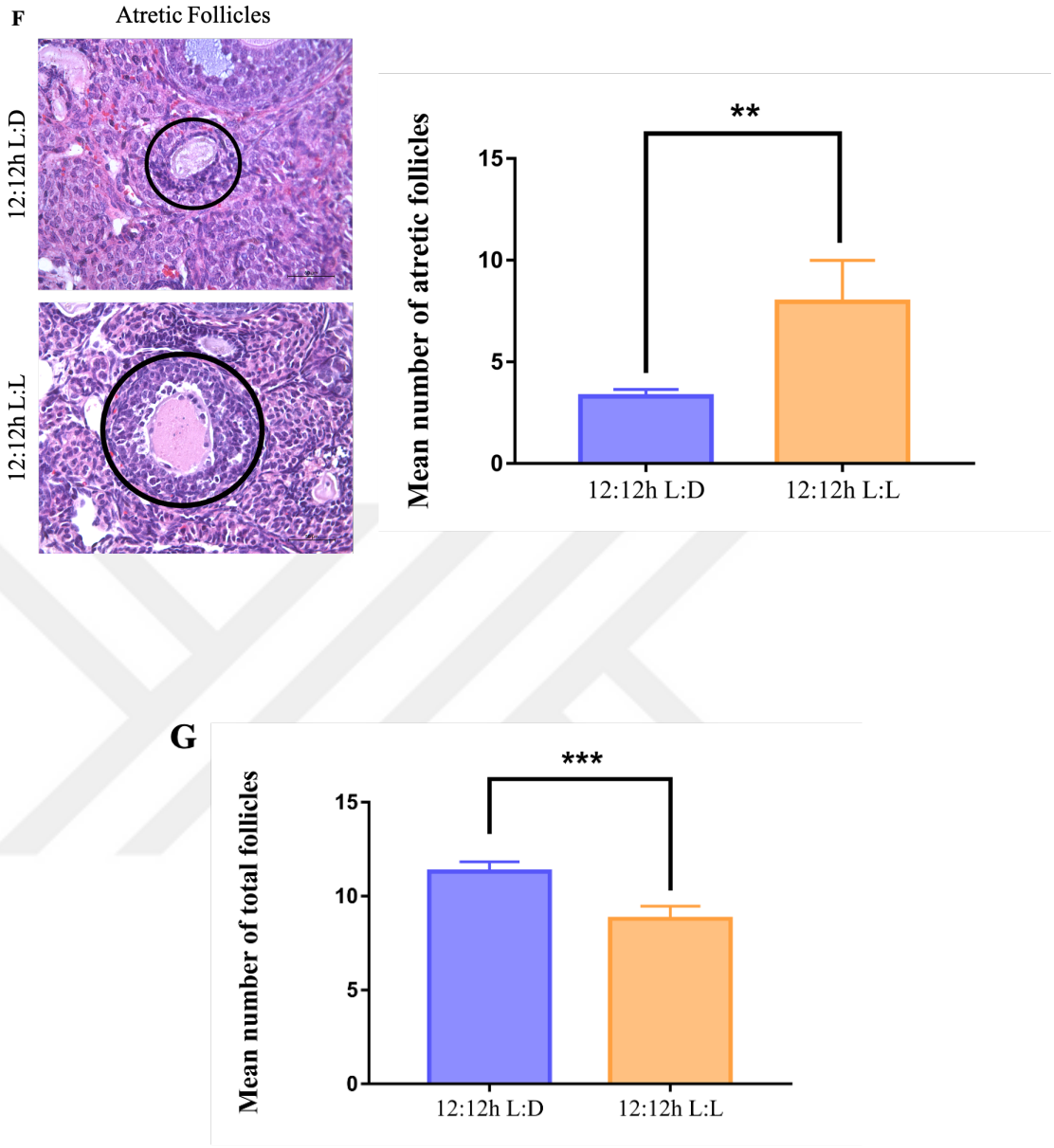


Figure 4.10. Follicle counting results. Mean number of primordial follicles and representative images of primordial follicles from both 12:12h L:D and 12:12h L:L group are shown (A). Mean number of primary follicles and representative images of primary follicles from both 12:12h L:D and 12:12h L:L group are shown (B). Mean number of pre-antral follicles and representative images of pre-antral follicles from both 12:12h L:D and 12:12h L:L group are shown (C). Mean number of antral follicles and representative images of antral follicles from both 12:12h L:D and 12:12h L:L group are shown (D). Mean number of corpus luteum and representative images of corpus luteum from both 12:12h L:D and 12:12h L:L group are shown (E). Mean number of atretic follicles and representative images of atretic follicles from both 12:12h L:D and 12:12h L:L group are

shown (F). Mean number of total follicle numbers from both 12:12h L:D and 12:12h L:L group is shown (G). Black circles indicate the follicles at different stages, corpus luteum and atretic follicles from 12:12h L:D group. Black circles indicate the follicles at different stages, corpus luteum and atretic follicles from 12:12h L:L group (n:5) ($p<0.05$).

4.6. Immunofluorescence Staining

4.6.1 Expression of nitrotyrosine (NTY)

In order to evaluate the oxidative stress, we performed immunofluorescence staining on paraffin sections of mouse ovaries from both 12:12h L:D and 12:12h L:L groups. While nitrotyrosine (NTY) was used for the detection of oxidative stress, DAPI was used for nucleus staining, and merged images taken by using confocal microscopy were evaluated. We observed primordial, primary, pre-antral and antral follicles in sections stained by immunofluorescence staining with NTY antibody in order to evaluate oxidative stress (Figure 4.11, 4.12, 4.13 and 4.14). When we compare the NTY localization, which was used as representation of oxidative stress, between the groups in general, we found that NTY signals were much stronger in the 12:12h L:L group compared to 12:12h L:D group (Figure 4.11, 4.12, 4.13, 4.14, 4.19).

When we evaluated the oxidative stress in the primordial follicles from 12:12h L:D and 12:12h L:L groups, we found that the NTY localization was stronger in the primordial follicles in 12:12h L:L group (Figure 4.11). We detected that oxidative stress is more in primordial follicles in 12:12h L:L group rather than 12:12h L:D group. We also noticed that oxidative stress occurred in granulosa cells rather than the oocytes in primordial follicles, and this accumulation was concentrated in the junctions of granulosa cells (Figure 4.11).

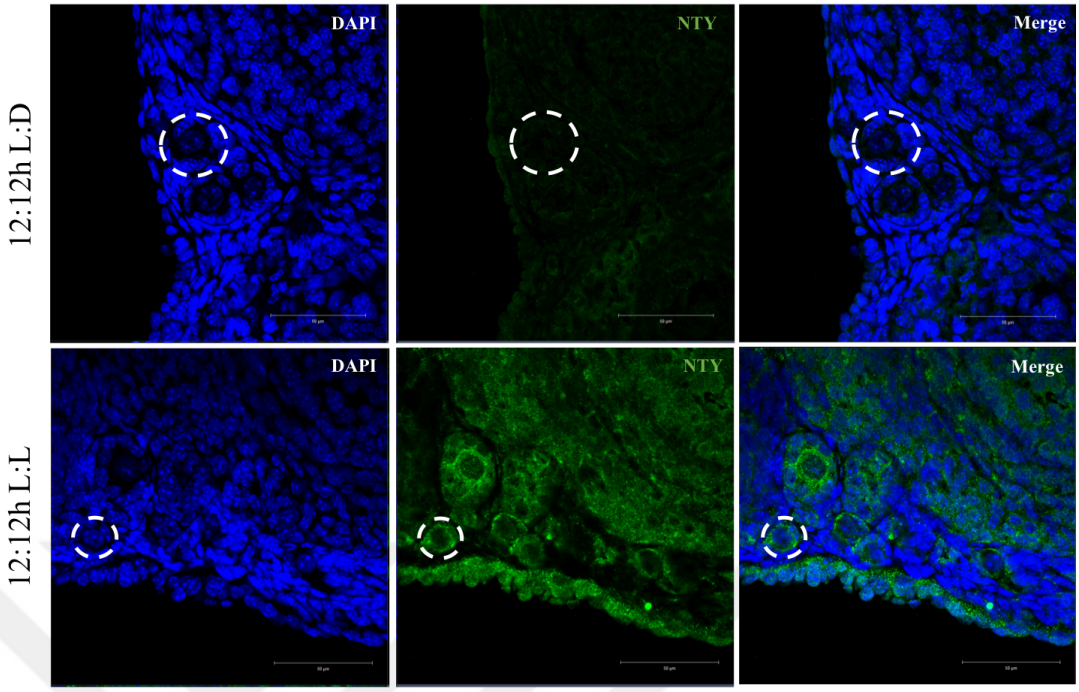


Figure 4.11. Oxidative stress in primordial follicles. NTY in primordial follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show NTY and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to primordial follicles in 12:12h L:D and 12:12h L:L group.

As we evaluated oxidative stress in primordial follicles, we also evaluated it in primary follicles from both 12:12h L:D and 12:12h L:L group. Generally, as in the primordial follicle evaluations, we found that oxidative stress was higher in the primary follicles from 12:12h L:L group. In addition to oxidative stress seen at junctions between granulosa cells, we have seen that unlike primordial follicles, oxidative stress was also observed in oocyte cytoplasm in 12:12h L:L group (Figure 4.12).

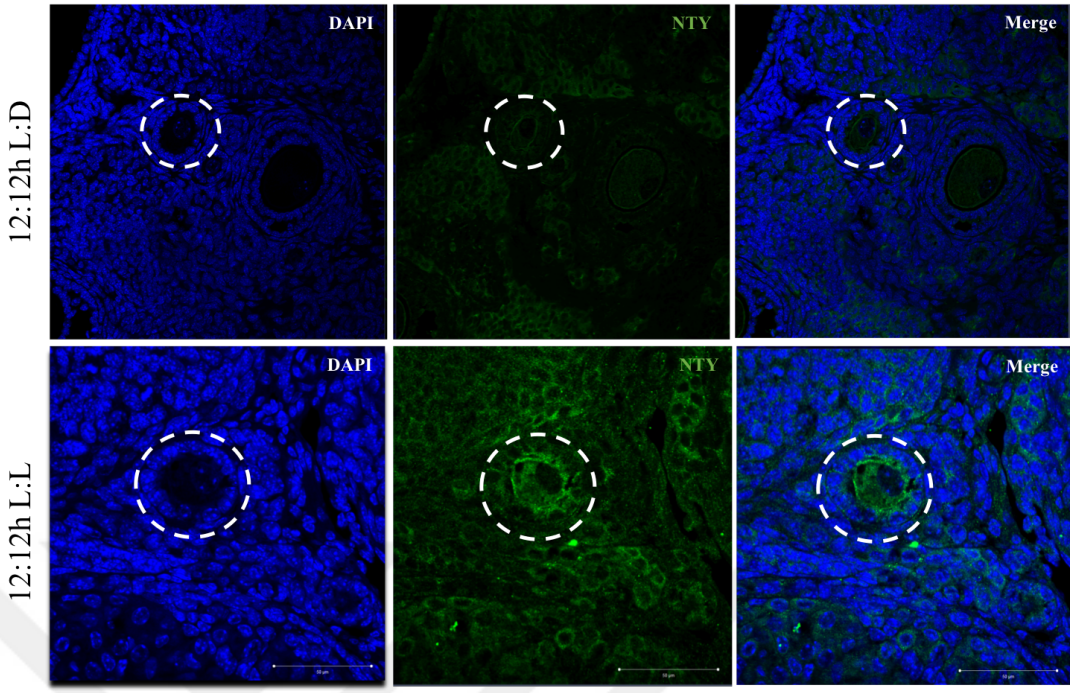


Figure 4.12. Oxidative stress in primary follicles. NTY in primary follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show NTY and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to primary follicles in 12:12h L:D and 12:12h L:L group.

After evaluating the primordial and primary follicles, we evaluated the pre-antral and antral follicles from both 12:12h L:D and 12:12h L:L groups in terms of oxidative stress. Similar to our previous results, we also found that oxidative stress in pre-antral and antral follicles from 12:12h L:L group were higher than those in 12:12h L:D group (Figure 4.13, 4.14). When the pre-antral and antral follicles were evaluated in terms of the localization of NTY; we observed that oxidative stress at the junctions of granulosa cells as well as in the oocyte cytoplasm. We also observed that oxidative stress in the follicles at these developmental stages was concentrated at the cortical areas of oocyte and especially in the zona pellucida (Figure 4.13, 4.14).

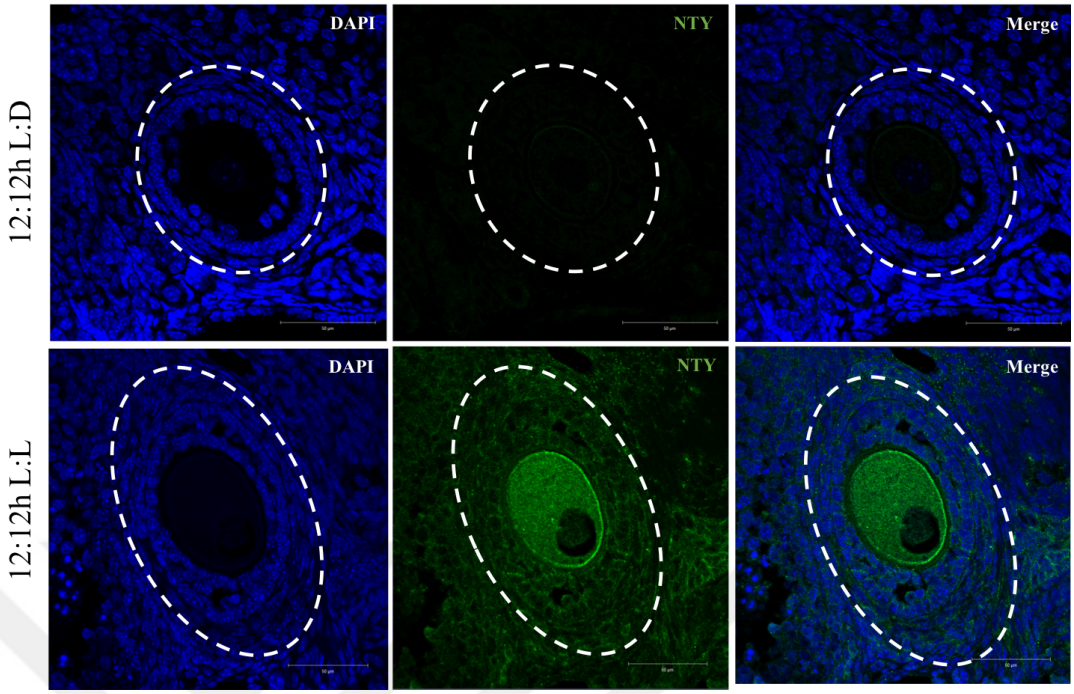


Figure 4.13. Oxidative stress in pre-antral follicles. NTY in pre-antral follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show NTY and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to pre-antral follicles in 12:12h L:D and 12:12h L:L group.

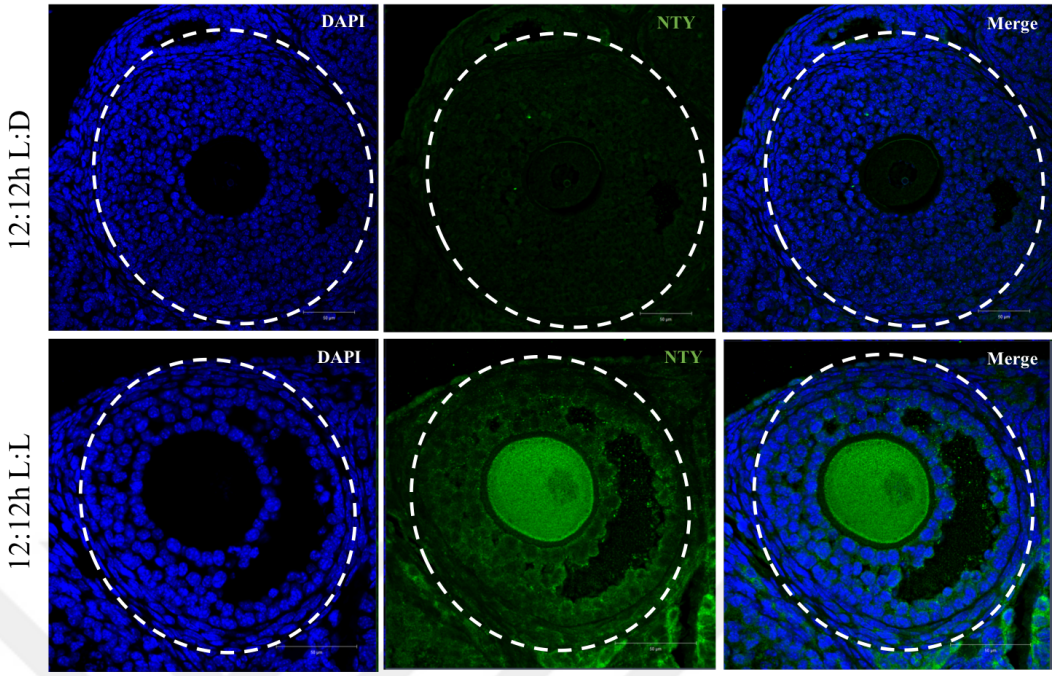


Figure 4.14. Oxidative stress in antral follicles. NTY in antral follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show NTY and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to antral follicles in 12:12h L:D and 12:12h L:L group.

4.6.2 Expression of zona pellucida protein ZP3

In order to evaluate the localization and strength of expression of zona pellucida protein ZP3, we performed immunofluorescence staining on paraffin sections of mouse ovaries from both 12:12h L:D and 12:12h L:L group whose circadian rhythm was disrupted by constant light for 1 week. ZP3 was used for the evaluation the difference between zona pellucida protein expression in groups. DAPI was used for nucleus staining, and merged images taken by using confocal microscopy were evaluated. We observed primordial, primary, pre-antral and antral follicles in sections stained by immunofluorescence staining with ZP3 antibody in order to evaluate the difference between zona pellucida structures of follicles in both 12:12h L:D and 12:12h L:L group (Figure 4.15, 4.16, 4.17 and 4.18). When we compare the ZP3 localization, which was used as representation of zona pellucida, between the groups in general, we found that ZP3 signals were much stronger in the 12:12h L:D group compared to the 12:12h L:L group (Figure 4.15, 4.16, 4.17, 4.18 and 4.19).

When we evaluated the ZP3 localization in the primordial follicles from 12:12h L:D and 12:12h L:L groups, we found that there are no signals for ZP3 in both 12:12h L:D and 12:12h L:L group as we expected because zona pellucida is not found in primordial follicles yet. It is known that zona pellucida is first observed and becomes a matrix which surrounds the oocyte when the granulosa cells start to proliferate (385). Therefore, within this information, we did not observe ZP3 expression in primordial follicles in both 12:12h L:D and 12:12h L:L group (Figure 4.15). Besides, we encountered a result that surprised us in 12:12h L:L group. Although we recorded almost no ZP3 signals in the growing follicles, the ZP3 signals in the zona pellucida structures which were disrupted and found in atretic follicles were quite strong (Figure 4.15, arrows).

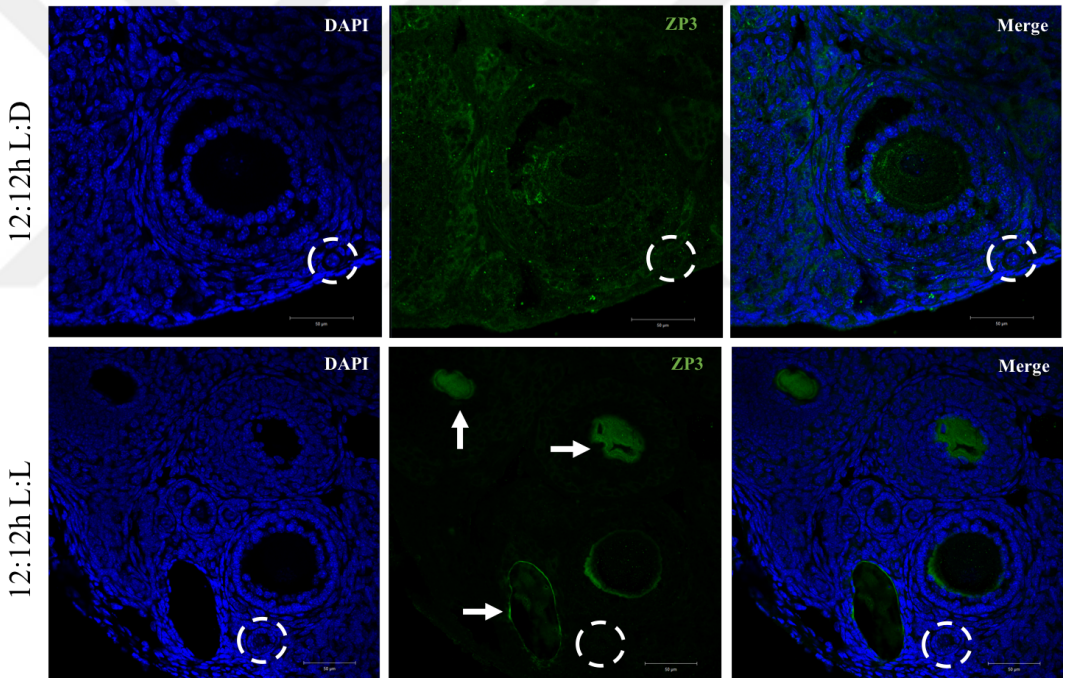


Figure 4.15. Expression of ZP3 in primordial follicles. ZP3 in primordial follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show ZP3 and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to primordial follicles in 12:12h L:D and 12:12h L:L group. Arrows represent the atretic follicles.

We evaluated ZP3 expression in primary follicles from both 12:12h L:D and 12:12h L:L groups. We found that there is no signal at the location of zona pellucida for ZP3 in both 12:12h L:D and 12:12h L:L group as we expected because zona pellucida is

not found in early primary follicles yet also, and can be seen in late primary follicles as granulosa cells initiate to proliferate (385) (Figure 4.16).

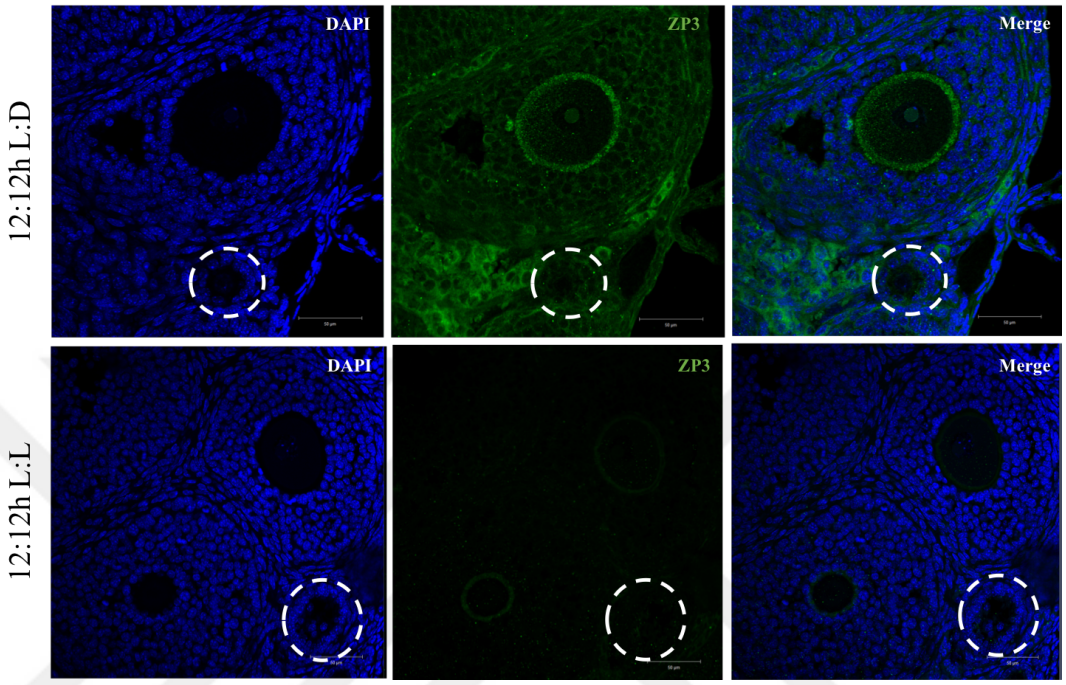


Figure 4.16. Expression of ZP3 in primary follicles. ZP3 in primary follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show ZP3 and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to primary follicles in 12:12h L:D and 12:12h L:L group.

After evaluating the primordial and primary follicles, we evaluated the pre-antral and antral follicles from both 12:12h L:D and 12:12h L:L group in terms of ZP3 localization (Figure 4.17, 4.18). In pre-antral and antral follicles from 12:12h L:D group, we observed strong ZP3 signals at zona pellucida surrounds the oocyte and a little weaker signal at oocyte cytoplasm (Figure 4.17, 4.18). In pre-antral and antral follicles, we also recorded signals of ZP3 in cytoplasm of granulosa cells which are known that they are not involve in synthesis of zona pellucida in mice rather than other species (386). Even it is known that major ZP glycoproteins which are called as ZP1, ZP2 and ZP3 are synthesized within growing oocyte itself and not with the surrounding follicular cells (386); we continued to observe ZP3 signals in granulosa cells in our experimental trials of immunofluorescence staining. On the other hand, in pre-antral and antral follicles from

12:12h L:L group, we recorded very weak or no signal of ZP3 at both zona pellucida, oocyte and granulosa cells (Figure 4.17, 4.18).

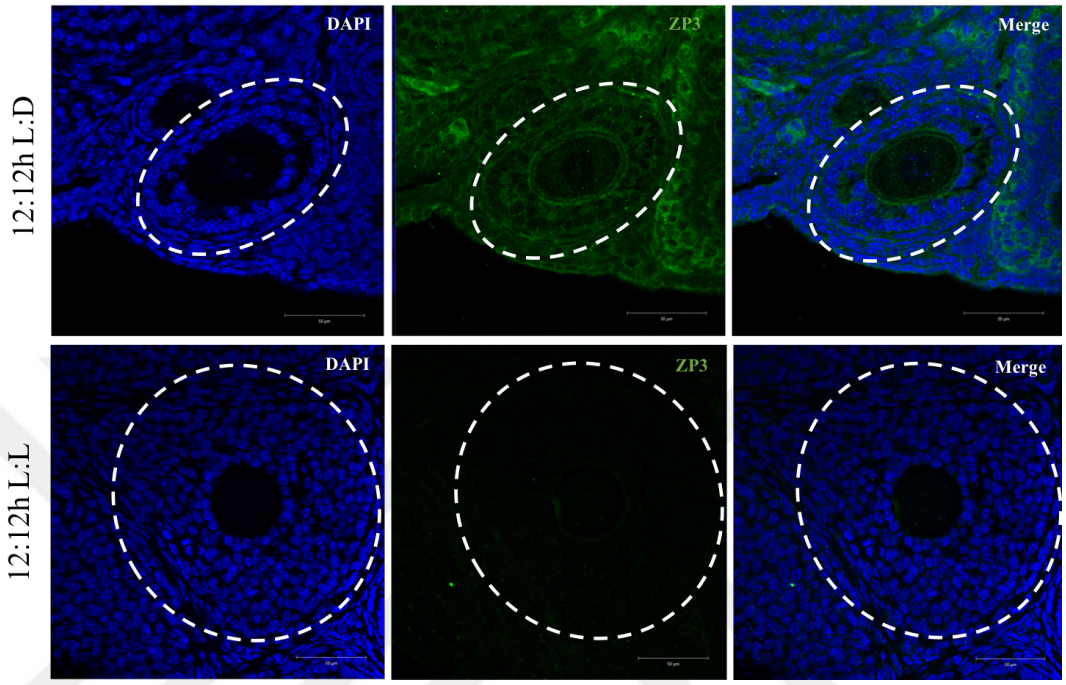


Figure 4.17. Expression of ZP3 in pre-antral follicles. ZP3 in pre-antral follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show ZP3 and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to pre-antral follicles in 12:12h L:D and 12:12h L:L group.

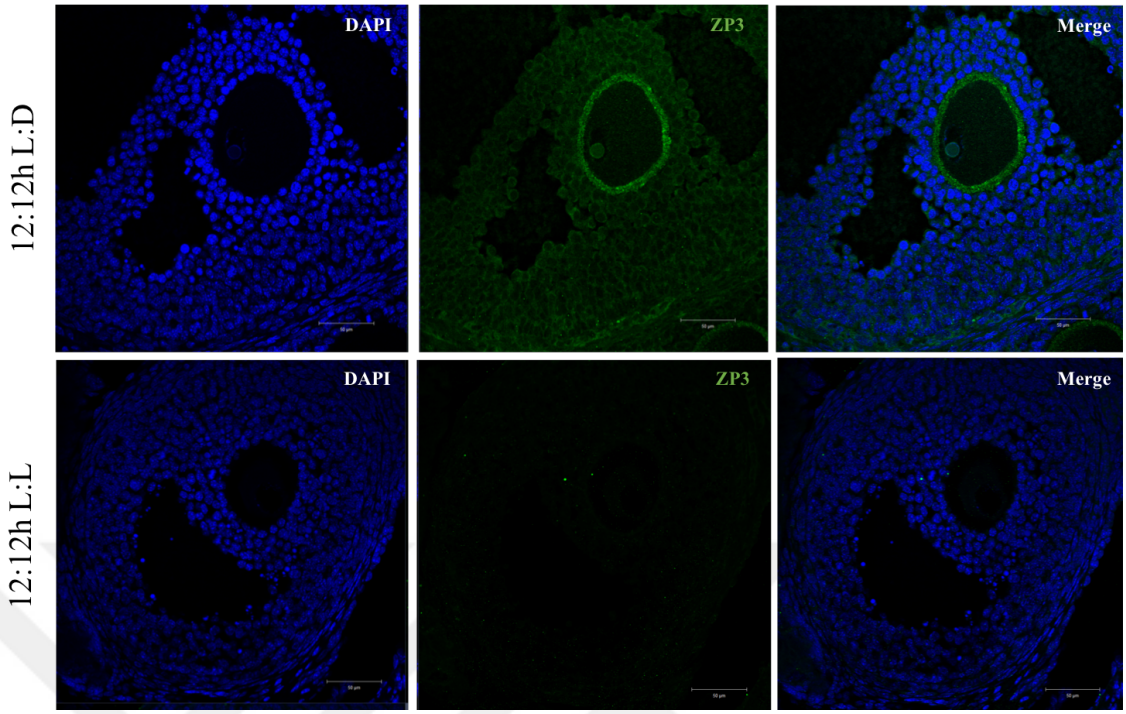


Figure 4.18. Expression of ZP3 in antral follicles. ZP3 in antral follicles from both 12:12h L:D and 12:12h L:L groups is shown by immunofluorescence staining. Green stained areas show ZP3 and blue stained areas show nucleus. Nucleus imaging was performed by DAPI. White circles refer to antral follicles in 12:12h L:D and 12:12h L:L group.

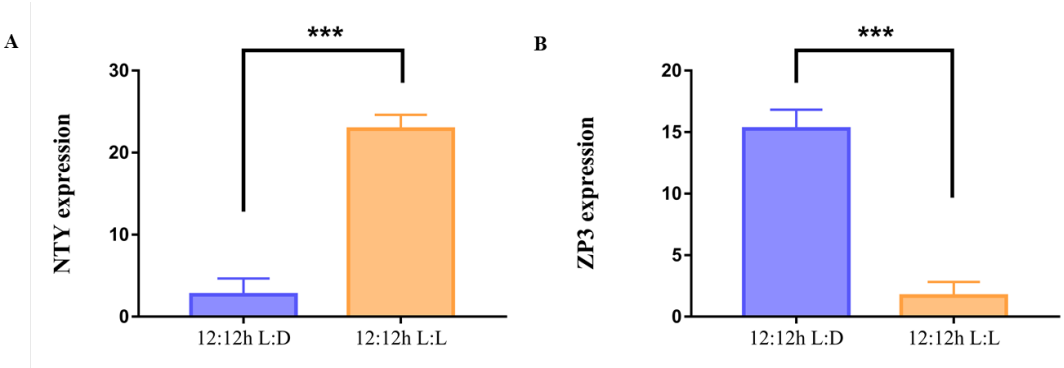


Figure 4.19.Quantitative analysis of expression of NTY and ZP3. NTY expression is significantly higher in 12:12h L:L group compared to 12:12h L:D group ($p<0.05$) (A). ZP3 expression is significantly lower in 12:12h L:L group compared to 12:12h L:D group ($p<0.05$) (B).

4.7 Immunohistochemistry Staining

To verify our results in immunofluorescence staining, we also performed immunohistochemistry staining in order to evaluate oxidative stress. We performed immunohistochemistry staining on paraffin sections of mouse ovaries from both 12:12h L:D and 12:12h L:L group whose circadian rhythm was disrupted by constant light for 1 week. While nitrotyrosine (NTY) was used for the detection of oxidative stress, counterstaining was performed with hematoxylin and images taken by using light microscopy were evaluated. We observed primordial, primary, pre-antral and antral follicles in sections stained by immunohistochemistry staining with NTY antibody in order to evaluate oxidative stress (Figure 4.20). When we compare the NTY localization, which was used as representation of oxidative stress, between the groups in general, we found that NTY signals were much stronger in the 12:12h L:L group compared to 12:12h L:D group, as similar as our results from immunofluorescence staining (Figure 4.20).

When we evaluated oxidative stress in the primordial follicles from 12:12h L:D (Figure 4.20A) and 12:12h L:L groups (Figure 4.20A'), we found that the NTY localization was stronger in the primordial follicles in 12:12h L:L groups (Figure 4.20A'). We detected that oxidative stress is found more in primordial follicles in 12:12h L:L group rather than 12:12h L:D group (Figure 4.20A and 4.20A').

As in primordial follicles, we also oxidative stress in primary follicles from both the 12:12h L:D (Figure 4.20B) and 12:12h L:L group (Figure 4.20B'). Generally, as in the primordial follicle evaluations, we found that NTY signals was higher in the primary follicles from 12:12h L:L group (Figure 4.20B and 4.20B'). NTY signals was strongly observed in oocyte cytoplasm in 12:12h L:L group, while weak NTY signals were found in cortical area and zona pellucida in 12:12h L:D group. (Figure 4.20B and 4.20B').

After evaluating the primordial and primary follicles, we evaluated the pre-antral (Figure 4.20C and 4.20C') and antral follicles (Figure 4.20D and 4.20D') from both 12:12h L:D and 12:12h L:L group in terms of oxidative stress. Similar to our previous results, we also found that oxidative stress in pre-antral follicles were higher those in 12:12h L:L group (Figure 4.20C and 4.20C'). We observed that in pre-antral follicles from 12:12h L:L group, NTY signals were obvious at the junctions between granulosa cells and also in oocyte cytoplasm (Figure 4.20C'). In pre-antral follicles from 12:12h L:D group, we also observed very weak NTY signals but not the junctions of granulosa cells and oocyte

cytoplasm rather at the cortical are of oocyte (Figure 4.20C). Antral follicles from the 12:12h L:L group showed higher NTY signals than those in 12:12h L:D group (Figure 4.20D and 4.20D'). When antral follicles were evaluated in terms of oxidative stress; we observed that more NTY signals at the junctions of granulosa cells as well as in the oocyte cytoplasm from 12:12h L:L group (Figure 4.20D'). We also observed that NTY signals in the antral follicles from 12:12h L:L group at these developmental stages were concentrated at the cortical areas of oocyte and especially in the zona pellucida (Figure 4.20D').

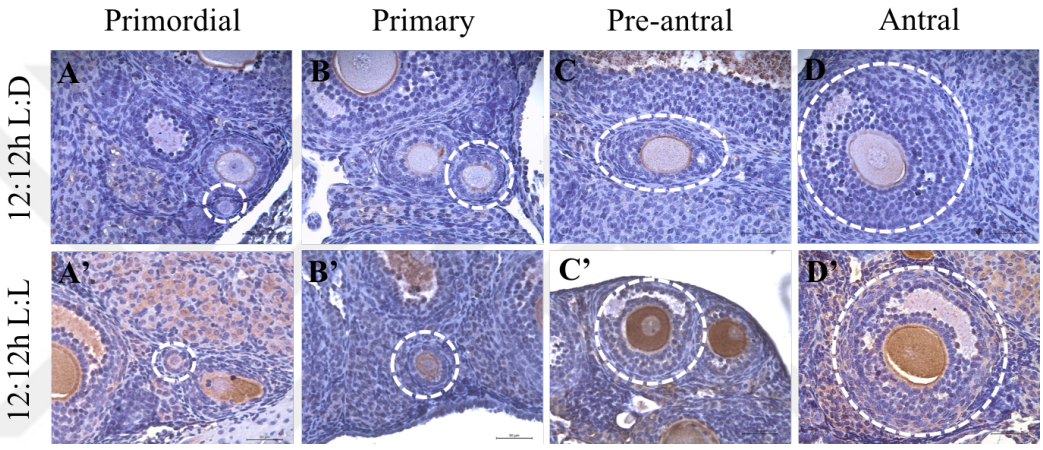


Figure 4.20. Oxidative stress in follicles in different stage of development. NTY in follicles in different stage of development from both 12:12h L:D and 12:12h L:L groups is shown by immunohistochemistry staining. White circles refer to follicles in 12:12h L:D and 12:12h L:L group. Brown stained areas show NTY and blue stained areas show nucleus. Nucleus staining was performed by hematoxylin. Primordial follicles in 12:12h L:D (A) and 12:12h L:L group (A') are shown here. Primary follicles in 12:12h L:D group (B) and 12:12h L:L group (B') are shown here. Pre-antral follicles in 12:12h L:D (C) and 12:12 L:L group (C') are shown. Antral follicles in 12:12h L:D (D) and 12:12h L:L group (D') are shown.

4.8 Western Blot Results

We performed western blot to evaluate the protein levels of the PER2, one of the circadian rhythm components and mTOR, which we predicted to be associated with PER2 in the ovary, in the ovaries of mice whose circadian rhythm was disrupted by continuous

light for a week. Besides, we also performed western blot on ovaries of mice for phosphorylated form of mTOR (p-mTOR) and Caspase-3.

We observed significant difference between the mTOR protein levels of 12:12h L:D and 12:12h L:L group ($p<0.05$) (Figure 4.21A). We recorded that mTOR protein levels were higher than the levels of 12:12h L:L group ($p<0.05$) (Figure 4.21A). Similar to mTOR protein levels of both 12:12h L:D and 12:12h L:L group, we also demonstrated that the p-mTOR levels were significantly higher in 12:12h L:D group than 12:12h L:L group ($p<0.05$) (Figure 4.21B). We showed that constant light for a week caused mTOR and p-mTOR protein levels to decrease in 12:12h L:L group (Figure 4.21A, 4.21B).

PER2 protein levels were also evaluated by western blot analysis (Figure 4.21C). We showed that PER2 protein levels in 12:12h L:D group were significantly higher than those in 12:12h L:L group (Figure 4.21C). PER2 protein levels decreased in ovaries of mice that housed for a week with constant light, as we expected ($p<0.05$) (Figure 4.21C).

To evaluate apoptosis levels found in ovaries of both 12:12h L:D and 12:12h L:L group, we performed western blot analysis for Caspase-3 which are known as one of the crucial mediators of programmed cell death (apoptosis) (387). We reported no significant difference between Caspase-3 protein levels of ovaries from 12:12h L:D and 12:12h L:L group even we observed more apoptotic cells in morphological analysis of ovaries from 12:12h L:L (Figure 4.9A and 4.9A').

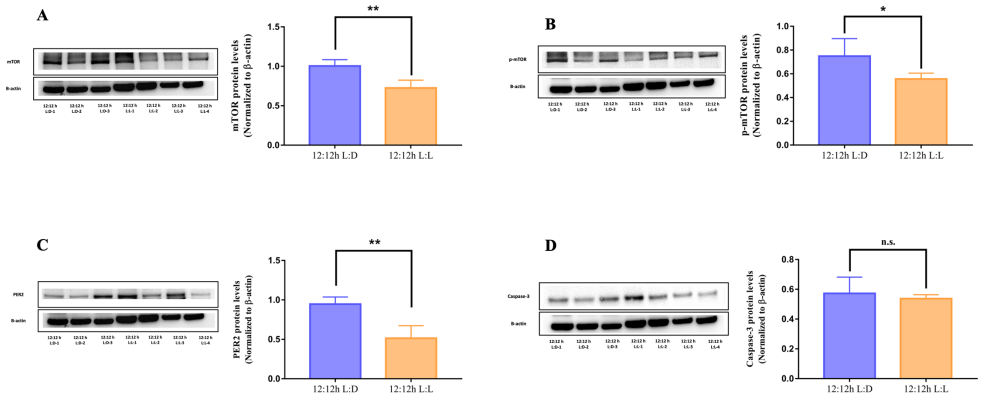


Figure 4.21. Western blot analysis. mTOR, p-mTOR, PER2 and Caspase-3 protein levels of 12:12h L:D and 12:12h L:L group are shown here. Significant difference between mTOR protein levels of 12:12h L:D and 12:12h L:L group is shown ($p<0.05$) (A).

Significant difference between p-mTOR protein levels of 12:12h L:D and 12:12h L:L group is shown ($p<0.05$) (B). Significant difference of PER2 protein levels of 12:12h L:D and 12:12h L:L group is shown ($p<0.05$) (C). No significant difference is found between Caspase-3 protein levels of 12:12h L:D and 12:12h L:L group (D) (n:5).

5. DISCUSSION AND CONCLUSION

In order to create circadian rhythm disruption, we housed mice which were selected as the 12:12h L:L in a constant light conditions for one week. The mice which were selected as the 12:12h L:D were housed in the normal light-dark cycle for one week. Symptoms that may arise due to circadian rhythm disruption began to be monitored with the start of the experiment. It is known that there is a direct relationship between the control of food intake and the biological clock (388). Because food consumption is temporally coordinated by the brain through the circadian rhythm (389) and strongly influenced by circadian rhythm alterations (390), we measured food intake of the mice at 07.00 am and 19.00 pm for each day for one week.

Feeding is known to be a highly cyclic condition and can not only be affected by the alterations in circadian rhythm but also alter it (388, 391). In the previous studies, it has been shown that mice with disrupted circadian rhythms may also experience altered feeding-fasting cycles. Global deletion in *Per1*, *Per2*, *Cry1* and *Cry2* caused damped day-night variations of food intake (392-394). Even mice consume almost 75 percent of their food during the day dark period, it has been shown that *Clock*-mutant and *Per2*-mutant mice consume almost as much food during the light period as the dark period (392, 395). It is known that suprachiasmatic nucleus (SCN) is the major controller of the daily timing of food intake (396, 397) and photic cues can reset the SCN clock (398). Besides, it is also known that the master clock is mostly entrained by light (399, 400), therefore; it is a predictable situation that the circadian rhythm, which is disrupted with the altered exposure to light, changes the feeding pattern. Although some other studies in male rats and male mice have shown that exposure to light at night does not cause a significant difference in food intake (401, 402), we found a significant difference in food intake in female mice. We observed that daytime food intake (07.00-19.00) of 12:12h L:L group is significantly higher than the 12:12h L:D group ($p<0.05$) when there is no significant difference in nighttime food intake (19.00-07.00). Furhermore, we also observed a significant increase in total food intake in 12:12h L:L group due to the increase in the amount of daytime food intake.

Previous studies have shown that exposure to light at night increases the body weight (401, 403, 404). Againist this informations we observed that mice in 12:12h L:L

group gained significantly less weight than those in the 12:12h L:D group ($p<0.05$). During the food intake measurements, we observed that the mice that would be normally resting during the daytime period were awake and active. We indicate that constant light at night may cause disruption in their rest-activity cycles of mice in 12:12h L:L group. The disruption in their rest-activity cycle may cause disruption in their fasting-feeding cycle too. Thus, we suggested that longer active hours of the 12:12h L:L mice that cannot distinguish between day and nighttime may have caused them to consume more food due to energy needs. Although mice in 12:12h L:L group consumed more food, they gained less weight because they were more active and spent more energy without resting during the daytime. Thus, probably the energy they received could not meet their energy need, and they gained less weight.

Previous studies have demonstrated the relationship between circadian rhythm and behavior-mood regulation in wild animals or laboratory animals including human (125, 405-407). It has been shown that the circadian rhythm disruptions are associated with the changes in mood-related behaviors, depression, emotional and psychotic attacks, and psychiatric disorders (125, 408). Disruptions in sleep or circadian rhythms are also thought to be associated with increases in anxiety (409).

According to the open field test results that we performed to measure locomotor and anxiety-like behavior in mice, we demonstrated that the number of fecal boli which might indicate increased anxiety and emotionality of mice were increased in 12:12h L:L group. On the other hand, we observed no difference between total distance, velocity, time spent in inner, outer or transition zone. It is known that increased fecal boli number in the open field test suggest a depressive-like behavior and a high anxiety response (410, 411). Therefore, the fact that we demonstrated no significant differences in locomotor activity markers in the 12:12h L:L group in which we showed a significant increase in the number of fecal boli can be interpreted as that constant light for one week may trigger anxiety-like behavior in mice but is not sufficient to disrupt their locomotor activities. In support of our results, there are also other studies showing that circadian rhythm disruptions with constant light for one week or different combinations of lightning conditions may not cause any change in total locomotor activity (9, 401, 403, 412, 413).

Previous studies have shown that the circadian rhythm plays a role in the regulation of the estrus cycle in female reproductive system and there is a strong relationship between them (140, 414). *Per1*, *Per2*, *Bmal1* and *Cry1* knockout mice showed irregular estrus

cycles (129, 230, 415). Apart from genetically disrupted circadian rhythms, disruption of circadian rhythm by light has also been reported to affect the estrus cycle. Mice housed in constant darkness and mice or rats housed in constant light have been demonstrated to develop disrupted/irregular estrus cycles (226, 416). In the studies on humans, it has been also reported that shift-working which increase the extent or duration of light exposure may be associated with the irregularities of menstrual cycles (134, 137, 417, 418). In our study, we showed that all of the mice in 12:12h L:L group were in pro-estrus cycle and some of the mice in the12:12h L:D group were in pro-estrus and the other part was in the metestrus.

When vaginal smear results were examined, we did not observe any noticeable irregularity in their estrus cycles between the groups. We did not see also any difference in stages of estrus cycle in the mice in 12:12h L:L group. In this study, since we aimed to examine the anxiety-like behaviors that may cause by circadian rhythm disruption with constant light, we did not want to perform any extra procedure that would cause extra anxiety and stress on animals. We applied vaginal smear only at the end of the experiment. We did not choose to take vaginal smear samples at regular intervals in order not to create an extra stress on them. Therefore, according to these results, although it seems that there is no irregularity on estrus cycles, there may be missed, prolonged or shortened stages of estrus cycle. As a previous study on rats demonstrated that constant light may cause persistent estrus (419) in mice, constant light may have triggered the persistence, lengthening or shortening of some stages of estrus cycle. The fact that each mouse in 12:12h L:L group at the same stage may not mean that they reached these stages as a result of regular and healthy estrus cycle.

Besides the relationship between circadian rhythm and estrus cycle, it has been shown that in previous studies that circadian rhythm affects many processes related to the female reproductive system. It is known that the circadian rhythm plays role in ovulation time, mating time, sexual receptivity as well as follicular development (8, 168, 203). The effect of circadian rhythm on the ovary is mostly on steroidogenesis, cellular differentiation, responsiveness to gonadotropins, ovulation and folliculogenesis (172). Studies have shown that circadian rhythm disruptions may adversely affect the female reproductive system. For instance, premature depletion of ovarian follicle reserve was observed in *Per1* and *Per2* knockout mice (225). Likewise, it is known that impaired *Per1* and *Per2* genes may cause a decrease in follicle numbers, especially in aged mice (225). It

was stated in another study that the ovaries of *Bmal1* knockout mice were smaller (230, 231). It has been also shown that the *Clock* gene may be associated with PCOS (240, 420). Although the negative effects of circadian rhythm disruption on ovary and follicular development have been explained with studies conducted on circadian rhythm genes, there are not many studies on the effect of circadian rhythm disruption by constant light on ovary and follicle development. In epidemiological studies, it has been reported that women working in shifts have higher risk for abnormal reproductive cycles, endometriosis and miscarriages due to disruption of circadian rhythm with irregular light-dark cycles (135, 136, 420). In the light of this literature information, it can be thought that light can disrupt the circadian rhythm and cause damaging effects on the organs and processes related to female reproductive system. Therefore, we aimed to determine the effects of disrupted circadian rhythm due to constant light on mice ovaries and follicular development. Although, we observed no difference in ovary sizes between groups, we demonstrated more lipid tissue around the ovaries of mice in 12:12h L:L group compared to 12:12h L:D group. In addition, we identified fibrous structures in the stroma of the ovaries of mice in 12:12h L:L group. With the follicle counting performed, we reported that the number of primordial follicles in the 12:12h L:L group decreased significantly compared to 12:12 L:D group ($p<0.05$). We have shown that the circadian rhythm disruption by constant light may cause reduction in follicle reserve. When this result is interpreted with the increase we observed in the number of pre-antral follicles in 12:12h L:L group, it may be said that the circadian rhythm, which is disrupted by constant light, may cause primordial follicle activation to occur faster and may result in consequences similar to the symptoms seen in premature ovarian insufficiency (POI) (380, 421). POI is thought as the subclass of ovarian dysfunction and, even the mechanism of it is remained unclear, it is thought to lead premature exhaustion of primordial follicle reserve (330). It is known that POI may also be associated with premature menopause that emerge before the age of 40 and both POI and premature menopause are known as the last stage of ovarian aging (336, 380). Premature menopause is characterized with the cessation of menstruation before the expected age of menopause (422). Menopause also means the cessation of ovulation and in cases of premature menopause, ovulation stops taking place before it should be ceased (423). Although, when we compared 12:12h L:L and 12:12h L:D groups, we observed no significant difference in the number of corpus luteum, which is one of the ovulation markers in ovary, the decreased number of corpus luteum in 12:12h L:L group

supports the idea that circadian rhythm disruption by constant light may trigger ovarian aging, POI or premature menopause.

When we evaluated the groups in terms of the number of atretic follicles, we observed that the number of atretic follicles in 12:12h L:L group increased and the atretic follicles were more depressive ($p<0.05$). Generally, follicular atresia which is known as the breakdown of ovarian follicles occurs throughout the lifetime of females and plays a role ovarian remodeling in each estrous cycle (424). When the atresia is not properly regulated in the ovary, it may adversely affect the primordial follicle pool and cause rapid reduction in primordial follicle reserve (425). The reduction in the number of primordial follicles in our study may be caused by rapid activation of primordial follicles as mentioned previously or increased atresia. Diseases associated with infertility such as POI may trigger follicular atresia and premature menopause may also emerge as result of increased atresia in follicles (426). In addition to the increase in the number of atretic follicles, we observed atresia in both pre-antral and pre-ovulatory follicles. However, it has been previously reported that pre-antral and pre-ovulatory follicles rarely undergo atresia (334, 335). These results made us to think that ovary could not perform its function properly and its regulation on processes performing in ovary altered. As a result of our evaluations on ovarian morphology and follicular development, we suggest that circadian rhythm disruptions by constant light may cause dysfunction in follicular development and negatively affect the regulation of atresia, thus we detected disrupted follicular development and decreased primordial follicle reserve. We suggested that the circadian rhythm disruption by constant light may trigger ovarian aging based on its effects on ovaries.

After determining the effects of circadian rhythm disruption by constant light on ovarian morphology and follicular development, we aimed to evaluate its effects on oocytes. In previous studies, it has been reported that expressions of clock genes are found in the rabbit, bovine and mice oocytes and mRNA of clock genes fluctuates with the function of developmental stage (208-211). Therefore, it is thought that the circadian rhythm is one of the regulators of the healthy oocyte maturation (172). *Cry1* or *Bmal1*, are very important for oocyte maturation and it has been shown that knockout of *Cry1* or *Bmal1* have damaging effects on oocyte maturation (208, 222). However, there has been no study showing the effect of circadian rhythm disruption on oocytes from mice whose circadian rhythm is disrupted by constant light. After demonstrating the damaging effects

of circadian rhythm disruption by constant light on ovarian morphology and follicular development, we aimed to determine the effects of the circadian rhythm disruption by constant light on oocytes in our study. Since we demonstrated that circadian rhythm disruption by constant light caused premature ovarian aging, we thought that it may cause premature aging in oocytes. It is known that oocyte quality is affected because of ovarian aging and during oocyte aging some morphological alterations in polar body, cytoskeleton and zona pellucida start to be observed (350). Zona pellucida hardening is observed in oocyte with age. Granulofibrillar zona pellucida which is interconnected with pores is observed in fresh oocytes but in aged oocytes, zona pellucida becomes harden and get cobblestone appearance (368, 370, 427). Therefore; we evaluated ZP3 by immunofluorescence to evaluate whether circadian rhythm disruption by constant light caused premature aging in oocytes or not. We observed very weak, almost no ZP3 expression in the oocyte and zona pellucida structures of growing follicles in ovaries of mice in 12:12h L:L group. We reported that ZP3 expressions were much stronger in the 12:12h L:D group compared to the 12:12h L:L group. We have shown that circadian rhythm disruption by constant light caused decrease in ZP3 expression in oocyte and zona pellucida structures in the mice ovary. When these findings is evaluated with the previous study showing significantly decreased ZP3 mRNA levels in aged mice oocytes (428), we can indicate that disruption of the circadian rhythm by constant light may cause oocyte aging.

We also evaluated the oxidative stress, which is the one of the markers of oocyte aging, to determine whether the circadian rhythm disruption by constant light causes oocyte aging. It is known that increased ROS damages are usually found in the oocytes whose quality decreased because of age, thus ROS levels are thought to be a marker for aged oocytes (334, 350). In other studies, it has been also demonstrated that oxidative stress is accompanied by the pathological process of aging and may promote ovarian aging (429, 430). In addition, the relationships between oxidative stress and circadian rhythm have been shown in studies and it has been shown that circadian rhythm disruptions may cause oxidative stress as well as irregularities in antioxidant mechanisms (125, 431, 432). In order to evaluate, the oxidative stress between the groups, we performed immunofluorescence staining for NTY which is known as oxidative stress, inflammation and cell damage marker (433, 434). We demonstrated that NTY signals were more

stronger in 12:12h L:L group compared to 12:12h L:D group. We observed gradual increase in oxidative stress following the follicular developmental stages.

In our study, which determines the negative effects of circadian rhythm disruption by constant light on ovarian function, ovarian follicles and oocyte, we aimed to evaluate the protein levels of PER2 which is expressed in ovarian cells and protein levels of mTOR which functions in various roles in ovary in order to elucidate the molecular mechanism between circadian rhythm disruption and its damaging effects on mice ovary. In previous studies, circadian clock genes were determined in the ovaries of rats, ruminants and mice (6). mRNA expression of *Per2* have been demonstrated in interstitial glandular tissue, granulosa cells of pre-antral and pre-ovulatory follicles and corpus luteum (8). It is also known that PER2 functions in steroid production and cell proliferation in granulosa cells (6). Besides, studies on PER2 have shown that the circadian rhythm disruptions by altering lightning conditions may changes its rhythmicity, mRNA and protein levels. It has been demonstrated that light exposure at night causes a decrease in the rhythmic expression of *Per2* gene and its mRNA levels in peripheral tissues such as liver and adipose tissue (7). It has been reported that constant light may cause attenuated circadian rhythm of *Per2* mRNA and protein levels in suprachiasmatic nucleus of mice (9). In another study supporting the relationship between light exposure at night and *Per2* mRNA and protein levels; it has been reported that dim light at night caused attenuation in the amplitude of *Per2* rhythm (404). In support of previous studies, we showed that the circadian rhythm disruption by constant light caused PER2 protein levels to decrease also in mouse ovary. Therefore, we suggest that the circadian rhythm disruption by constant light may cause negative consequences on the mice ovaries through PER2.

It is known that the mTOR signaling pathway has many different roles in the ovary and is very important for the regulation of ovarian functions. In previous studies, it has been demonstrated that mTOR plays an important role during folliculogenesis (14), oocyte maturation (305), ovarian somatic cell proliferation (306) and ovarian aging (15). According to our results of ovarian morphology and follicle counting, we have shown that circadian rhythm disruption by constant light caused damaging effects on follicular development and triggered consequences that can be associated with ovarian aging. This situation suggests that there may have been dysfunction or alteration in mTOR signaling pathway in ovaries of mice in 12:12h L:L group. When a relationship between PER2 and mTOR in liver tissue was shown in a recent study (1), it suggested that there may also be

an association between mTOR and PER2 in mice ovaries too. Until now, the possible relationship between mTOR and PER2 has not been demonstrated in the ovary. Thus, we suggested that circadian rhythm disruption by light may cause damaging effects on ovary, follicular development and oocytes through a possible relationship between PER2 and mTOR in mice ovary. Unlike the suppressive effect of PER2 on mTOR signaling pathway shown in mice liver (1), we demonstrated decreased protein levels of mTOR in the ovaries of mice in 12:12h L:L group in which protein level of PER2 also decreased.. It was reported that increased mTOR signaling causes activation of primordial follicle (312). Therefore, from this point of view, an increased amount of mTOR protein levels are expected in ovaries which show ovarian-aging like morphology such as decreased primordial follicle numbers. At the same time, it has been supported by previous studies showing the increasing number of primordial follicles when mTOR signaling is inhibited by rapamycin (15, 339). However, in our study, we detected decreased number of primordial follicles with decreased mTOR protein levels. This suggests that constant light may not be able to cause primordial follicle pool to decrease by triggering primordial follicle activation but affecting the atresia mechanism.

mTOR is phosphorylated by its serine residue 2448 and phosphorylated mTOR (p-mTOR) is known as activated form of mTOR (316). We observed decreased p-mTOR protein levels in ovaries from 12:12h L:L group. It is known that mTOR is expressed in both granulosa cells and oocyte cytoplasm and p-mTOR is expressed in nuclei in granulosa cell during mitosis (316, 379).

In this study we aimed to disrupt ovarian peripheral clock by disrupting central clock with 12:12h L:L exposure. We showed that 12:12h L:L triggered decreased phosphorylation of mTOR (p-mTOR (serine 2448)) which has very important during follicular development with join to mitotic division of granulosa cell.

Decrease protein expression of the mTOR and p-mTOR (2448) by circadian rhythm disruption for one week may mediates decrease protein expression of PER2. Therefore we suggested that mTOR could stimulate PER2 activity by several routes: firstly, we showed that the number of atretic follicles are higher in 12:12h L:L group than 12:12h L:D group. Furthermore, we also detected decreased Caspase-3 protein level in 12:12h L:L group. As we know, *Per2* reduces de novo protein synthesis but increase autophagy (1). Therefore we suggested that mTOR may trigger phosphorylation-dependent activation of P70S6K and cause autophagy of ovarian follicles during atresia mediated *Per2* or phosphorylation-

dependent inhibition of 4EBP1 in inhibition of protein synthesis.

Secondly, mTOR signaling pathway has very crucial role in regulation of energy metabolism with protein anabolism and catabolism. Additionally, circadian clock and mTOR pathway play critical role in regulation of metabolism (435). The interaction between mTOR signaling pathway and circadian clock also have been implicated in the control of aging in different organisms (435).

Interestingly, we observed increased total food intake againist less weight gain in 12:12h L:L group. In this point, we suggested that metabolic energy homeostasis may disrupt in 12:12h L:L group and circadian rhythm disruption may change mTOR and PER2 expression in linking metabolic states to response to circadian clock functions of ovary. Therefore, we think that premature aging of ovary may ocur through increased oxidative stress with high expression of NTY in oocyte cytoplasm, decreased oocyte ZP3 expression in zona pellucida and increased number of atretic follicles in circadian rhythm disrupted group.

Within the scope of all this literature and our results, we can say that constant light may reduce follicle reserve, cause follicles to go rapidly atresia and disrupt the oocyte quality, thus it may be a risk factor for female reproductive diseases that adversely affect fertility in females such as premature ovarian failure and early menopause. With the future studies on the relationship between the circadian rhythm disrupted by light and ovarian function, and with the explanation of molecular or hormonal mechanism underlying this relationship may lead the usage of circadian rhythm-based or light-based therapies currently using to treat some diseases or reduce the symptoms on also female reproductive system related diseases. Circadian rhythm-based therapies which can be used to treat female reproductive diseases that may result in infertility or to relieve symptoms may be cheaper, more accessible and less stressful than the medications. It should not be forgotten that in today's developing conditions, irregular working schedules, shift-working, frequent travels and usage of electronic devices (especially in the evening) cause the circadian rhythm to be disrupted, especially by the light exposure. This situation affects not only the females' reproductive system functions and fertility, but also affects the offspring that even from the pregnancy (125). Thus, the effect of disrupted circadian rhythm on reproduction continue throughout the generations. Giving importance to the circadian rhythm as much as other factors in studies to prevent this situation will lead to improve new treatments.

Within this study, we wanted to demonstrate once again the importance of circadian rhythm, especially light exposure, on the female reproductive system and contribute to development of new treatment methods for female reproductive system related diseases.

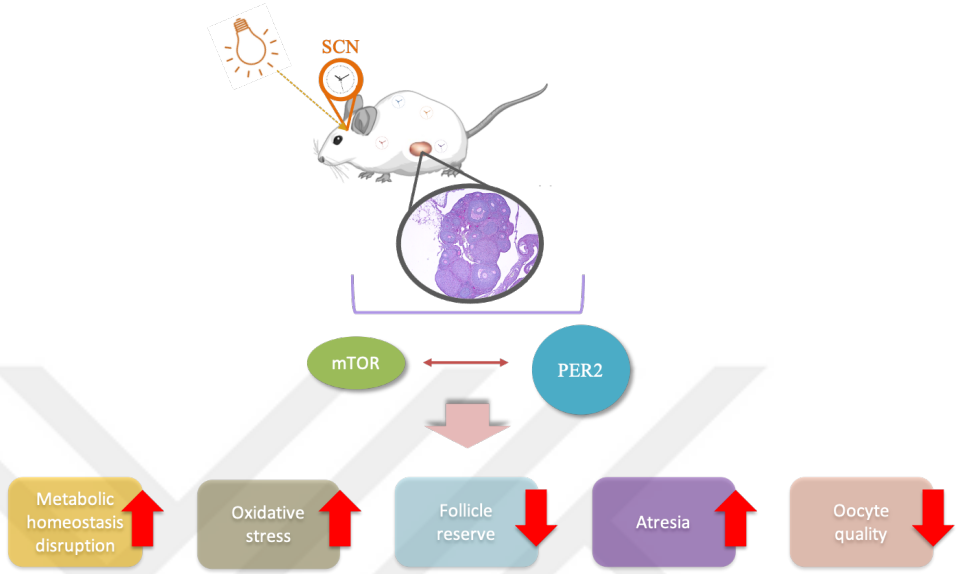


Figure 5.1. Circadian rhythm disrupted by constant light and ovarian function in mice**.** Circadian rhythm disruption may cause ovarian reserve to decrease by decreasing primordial follicle numbers in mice ovary. Circadian rhythm disruption by constant light may also increase follicular atresia and may lead premature oocyte aging. Underlying mechanism of relationship between circadian rhythm disruption by constant light and ovary function may include the possible relationship between PER2 and mTOR signaling pathway. There may be also some other molecular or hormonal mechanisms underlying the relationship between circadian rhythm disruption by constant light and ovarian function.

We concluded that the crosstalk between mTOR and PER2 may regulate ovarian circadian clock function including and ovarian physiological functions include preservation of follicle reserve, oocyte maturation and quality. It may be worthwhile to investigate the potential contribution of PER2-mTOR interaction to ovarian reproductive clock. We suggested that our findings can provide exciting new insights the mechanisms underlying diminished ovarian reserve and oocyte quality with circadian rythm disruption. Further studies may shed light on possible effects of light disruption on ovarian aging which SCN clock regulates with ovarian clock as a peripheral organ clock and mTOR-PER2 connection might help to understand underlying mechanisms of premature ovarian aging.

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7. APPENDICES

7.1. Ethical Approval

T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2019-788	22.10.2019	2019/10	2019/10-5	Doç. Dr. Aylin YABA UÇAR

'Sirkadiyen Ritmin Işık ile Bozulmasının mTOR Sinyal Yolağı Üzerinden Oosit Yaşlanmasına Olan Etkisinin Belirlenmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.

Hayvan Türü / Irkı	Toplam Hayvan Sayısı	Hayvanın Cinsiyeti
Fare / BALB/c	20	Dişi

Görevi	Adı Soyadı
Başkan	Prof. Dr. Bayram YILMAZ
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA
Üye	Veteriner Hekim Engin SÜMER
Üye	Prof. Dr. M. Ece GENÇ
Üye	Prof. Dr. Rukset ATTAR
Üye	Prof. Dr. Gamze TORUN KÖSE
Üye	Doç. Dr. Ediz DENİZ
Üye	Doç. Dr. Aylin YABA UÇAR
Üye	Hakan GÖKSEL
Üye	Ahmet ŞENKARDEŞLER

T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2020-829	18.02.2020	2020/02	2020/02-11	Doç.Dr. Aylin YABA UÇAR
'Sirkadiyen Ritmin Işık ile Bozulmasının mTOR Sinyal Yolağı Üzerinden Oosit Yaşlanmasına Olan Etkisinin Belirlenmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				

Hayvan Türü / Irkı	Toplam Hayvan Sayısı	Hayvanın Cinsiyeti
Fare / BALB/C	12	Erkek ve Dişi

Görevi	Adı Soyadı
Başkan	Prof. Dr. Bayram YILMAZ
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA
Üye	Veteriner Hekim Engin SÜMER
Üye	Prof. Dr. M. Ece GENÇ
Üye	Prof. Dr. Rukset ATTAR
Üye	Prof. Dr. Gamze TORUN KÖSE
Üye	Doç. Dr. Ediz DENİZ
Üye	Doç. Dr. Aylin YABA UÇAR
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL
Üye	Hakan GÖKSEL
Üye	Ahmet ŞENKARDEŞLER

8. CURRICULUM VITAE

Personal Informations

Name	Gizem	**Surname**	BORA
Place of Birth		**Date of Birth**	
Nationality		**TR ID Number**	
E-mail		**Phone number**	

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate			
Master			
University	Koç University	Molecular Biology and Genetics	2015
High school	Cağaloğlu Anatolian Highschool		2009

Languages	**Grades (#)**
English	
German	

All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Work Experience (Sort from present to past)

Position	**1.1.1. Institute**	**Duration (Year - Year)**
		-
		-

Computer Skills

Program	**Level**
MS Office Programs	Good

***Excellent , good, average or basic**

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Articles published in other journals

Proceedings presented in international scientific meetings and published in proceedings book.

Journals in the proceedings book of the refereed conference / symposium

Others (Projects / Certificates / Rewards)

Poster Presantation in 24th National Congress of Electron Microscopy (2019)
3rd Prize of Oral Presentation in 4th International Congress of Turkish Neuroendocrinology Society (2020)