Regulatory Role of the Circadian Clock System in Luteal Regression and Cell Death Pathways in Cattle

(ウシにおける概日時計システムの黄体退行および細胞死経路に対する制御機構)

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Contents

Abbreviations

WHO	The World Health Organization
FAO	The Food and Agriculture Organization of the United Nations
CL	Corpus Luteum
P4	Progesterone
CC	Circadian Clock
WB	Western Blotting
cDNA	Complementary DNA
IF	Immunofluorescence
mRNA	Messenger RNA
$PGF_{2\alpha}$	Prostaglandin F2α
ELISA	Enzyme-linked Immunosorbent Assay
PBS	Phosphate Buffered Saline
qPCR	Quantitative real-time PCR
LH	Luteinizing Hormone
CLOCK	Output Cycles Kaput
BMAL1	Brain and Muscle Arnt-Like 1
CRYs	Cryptochrome
PERs	Period
NR1D1	Nuclear Receptor Subfamily 1 Group D Member 1
RORα	Retinoic Acid-Related Orphan Receptors
SEM	Standard Error of The Mean
CCGs	Clock-Controlled Genes
LD	Light-Dark Cycles Constant
DD	Constant Darkness
R.T.	Room Temperature
ROS	Reactive Oxygen Species
SCN	Suprachiasmatic Nucleus
STAR	Steroidogenic Acute Regulatory Protein
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
HSD3β	Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase
PVDF	Polyvinylidene Difluoride
TBST	Tris Buffered Saline Tween-20
CTSB	Cathepsin B

MEFs	Mouse Embryonic Fibroblasts
BGCs	Bovine Granulosa cells

Unit measures abbreviations

%	Percentage
× g	Times gravity
°C	Degree Celsius
h	Hour
min	Minute
s	Second
g	Gram
mg	Microgram
ng	Nanogram
mL	Milliliter
µL	Microliter
µm	Micrometer

Abstract

The corpus luteum (CL) is a transient endocrine structure that secretes progesterone (P4) following ovulation and plays a crucial role in regulating the estrous cycle and maintaining pregnancy in mammals. In livestock species—particularly in cattle—appropriate CL function is essential for the precise timing of the estrous cycle and successful reproduction. Disruptions in luteal function can lead to irregular estrous cycles, impaired implantation, and reduced fertility, ultimately decreasing reproductive efficiency in herd management systems.

Luteolysis, the structural and functional degradation of the CL, is mediated by programmed cell death pathways such as apoptosis and autophagy. The circadian clock (CC) system has been extensively studied as a mechanism for regulating physiological functions in a rhythmic and time-dependent manner. However, its involvement in rapid and acute tissue remodeling processes, such as CL regression, remains poorly understood. In particular, whether and how core clock components like the nuclear receptor subfamily 1 group D member 1 (*NR1D1*) contribute to the regulation of apoptosis and autophagy during the short timescale of luteolysis has yet to be elucidated.

To address this gap, the present study aimed to clarify the regulatory role of NR1D1 in bovine CL regression, particularly its interaction with autophagy- and apoptosis-related mechanisms. Across four stages of the estrous cycle—early, mid, late, and regression—I investigated the dynamic expression patterns of P4, steroidogenic enzymes, and CC components (Chapter II). An *ex vivo* model of $PGF_{2\alpha}$-induced

luteolysis was then established to evaluate functional and molecular markers of CL regression, including apoptotic and autophagic pathways (Chapter III). To examine the specific involvement of NR1D1, pharmacological activation or inhibition using GSK4112 and SR8278 was applied, revealing opposing effects on P4 synthesis and cell death markers (Chapter IV). Finally, the combined treatment of $PGF_{2\alpha}$ with SR8278 was used to assess whether *NR1D1* antagonism could counteract luteolysis (Chapter V). Collectively, this dissertation aimed to elucidate how the CC system, and NR1D1 in particular, regulates bovine CL function and regression, offering insights for improving reproductive efficiency in cattle.

In Chapter II, I investigated the dynamics of P4 content, the expression of steroidogenesis-related genes, and the gene and protein expression of CC system components in the bovine CL across four luteal stages. The results showed that P4 secretion followed a distinct unimodal pattern throughout CL development, peaking at 1.91 ng/mg during the mid-luteal stage and subsequently declining to 0.144 ng/mg in the regression stage, representing a 63.4% reduction from the peak. These changes paralleled the expression profiles of key steroidogenic genes, including *STAR*, *CYP11A1*, and *HSD3β*. I used immunofluorescence staining to localize NR1D1 and BMAL1 proteins in nuclear and cytoplasmic compartments. Combined with qPCR and Western blot analyses, the data indicated that NR1D1 expression was significantly elevated during the late luteal phase, while other core CC genes (*CLOCK*, *PER2*, *CRY1*) also peaked in this stage. In contrast, *BMAL1* and *DBP* expression reached their maximum during the mid-luteal stage, aligning with peak P4 and steroidogenic gene

expression. These findings suggest that the *NR1D1/BMAL1* axis may be involved in the intrinsic timing mechanism regulating bovine luteal phase transitions.

In Chapter III, I developed an *ex vivo* model of induced CL regression by treating mid-luteal tissues with 1 μM $PGF_{2\alpha}$ for 24 h. I observed significant reductions in P4 content in both the luteal tissue and the culture medium, confirming the successful establishment of the regression model. Consistent with functional regression, I detected increased expression of apoptotic markers (*CTSB*, *CASPASE8*) and autophagic markers (*LC3B*, *ATG5*), with CTSB showing the most marked upregulation. I also analyzed the expression of CC-related genes and proteins, and although $PGF_{2\alpha}$ treatment tended to suppress *NR1D1* gene expression and BMAL1 protein levels, these changes were not statistically significant. Overall, these results indicate that the apoptotic pathway is preferentially activated and that the CC system may be involved in $PGF_{2\alpha}$-induced luteolysis.

In Chapter IV, I established a model to disrupt the CC system by treating mid-luteal tissues with the NR1D1 agonist GSK4112 and antagonist SR8278. Both treatments significantly reduced P4 levels, and GSK4112 treatment led to downregulation of *CYP11A1* expression. I found that *NR1D1* expression was significantly increased following GSK4112 treatment, along with upregulation of the autophagy-related gene *ATG5* and the apoptosis-related gene *CTSB*. In contrast, SR8278 treatment produced the opposite trends in these markers, suggesting that SR8278 may possess the potential to counteract luteal regression.

In Chapter V, I examined whether SR8278 could mitigate $PGF_{2\alpha}$-induced CL

regression by co-treating mid-luteal tissues with $PGF_{2\alpha}$ and SR8278. I observed that the co-treatment significantly restored P4 synthesis, particularly in the culture medium, and increased the expression of the steroidogenic gene *STAR*. Importantly, the lysosomal protease CTSB showed an opposite trend in the PG + SR-6h and PG + SR groups, while LC3 expression increased, suggesting modulation of autophagic activity. These findings imply that SR8278 may reverse $PGF_{2\alpha}$-induced luteal regression by restoring steroidogenic capacity and suppressing apoptotic activity.

In conclusion, the results presented in Chapters II to V suggest that the CC system exhibits a dynamic regulatory pattern during luteal development and that NR1D1 interacts with $PGF_{2\alpha}$ to modulate CL regression. These new findings highlight the CC system, particularly NR1D1, as a novel potential molecular target for improving reproductive efficiency in cattle.

Key words: Corpus luteum regression, Cow, Circadian clock system, autophagy, apoptosis

Chapter I

General Introduction

The British classical economist Thomas Robert Malthus predicted that a food and ecological crisis was inevitable because the population would grow exponentially, but food resources would only grow arithmetically [1]. With the exponential growth of the global population, coupled with agricultural declines attributed to climate change, driving global hunger rates back to decadal highs [2]. Tackling hunger is one of the greatest challenges of our time [3]. It is predicted that the global population will reach 9.9 billion by 2050. This demographic surge imposes unprecedented stress on Earth's finite resources, particularly as rising national incomes fuel escalating food consumption. For instance, the World Health Organization (WHO) reported that France's animal product intake exceeds international health recommendations [4]. While Rachmawati et al. [5] highlight that in Indonesia, the national demand for beef reached 680,000 tons in 2019, but the production was only 500,000 tons, resulting in a shortage of approximately 210,000 tons of beef supply in 2021. The Food and Agriculture Organization of the United Nations (FAO) forecasts that global demand for animal-sourced foods will reach 550 million tons by 2050 [6]. Against this backdrop, beef and dairy products serve as critical sources of high-quality protein amid dietary transitions. Cattle, as one of the most important domestic animals, occupy an irreplaceable position in human society. Globally, prized for their genetic versatility,

production efficiency and economic value, and support global food security. According to statistics from the FAO, global beef consumption reached 49 million tons in 2024, growing at 2.3% annually, while dairy cows must produce over 1 billion tons of milk annually to meet market demand. However, currently, cattle reproductive inefficiency threatens supply stability. Approximately 30% of global cows fail to reproduce due to abnormal luteal function (such as delayed or incomplete degeneration), causing over $20 billion in annual livestock losses, particularly in intensive breeding systems. Improving productivity and preventing reproductive decline in livestock requires effective reproductive management practices, including nutritional regulation, estrus detection, appropriate artificial insemination or embryo transfer, and pregnancy diagnosis. To facilitate these strategies, basic scientific research is critically needed to establish a robust foundation for evidence-based reproductive management.

In the reproductive process of mammals, luteolysis represents a crucial physiological event, with its precise regulation playing a decisive role in the establishment and maintenance of a normal reproductive cyclicity and the successful pregnancy [7,8]. As a cornerstone of global livestock agriculture, the 240-day gestation cycle hinges on the precise timing of luteolysis, a process central to estrus synchronization and pregnancy outcomes in cattle. Deciphering the exploration of the molecular mechanism underlying bovine luteolysis not only helps to reveal the fundamental understanding of mammalian reproductive regulation but also provides the crucial theoretical basis and practical guidance for enhancing the reproductive management of cattle. Importantly, the regulatory mechanisms governing luteolysis

exhibit cross-species conservation. As a quintessential monotocous model, the bovine shares striking similarities in the luteal regression process with humans, sheep, pigs, mice, and other mammals [9–12]. This makes cattle an ideal model to dissect the synergistic interplay of apoptosis, autophagy, and hormone signaling during reproductive cycling [13–16]. Additionally, it provides an important reference for understanding the pathological mechanisms of reproductive diseases in mammals. Particularly in the field of human medicine, this model offers a valuable significance for studying the pathogenesis of diseases such as luteal insufficiency and early miscarriage.

The corpus luteum (CL) is an essential transient endocrine structure in the mammalian ovary, typically existing for about 21 days in cattle [17–19], Similar to the 28-day human cycle [20]. The CL forms through luteinization of granulosa cells and theca cells under the influence of luteinizing hormone (LH) stimulation following the follicular wall's collapse after ovulation [7,21]. This transformation involves vascularization and tissue reorganization as the follicular membrane connective tissue and capillaries extend into the granulosa layer. It primarily secretes progesterone (P4), a hormone crucial for managing the estrous cycle and supporting pregnancy. Actually, the earliest clear description and illustration of the CL was documented by Regnier de Graaf, who referred to it as "globules"[22]. In 1681, Marcello Malpighi coined the term "corpus luteum" in a letter to Jacob Spon, and published in 1685 [22]. The accurate physiological functions of the CL were not systematically reported until 1901, while the discovery and characterization of "progesterone" as a key hormone were first

described in 1934 by Allen [23–25]. P4 orchestrates a series of physiological events critical for successful reproduction. It modulates the endometrium, inducing its transformation into a secretory state that supports embryo implantation and nourishment [26–28]. Additionally, P4 suppresses uterine smooth muscle contractions, creating a quiescent environment conducive to embryonic development, thereby serving as a linchpin in pregnancy maintenance [29–31]. In the absence of fertilization, the CL initiates a programmed regression process, which can be divided into two distinct phases: functional and structural luteolysis [32]. Functional luteolysis is characterized by a precipitous decline in P4 synthesis and secretion, signaling the termination of the CL's endocrine activity. This is followed by structural luteolysis, a cascade of events that includes luteal cell apoptosis, extracellular matrix degradation, and vascular regression, ultimately leading to the involution and disappearance of the CL and completing the reproductive cycle [33,34]. The lifecycle of the CL includes a series of well-orchestrated physiological phases-formation, maturation, maintenance, and luteolysis [35,36]. During its regression, numerous complex molecular and cellular mechanisms are tightly regulated, encompassing apoptosis, autophagy, and modulation by various signaling pathways, especially prostaglandins. Prostaglandins, particularly prostaglandin F2α ($PGF_{2\alpha}$), are physiologically active unsaturated fatty acids found in various tissues and body fluids, noted for their strong luteolytic effects [37,38]. It plays a core role in the luteolytic process of cattle and is recognized as the main luteinizing factor [39–41]. Studies indicate that the degradation of the CL at the end of the estrus cycle in ruminants begins with $PGF_{2\alpha}$ release from the uterus, transmitted to the CL

through the countercurrent exchange system between the uterine and ovarian arteries [42,43]. $PGF_{2\alpha}$ significantly influences steroidogenesis by altering steroid-related enzymes and gene expression, affecting luteolysis and determining the lifespan of the CL, both *in vivo* and *in vitro* [44–46]. Research has shown that $PGF_{2\alpha}$ triggers an increase in intracellular reactive oxygen species (ROS) and enhances receptor-mediated activation of the PINK-Parkin mitotic pathway [38], pointing to mitochondria as a novel target in the cellular response to $PGF_{2\alpha}$, thus providing new insights into the mechanisms of CL regression [47,48]. Inhibition of autophagosome formation has also been observed to decrease luteal cell apoptosis and cell death, particularly during CL regression [49,50]. Additionally, past findings from my laboratory have highlighted significant upregulation of Cathepsin B (CTSB) in the late stage of CL life [51], underlining the role of $PGF_{2\alpha}$ in luteolysis through both autophagy and apoptosis.

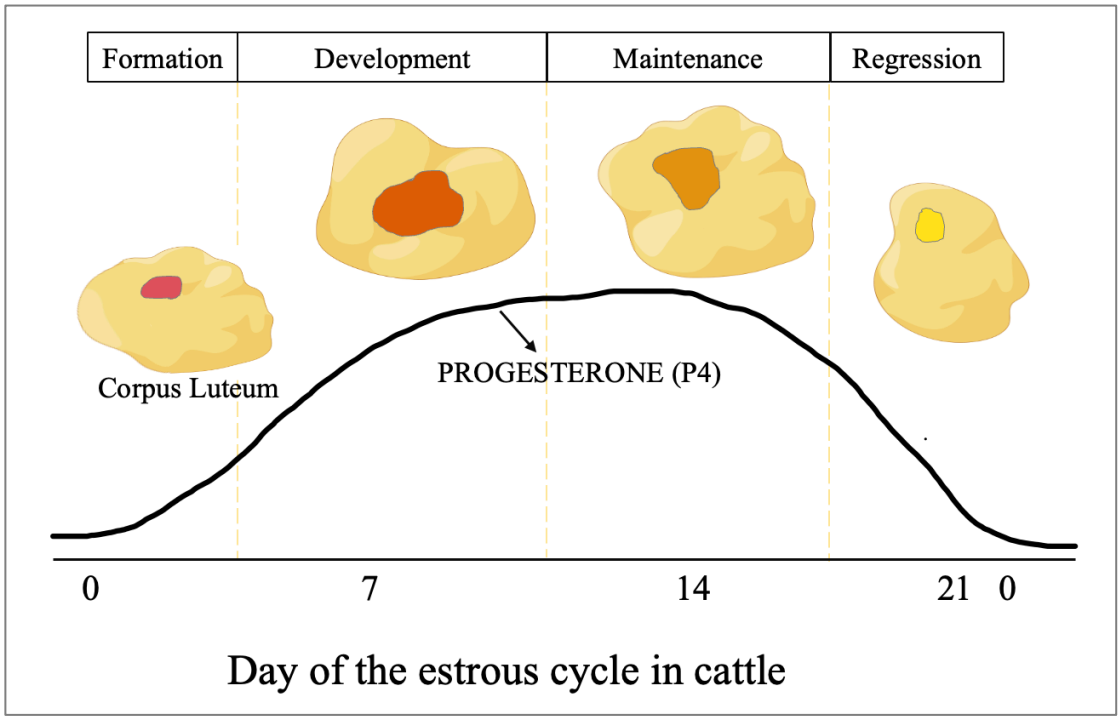


Figure. 1-1 The day of the estrous cycle in cattle

Autophagy, a self-degradative process essential for cellular homeostasis, is a generic term for all pathways by which cytoplasmic materials are delivered to the lysosome in animal cells or the vacuole in plant and yeast cells [52]. Autophagy is characterized by the formation of double-membraned autophagosomes that sequester and degrade damaged organelles, misfolded proteins, and protein aggregates [53–56]. Depending on the molecular mechanisms that are involved, autophagy is divided into macroautophagy, microautophagy, and chaperone-mediated autophagy [57,58]. Of these, multiple ATG proteins govern autophagosome formation. Among the 35 ATG proteins thus far identified in yeast, ATG1-10, 12-14, 16, and 18 are the "core ATG proteins"[59]. In brief, these are the ULK complex (ULK1, ULK2, ATG13, RBCC1/FIP200); the class III lipid kinase complex I producing phosphatidylinositol 3-phosphate (PIK3C3/VPS34, PIK3R4/p150, BECN1/Beclin 1, ATG14); 2 ubiqutin-like conjugation complexes (ATG3, ATG7, ATG10) [60]. They are highly conserved in mammals, and it has become clear that autophagy occupies a central position in the biology of most eukaryotes, interfacing with the regulation of core metabolism [61,62], damage control [63], cell death [64,65], and plays a key role during luteolysis in maintaining cellular homeostasis [66,67]. Apoptosis, a type of programmed cell death involving various pathways and mechanisms [68], is another critical mechanism in CL regression. During luteolysis, luteal cells display classical apoptotic features including nuclear condensation, chromatin margination, and DNA fragmentation, accompanied by altered expression of apoptosis-related genes/proteins [15,69,70]. Extensive research on both autophagy and apoptosis highlights their importance in this process [71–73].

Studies have demonstrated various forms of programmed cell death in CL cells, including caspase-dependent apoptosis, autophagic cell death mediated by the ATG family, and RIPK-dependent programmed necrosis [15]. A comprehensive review by Teeli et al. discusses the regulation of CL regression through the ubiquitin-proteasome and autophagy pathways, also considering the potential transcription factors involved in these processes [66]. Notably, both autophagy and apoptosis have emerged as central players in the $PGF_{2\alpha}$-induced luteal regression event.

The circadian clock (CC) system is well recognized in physiological processes such as hormone release, metabolism, and behavior, ensuring synchronization with the natural day-night cycle [74–77]. The master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus aligns these processes with environmental light cues. As an endogenous timing system in organisms, it is composed of a network of core clock genes and their encoded proteins [78–80]. The rhythmic oscillations of the mammalian CC arise from the interplay of transcriptional and translational positive-negative feedback loops involving multiple clock genes and their encoded proteins [81]. Key clock genes identified to date include Circadian Locomotor Output Cycles Kaput (*CLOCK*), Brain and Muscle Arnt-Like 1 (*BMAL1*), Cryptochromes (*CRYs*, *CRY1*/*CRY2*), Periods (*PERs*, *PER1*/*PER2*/*PER3*), Nuclear Receptor Subfamily 1 Group D Member 1 (*NR1D1*), and Retinoic Acid-Related Orphan Receptors (*RORs*, *RORα*/*RORβ*). The positive regulators *BMAL1* and *CLOCK* form heterodimers that drive transcription of *PERs* and *CRYs* genes, while the resulting PERs and CRYs proteins inhibit this activity, creating the core feedback loop. Beyond this core

mechanism, supplementary loops involving NR1D1 and RORα/β modulate clock rhythmicity: the *BMAL1-CLOCK* dimer promotes *NR1D1* and *RORα* transcription via E-box enhancer elements, while NR1D1 and RORα proteins competitively bind to retinoic acid-related orphan receptor response elements (ROREs) in the BMAL1 promoter to inhibit or enhance BMAL1 transcription, respectively [82,83]. Through cis-acting elements like E-box and RORE, the circadian system coordinates rhythmic expression of numerous clock-controlled genes to regulate diverse physiological processes, such as hormonal dysregulation and metabolic imbalance in cells. In mammalian reproductive systems, these genes are ubiquitously expressed in key tissues including the ovaries, uterus, and CL, where their oscillatory expression patterns are critically linked to normal reproductive function [81,84–87]. Disruption of the circadian rhythm can lead to various health problems, including reproductive disturbances [88,89]. In luteal cells, dynamic changes are observed throughout the estrous cycle and pregnancy, where light condition alterations have been shown to influence P4 synthesis and *Star* gene expression [90]. The involvement of the CC system in CL regression suggests that the precise timing of physiological events is crucial for optimal reproductive success. The CC system has been extensively studied as a mechanism for regulating physiological functions in a rhythmic and time-dependent manner. However, its involvement in rapid and acute tissue remodeling processes, such as CL regression, remains poorly understood. In particular, whether and how core clock components like the NR1D1 contribute to the regulation of apoptosis and autophagy during the short timescale of luteolysis has yet to be elucidated. Despite these established associations,

the precise molecular mechanisms by which the CC system regulates $PGF_{2\alpha}$-induced luteolysis in cattle remain unclear. In particular, the temporal coordination between clock gene oscillations and key luteolytic pathways requires further elucidation.

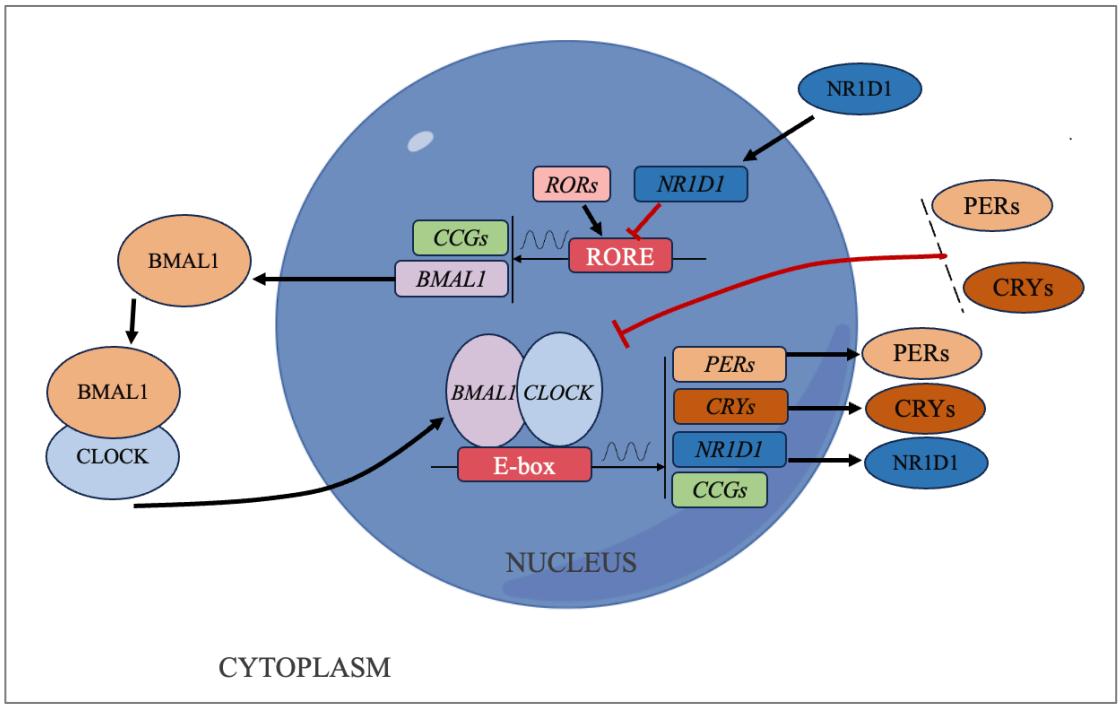


Figure. 1-2 The circadian clock system network in mammals

In this study, I aimed to elucidate the potential involvement of the CC system during bovine luteal formation and regression, with particular focus on the effects of the luteolytic hormone $PGF_{2\alpha}$ on CL regression in cattle. Through a combination of an *ex vivo* model using fresh CL tissues and the mid-CL models, I have obtained preliminary evidence establishing a functional relationship between circadian regulation and luteal regression processes. Furthermore, by elucidating the role of NR1D1 in the $PGF_{2\alpha}$-induced CL regression model, I aimed to: (1) establish a novel chronobiological framework for understanding luteolysis, (2) identify key molecular

interactions between the CC system and luteolytic pathways, and (3) explore potential applications of circadian modulation for enhancing reproductive efficiency in cattle.

Chapter II

Dynamic profiling of progesterone steroidogenic and circadian clock molecules during luteal development

2.1 Introduction

As the core endocrine organ regulating the reproductive cycle, the bovine CL functional state directly determines the synthesis efficiency of P4 and the success or failure of embryo implantation [7,38,91]. The dynamic secretion of P4 depends on the precise expression of steroid synthesis genes and is closely related to the formation, development, maintenance, and regression processes of the CL. The life cycle of the bovine CL can be divided into the formation stage (1-3 days after ovulation), the functional stage (4-17 days), and the regression stage (17 days to apoptosis), three stages [92]. During the formation period, follicular membrane cells and granulosa cells rapidly proliferate and differentiate into luteinizing cells driven by LH, initiating the process of P4 synthesis. The functional phase is the peak stage of P4 secretion. High levels of P4 create a microenvironment suitable for embryo implantation by acting on endometrial receptors. If conception does not occur, the CL enters a degenerative phase, with a sharp decline in P4 synthesis, accompanied by cell apoptosis and tissue structure disintegration. This dynamic secretion pattern of P4 is essentially the result of the phased activation and inhibition of the steroid synthesis gene network [93]. The core

steps of steroid synthesis include cholesterol transport, transmitochondrial membrane metabolism and cytochrome P450 catalytic reactions. This process mainly involves Steroidogenic Acute Regulatory Protein (STAR), Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1), and Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase (HSD3β) [94–96]. Existing studies have shown that the transcriptional levels of *STAR* and *CYP11A1* during the luteal formation period are significantly upregulated and positively correlated with the secretion of P4 [97,98]. Following the occurrence of the cyclical, the steroid synthesis pathway is blocked due to the transcriptional inhibition induced by $PGF_{2\alpha}$ [99]. In cattle, it is clear that a decline in P4 concentration by $PGF_{2\alpha}$ initiates apoptosis and autophagic cell death [15,100]. However, more pathways have been confirmed to regulate autophagy or cell death [101–104].

The CC system is an endogenous timing system that is widely present in the body. It ensures that various physiological processes have a circadian rhythm of nearly 24 h, including cell metabolism, division, apoptosis, and tumor formation [86,105,106]. In mice, the CC system regulates granulosa cell autophagy through NR1D1-mediated inhibition of ATG5 [107]. The mitochondrial function and E_2 synthesis are impaired following the alteration of *CLOCK* gene expression in porcine ovarian granulosa cells [108]. Additionally, the BMAL1 has a positive correlation with genes regulating steroid biosynthesis, and loss of *BMAL1* can lead to the failure of embryo implantation. [109–111]. However, few studies have examined how alterations in P4 or CL development can be attributed to the CC system in bovines.

In this chapter, the fresh bovine CL tissue samples were collected, intending to use Enzyme-linked Immunosorbent Assay (ELISA) to detect the P4 content in luteal tissue homogenates at different stages, and combine quantitative real-time PCR (qPCR), western blotting (WB), and immunofluorescence (IF) staining to analyze the expression of steroidogenic (*STAR*, *CYP11A1*, *HSD3β*) and CC system (*NR1D1*, *BMAL1*, *CLOCK, PER2*, *CRY1*, *DBP*) to analyze the gene expression characteristics at different luteal periods. An in-depth analysis of the temporal changes in P4 levels and steroid synthesis gene expression during bovine luteal development is not only a key scientific issue for revealing the reproductive rhythm of mammals, but also provides an important theoretical basis for clinical regulation of luteal function and optimization of concurrent estrus techniques.

2.2 Materials & Methods

2.2.1 Collection of Bovine CL Tissues

Fresh bovine ovaries were collected from a local slaughterhouse. The ovaries were freed from connective tissue and washed with 0.85% sterile saline solution. CL tissues at different stages were defined and collected based on the morphological characteristics of ovaries [17,92,112]. Simply, these tissues were categorized into four stages: early stage (1-3 days post-estrus), middle stage (4-10 days), late stage (10-17 days), and regressive stage (more than 17 days) [16,18], shown as in Figure 2-1. Then, about 50 mg of CL tissues were designated for mRNA detection, while 100 mg were

allocated for WB. Small pieces of CL were promptly frozen in liquid nitrogen using Tissue-Tek® O.C.T. compound (Sakura Finetek, Tokyo, Japan) for subsequent IF staining.

2.2.2 Determination of P4

As previously described [16], P4 was detected in fresh and treated small pieces of CL tissues at different stages as well as tissue culture medium, using a Progesterone ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA). Fifty mg of CL tissues were accurately weighed and added to an equal volume of ELISA buffer. Subsequently, the mixture was homogenized using a disposable homogenizer (BioMasher I, Nippi, Tokyo, Kapan). Then, the sample was diluted 5,000-fold with the ELISA buffer to be tested. For the measurement of P4 in the culture medium, the CL tissue culture medium was directly collected and diluted 2,000 times with the ELISA buffer. Following the dilution, all measurements were carried out strictly in accordance with the manufacturer's instructions. In detail, after sequentially adding the samples to the wells as per the instructions, all wells were sealed with plastic film and incubated at room temperature (R.T.) on an orbital shaker for 1 h to allow for proper reactions to occur. After incubation, the liquid was emptied, and wells were rinsed five times with washing buffer to remove any unbound substances. Then, 200 μL of Ellman's reagent was added to each well. Once again, all wells were covered with plastic film and incubated at R.T. away from light for another hour. Finally, the absorbance was measured at 405 nm using a microplate reader.

2.2.3 Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from fresh and treated CL using ISOGEN II (NIPPON GENE, Toyama, Japan) according to the manufacturer's protocol. After treatment, samples were fully homogenized using a disposable homogenizer. ISOGEN II was added to the homogenate, which was vigorously mixed and incubated at room temperature (R.T.) for 15 min. The mixture was centrifuged at 12,000 × g for 15 min. Subsequently, the supernatant was collected and mixed with an equal volume of Isopropyl Alcohol. The mixture was gently inverted several times and then left at R.T. for 10 min. After centrifugation at 12,000 × g for 10 min, the supernatant was discarded. The RNA pellet was washed with 75% ethanol, followed by centrifugation at 8,000 × g for 5 min. Then RNA pellet was air-dried at R.T. Finally, RNA was dissolved and diluted with RNase-free water. The concentration and purity of total RNA were measured using a NanoDrop instrument (Thermo Fisher Scientific, Wilmington, DE, USA). The extracted RNA was reverse transcribed into complementary DNA (cDNA) using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Then, the cDNA was established as a template for qPCR analysis with THUNDBIRD Next SYBR qPCR Mix reagent (TOYOBO, Osaka, Japan). The target genes included steroid synthesis-related genes (*STAR*, *CYP11A1*, *HSD3β*), CC system genes (*NR1D1*, *BMAL1*, *CLOCK*, *PER2*, *CRY1*, and *DBP*). primers specified for target genes, as listed in Table 1. Relative mRNA abundance was calculated using the ΔΔCt method, with *H2AFZ* as a reference gene.

2.2.4 Protein extraction and Western blotting

Total protein from fresh and treated CL tissues was extracted according to the previous study [16,113]. After collection of approximately 100 mg mid-CL, the samples were homogenized with lysis buffer by using a disposable homogenizer. The homogenate was kept at 4 °C and centrifuged at 12,000 × g for 5 min. Then, the supernatant, which was the protein sample, was prepared for electrophoresis by mixing with Sample Buffer Solution (2ME-) (×4) (FUJIFILM WAKO) and heating at 95°C for 5 min. The proteins were separated by electrophoresis on a 10%-20% e-PAGEL (ATTO, Tokyo, Japan) and transferred onto a polyvinylidene difluoride (PVDF) membrane using an iBlot 2 Gel Transfer Device (Thermo Fisher Scientific, Waltham, USA). After transfer, the membrane was immersed in TBST (Tris buffered saline Tween-20) buffer for 5 min, incubated with antibodies mixed with FastGene® Block & Go reagent at R.T. for 1 h with gentle agitation, and washed three times with TBST buffer. The developed images were captured using Amersham ECL Prime Western Blotting Detection Reagents (Cytiva, Buckinghamshire, UK) and analyzed with a Lumi Cube system (LIPONICS, Tokyo, Japan). Details about antibody information and reaction conditions are in the supplementary information.

2.2.5 Statistical analysis

All experimental results are presented as mean ± standard error of the mean (SEM). The statistical significance of differences in P4 secretion and the mRNA expressions of the CL tissues were assessed by Student's *t*-test and one-way analysis of ANOVA

implemented in GraphPad Prism® 10 Software (La Jolla, CA, USA). Statistical significance was set at $P < 0.05$. Different letters represent the significance. Data are representative of at least three independent experiments.

2.3 Results

2.3.1 The secretion levels of P4 in bovine different estrous stages

In the initial study, I aimed to elucidate the dynamic changes in P4 secretion across various stages of the bovine CL's development. ELISA was employed to quantify P4 levels in freshly homogenized CL tissue samples. These findings, as depicted in Figure 2-2, revealed a characteristic unimodal pattern of P4 secretion throughout CL development. The P4 level was relatively low in the early stage of the CL. As connective tissue and capillaries gradually extended to the granular layer, resulting in a significant enhancement in P4 synthesis. And it has shown a peak P4 secretion during the mid-stage of bovine CL. However, with the onset of luteolysis, a series of degenerative events ensued, leading to a decline in luteal function. Concomitantly, P4 production sharply decreased, eventually falling below the levels observed during the early developmental stage.

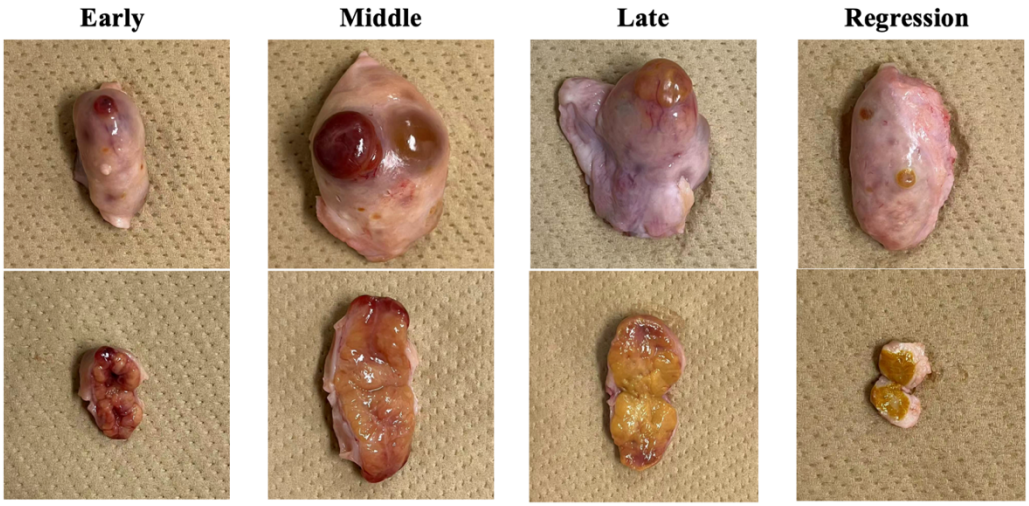


Figure 2-1. Morphological characteristics of bovine corpus luteum at different stages.

Bovine ovaries were collected from a slaughter at a local abattoir, and according to the morphological characteristics of the CL at different stages, they were divided into four periods: early stage, days 1-3; middle stage, days 4-10; late stage, days 10-17; regression stage, more than day 17.

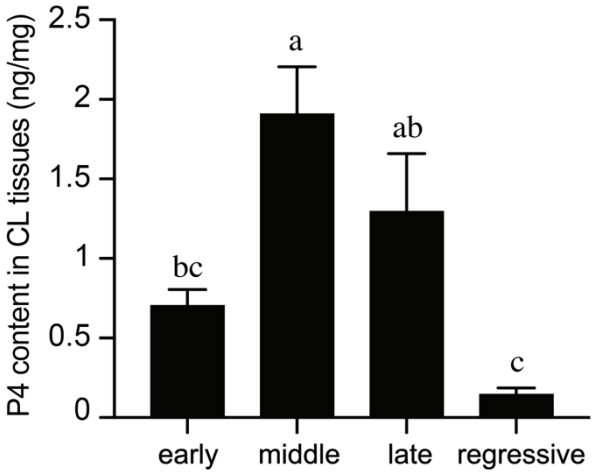


Figure 2-2. The secretion levels of P4 in bovine different estrous stages

The bovine CL at different stages was ground to form a homogenate, and the P4 content at each stage was detected using a Progesterone ELISA Kit. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters in lowercase indicate significant differences between groups.

2.3.2 The expression levels of related steroidogenic enzyme genes in bovine different estrous stages

To further elucidate the regulatory mechanisms underlying bovine CL function, I investigated the expression profiles of steroidogenesis-related genes across different developmental stages. As illustrated in Figure 2-3, the gene expression profiles of the steroidogenic enzymes *STAR*, *CYP11A1*, and *HSD3β* showed concordance among their respective expression patterns and closely mirrored the dynamic changes in P4 levels. Specifically, expression levels were elevated during the middle and late stages, peaking in the middle stage and declining during the regression stage.

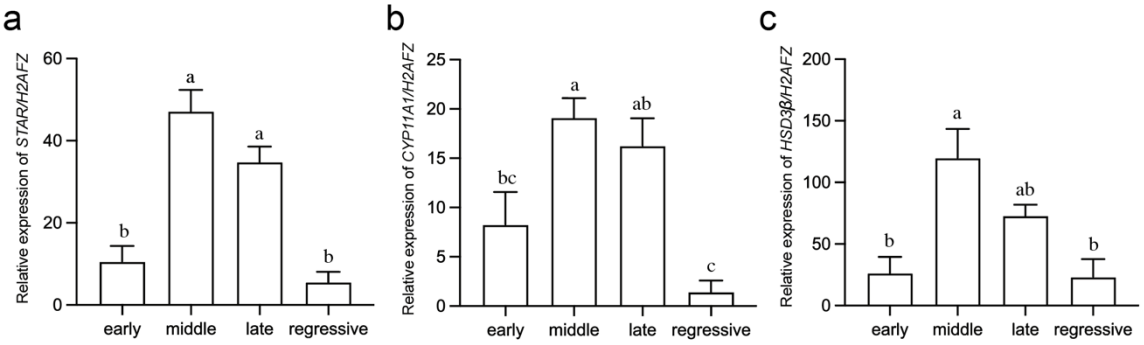
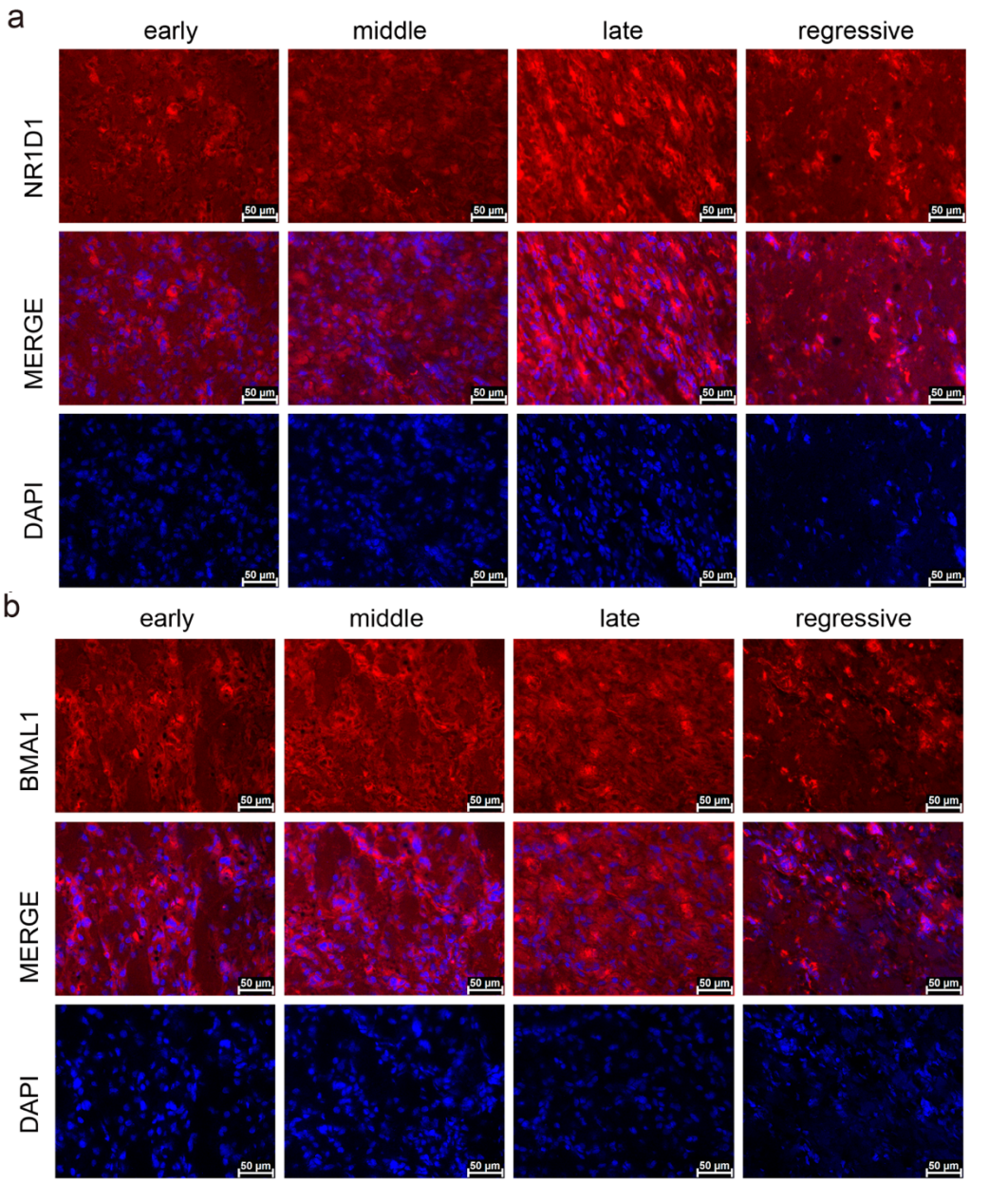


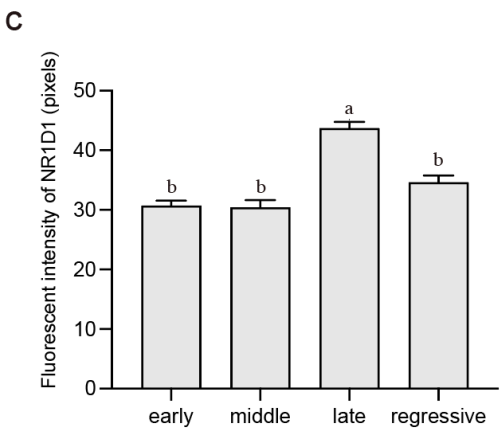
Figure 2-3. The expression levels of related steroidogenic enzyme genes in bovine different estrous stages

Gene expression levels of steroidogenic enzyme genes were detected using qPCR in CL at the different stages. a: *STAR*, b: *CYP11A1*, c: *HSD3β*. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters in lowercase indicate significant differences between groups.

2.3.3 Expression levels of circadian clock genes and proteins in bovine CL at different estrous stages

To assess the involvement of the CC system in CL regression, I analyzed the expression levels of key components of the CC system throughout the developmental stages of the CL. Firstly, IF staining was employed to localize the core factor of the CC system, NR1D1 and BMAL1 proteins were present in the CL development, as shown in Figure 2-4. Then, genomic analyses revealed that the core components of the CC system genes showed expression profiles that closely resembled those of genes involved in steroid synthesis in fresh CL tissues. Specifically, the *NR1D1*, *CLOCK*, *PER2*, and *CRY1* have a peak expression in the late stage than the early, middle, and regression stages. By contrast, the expression of *BMAL1* and *DBP* peaks in the mid-CL stage (Figure 2-5). Subsequently, I validated the expression of core CC system factors at the protein level. As depicted in Figure 2-6, the key clock proteins NR1D1 and BMAL1 exhibited a temporal expression pattern characterized by higher abundance in the mid-late stages compared to the early and regression stages of luteal development. This trend was further corroborated with IF staining analysis, which revealed distinct subcellular localization of NR1D1 and BMAL1 in both the cytoplasm and nucleus, particularly pronounced during the mid-late stages, where their spatial distribution was highly defined.


a
early
middle
late
regressive
NR1D1
MERGE
DAPI
50 μm
b
early
middle
late
regressive
BMAL1
MERGE
DAPI
50 μm


c
Fluorescent intensity of NR1D1 (pixels)
0
10
20
30
40
50
b
b
a
b
early
middle
late
regressive

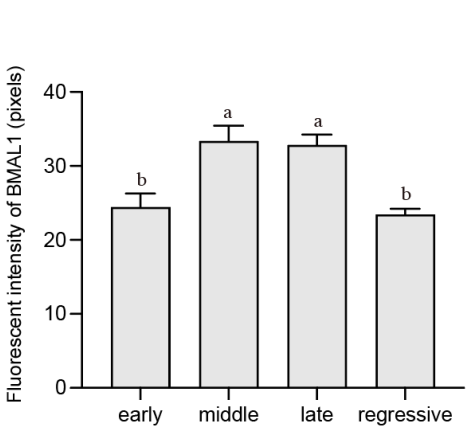

d
Fluorescent intensity of BMAL1 (pixels)
0
10
20
30
40
b
a
a
b
early
middle
late
regressive

Figure 2-4. The expression of CC system proteins in bovine CL at different stages

The location of NR1D1 and BMAL1 was detected using IF staining in CL at the different stages. a: NR1D1, b: BMAL1, c-d, relative level of NR1D1and BMAL1 by calculating fluorescence intensity. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters in lowercase represent significant differences. Each value represents the mean ± SEM in at least three independent experiments.

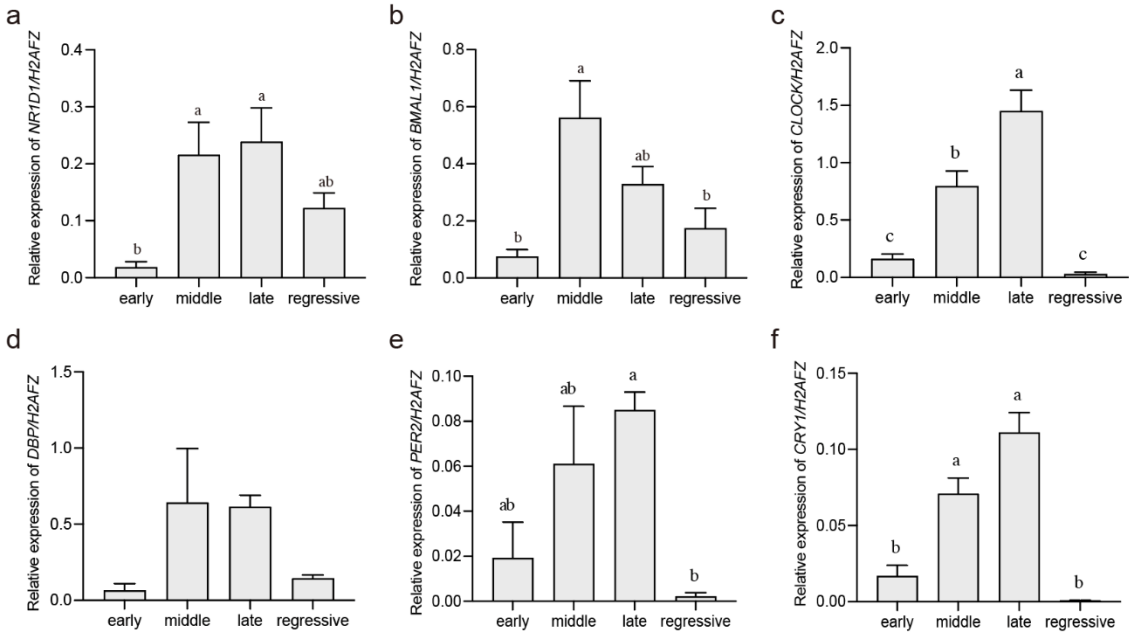


Figure 2-5. The expression of CC system genes in bovine CL at different stages

Expression levels of the CC system core factors in different stages of bovine CL were analyzed by qPCR. a-f, *NR1D1*, *BMAL1*, *CLOCK*, *DBP*, *PER2*, *CRY1*, the relative expression normalized to *H2AFZ*. Data are expressed as mean ± SEM, and Student's t-test was used to analyze at least three replicated experiments. Value with Different letters in lowercase indicates significant differences.

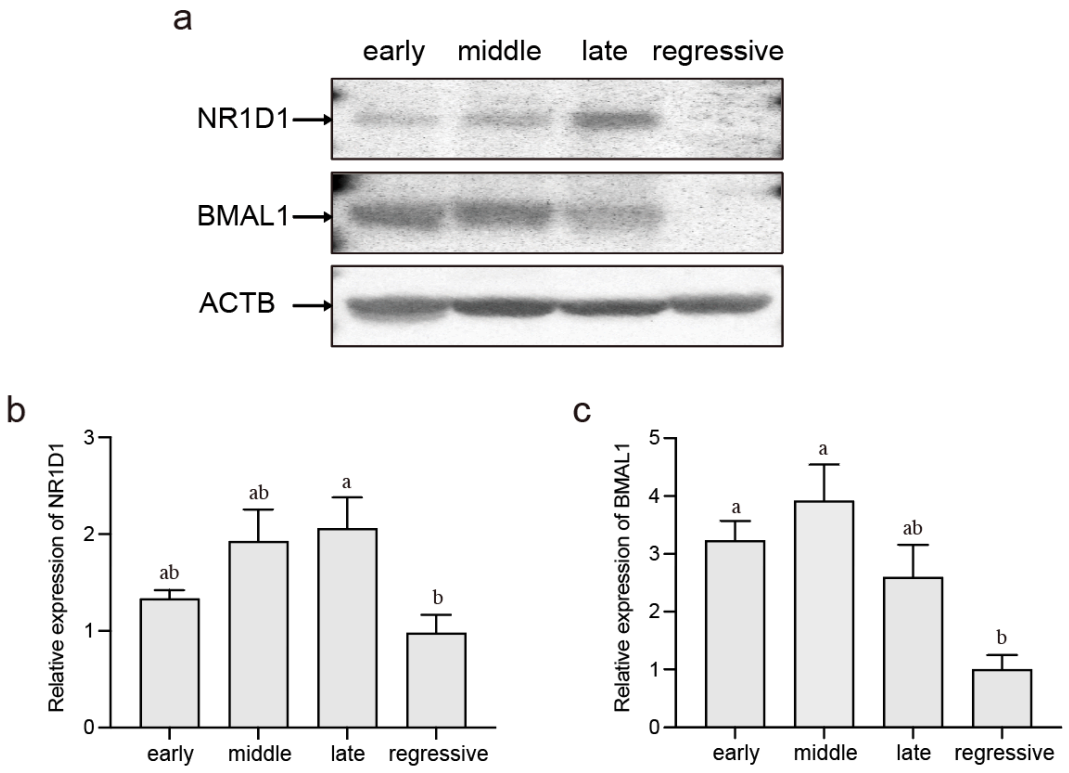


Figure 2-6. The expression of NR1D1 and BMAL1 proteins in bovine CL at different stages

WB analysis of the protein levels of NR1D1 and BMAL1 at different stages of CL tissues (a). (b-c) Relative levels of NR1D1 and BMAL1 normalized to ACTB. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters in lowercase represent significant differences. Each value represents the mean ± SEM in at least three independent experiments.

2.4 Discussion

A thorough understanding of CL development mechanisms is critical for enhancing reproductive efficiency in mammals. In this study, by collecting bovine CL tissues at distinct developmental stages and analyzing P4 secretion, steroid synthesis-related gene expression, and key factors in the autophagy-apoptosis pathway, I systematically characterized the molecular dynamics underlying CL progression. These findings reveal that P4 secretion and steroidogenic gene expression exhibit highly synchronized unimodal patterns during CL development, reflecting the functional integration of hormone synthesis and genetic regulation. This observation aligns with prior work by [8,21,48,114,115], who similarly reported the secretion of P4 and steroidogenic genes (*STAR*, *CYP11A1*, *HSD3β*) across the bovine luteal phases, supporting their functional interdependence in maintaining CL homeostasis. Meanwhile, the consistently reported congruent results reinforce the reliability of these observations. In addition, the CC system results emerge as increasingly prominent regulators across the CL lifecycle, with their peak activity occurring during bovine CL middle and late stages. The similar pattern of the CC system factors underscores their indispensable roles in orchestrating CL structural and functional involution.

Studies on bovine reproductive biology have remained a focal point for researchers worldwide, given cattle's economic significance in livestock production and its relevance as a model for human reproductive physiology. Among the myriad regulatory mechanisms and pathways governing luteal function, although the CC system has long

been implicated in it, its precise roles during this process remain incompletely understood. In this study, I performed homogenization-based assays on bovine fresh CL tissues across distinct developmental stages and characterized the roles of these pathways through gene and protein expression profiling. It is expected that the CC system and steroidogenic genes have similar expression patterns during the luteal progression. This phenomenon suggests that the CC system may be involved in programmed cell death mechanisms during luteinization and the hormonal signaling withdrawal, particularly decline of P4 levels [116–118]. In addition, the accumulation of oxidative stress and the remodeling signals mediated by immune cells may also be regulated by the CC system [103,119].

In summary, in this chapter, I systematically characterized the dynamic expression patterns of P4, steroidogenic genes (*STAR*, *CYP11A1*, *HSD3β*), and key regulators (NR1D1, BMAL1) of the CC system during bovine CL development. These findings demonstrate that the luteal formation, maintenance, and ultimately, programmed regression are precisely coordinately regulated through the integrated action of hormonal signaling, gene expression modulation, and cellular programmed death pathways. The temporally regulated interplay between steroidogenic activity and cellular fate decisions reveals a sophisticated biological timer that ensures proper luteal tissue turnover and maintains reproductive cyclicity.

2.5 Conclusion

This chapter presents results demonstrating dynamic P4 synthesis in fresh luteal

tissue, coupled with stage-specific expression patterns of steroidogenic genes. P4 levels and *STAR*, *CYP11A1*, and *HSD3β* gene expression peaked at the mid-luteal stage, with lower abundance in the early and regression phases. Notably, the CC system components exhibited distinct temporal dynamics: NR1D1 expression was highest in the late stage, whereas BMAL1 reached maximal levels at mid-stage. These findings imply a functional link between the CC system and luteal developmental phases, particularly in regulating P4 synthesis and steroidogenic gene networks. It also provides a theoretical foundation for exploring circadian-based strategies to optimize bovine reproductive performance.

Chapter III

Effect of $PGF_{2\alpha}$ on the expression of the NR1D1 and BMAL1 genes and proteins in mid-CL tissue

3.1 Introduction

As shown in the previous chapter, the mid-luteal phase represents the peak period of P4 secretion and steroidogenic gene expression, serving as a key juncture that determines whether the luteal phase continues to maintain or initiates regression. During this stage, luteal cells must sustain an efficient secretion of P4 to support endometrial decidualization while resisting premature activation of degenerative processes [120,121]. In most mammalian species, endometrium-derived prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is widely recognized as the primary luteolytic factor, with classic mechanisms of action including induction of luteal cell apoptosis, inhibition of P4 synthesis, and promotion of vascular degeneration [20,122,123]. Multiple studies have shown that $PGF_{2\alpha}$ treatment ultimately reduces the production of P4, whether in mixed luteal cells or co-cultures of luteal-endothelial cells. However, exogenous $PGF_{2\alpha}$ is most effective as a luteal regression factor when administered *in vivo* [124]. Luteal regression proceeds in two distinct phases: the decline in P4 production is termed functional luteolysis, followed by structural luteolysis, the physical involution of CL [125–127]. Evidence indicates that endometrial or extrauterine-derived $PGF_{2\alpha}$

triggers functional luteolysis, whereas locally produced luteal $PGF_{2\alpha}$ may mediate the transition to structural luteolysis [124]. In this chapter, fresh mid-stage CL were collected, minced, and utilized as an *ex vivo* tissue culture model for $PGF_{2\alpha}$ treatment.

Prostaglandins (PGs) belong to the eicosanoid family of biologically active endogenous bioactive lipids derived from arachidonic acid, synthesized ubiquitously across diverse tissues [128,129]. Each PGs subtype binds to specific receptors expressed in systemic organs, enabling them to regulate multiple physiological systems, including the central nervous system, cardiovascular system, and immune system by mediating downstream signaling pathways [130]. $PGF_{2\alpha}$, a prominent PGs subtype, acts on blood vessels surrounding the glomerular apparatus and retinal arteries [131]. Its cognate receptor, prostaglandin F receptor (PTGFR) is localized in the eye and uterus, where ligand binding activates signaling cascades involving protein kinase C (PKC) and AMP-activated protein kinase (AMPK) [132]. While previous studies have established that plasma concentrations of certain PGs exhibit circadian rhythmicity [133] and affect the CC system gene expression [134], no reports to date have investigated whether $PGF_{2\alpha}$ modulates the CC system in the bovine mid-CL. Furthermore, studies have shown that the CC system is involved in autophagy and apoptosis pathways in numerous tissues and organs [107,111,135]. It is necessary to explore the relationship between $PGF_{2\alpha}$ and the CC system for luteal development.

In this Chapter, the bovine mid-CL small pieces were applied with $PGF_{2\alpha}$ to establish an induced luteal regression model in *ex vivo*. Combined with qPCR, WB, and ELISA techniques, the dynamics in P4, steroidogenic, autophagy, apoptosis, and the

CC system gene and protein were analyzed. Investigate the changes of the circadian CC system during luteal regression and its association with $PGF_{2\alpha}$.

3.2 Materials & Methods

3.2.1 Collection of Bovine CL Tissues

The bovine CL tissue samples were prepared as described in Chapter II.

3.2.2 Determination of P4

The detection of P4 content was described in Chapter II.

3.2.3 Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA extraction and qPCR implementation were prepared as described in Chapter II.

3.2.4 Protein extraction and Western blotting

Total protein was prepared as described in Chapter II.

3.2.5 *Ex vivo* $PGF_{2\alpha}$-induced regression model of CL tissue

$PGF_{2\alpha}$ is a hormone known for its crucial role in controlling a myriad of essential biological processes [136]. It is well-documented that $PGF_{2\alpha}$ induces CL regression by acting on luteal tissue. To simulate bovine CL regression, middle-stage CL tissues (mid-CL) were collected and induced with $PGF_{2\alpha}$, as mentioned in prior literature [137,138]. The mid-CL was divided into two groups: the control group received normal DMEM (Fujifilm Wako Pure Chemical Co, Osaka, Japan) culture medium containing 5% FBS, 1% penicillin G potassium (NACALAI TESQUE, Kyoto, Japan), and 0.06% streptomycin sulfate (NACALAI TESQUE); the experimental group additionally received 1 μM $PGF_{2\alpha}$ (SIGMA-ALDRICH, St Louis, USA). The tissues were preincubated in a humidified atmosphere of air with 5% CO_2 at 38.5°C for about 24 h. Afterward, small pieces of mid-CL were collected and homogenized using a disposable homogenizer as previously described, the levels of mRNA, protein, and P4 content were detected.

3.2.6 Statistical analysis

All data are presented as mean ± SEM and represent at least three replicates. Statistically significant differences were identified using one-way analysis of variance (ANOVA)-Tukey's multiple range test implemented in GraphPad Prism® 10 Software (LA Jolla, CA, USA). Statistical significance was set at $P < 0.05$. Different letters

indicated significance.

3.3 Results

3.3.1 The secretion levels of P4 and the expression levels of related steroidogenic enzyme genes in bovine mid-CL

The bovine mid-CL was collected and made into small pieces. $PGF_{2\alpha}$ was employed to induce mid-CL regression in an *ex vivo* model for 24 h. First of all, I verified whether the model was successfully induced, as illustrated by P4 synthesis and steroid gene expression by ELISA and qPCR. The results demonstrated significant reductions of P4 content in both mid-CL tissue and the tissue culture medium (Figure 3-1), accompanied by downregulation of the steroidogenic gene *STAR* ($P = 0.0512$) and *HSD3β*, as shown in Figure 3-2a, c. The expression level of *CYP11A1* is not obvious (Figure 3-2b).

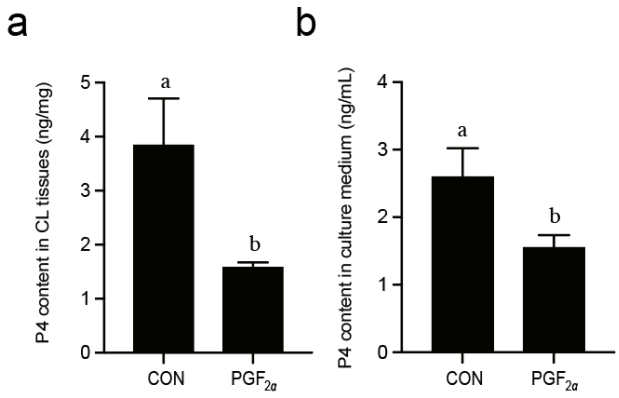


Figure 3-1. The secretion levels of P4 in bovine mid-CL treated with $PGF_{2\alpha}$ for 24 h

The bovine mid-CL treated with $PGF_{2\alpha}$ for 24 h was ground to form a homogenate, and the P4 content at each stage was detected using a Progesterone ELISA Kit. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters in lowercase indicate significant differences between groups.

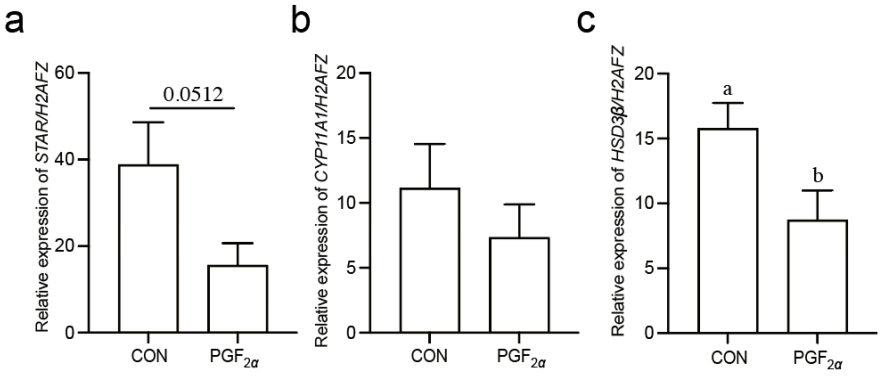


Figure 3-2. The expression levels of related steroidogenic enzymes in bovine mid-CL treated with $PGF_{2\alpha}$ for 24 h

Gene expression levels of steroidogenic enzymes were detected using qPCR in mid-CL treated with $PGF_{2\alpha}$. a: *STAR*, b: *CYP11A1*, c: *HSD3β.* The relative expression standard to *H2AFZ*. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters in lowercase indicate significant differences between groups.

3.3.2 The expression levels of related autophagy and apoptosis genes in bovine mid-CL

As previously elucidated, $PGF_{2\alpha}$-induced luteal regression is intricately linked to autophagic and apoptotic pathways. To further investigate this mechanism, I measured the expression of autophagy- and apoptosis-associated factors in the collected mid-CL samples by mRNA detection. The result is shown in Figure 3-3, although treatment with $PGF_{2\alpha}$ induced changes in both autophagy (*LC3B*, *ATG5*) and apoptosis (*CASPASE8*) markers, these alterations did not achieve statistical significance. In contrast, the expression level of the *CTSB* gene was markedly upregulated, as shown in Figure 3-3d. Furthermore, the WB analysis revealed that compared to the CON group, the CTSB protein level was significantly elevated in the $PGF_{2\alpha}$-treated group, without obvious changes in LC3 expression (Figure 3-4).

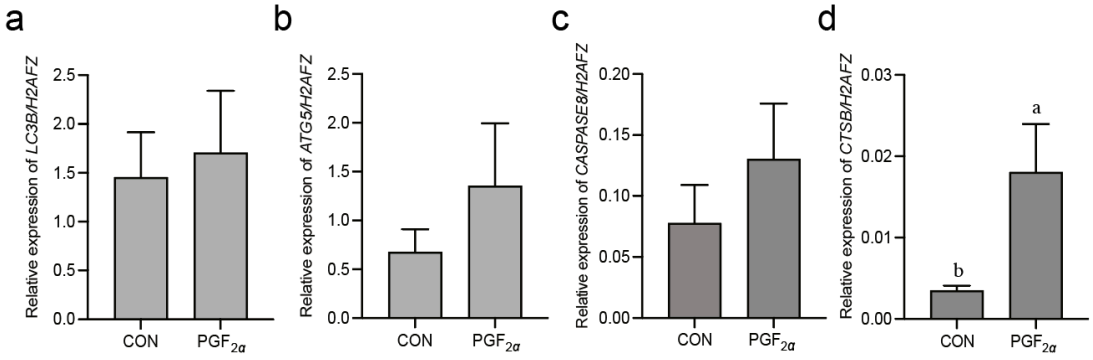


Figure 3-3. The expression levels of autophagy- and apoptosis-related genes in bovine mid-CL treated with $PGF_{2\alpha}$ for 24 h

The expression levels of autophagy- and apoptosis-related genes were detected using qPCR in mid-CL treated with $PGF_{2\alpha}$. a: *LC3B*, b: *ATG5*, c: *CASPASE8*, d: *CTSB.* The relative expression standard to *H2AFZ.* All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters indicate significant differences between groups.

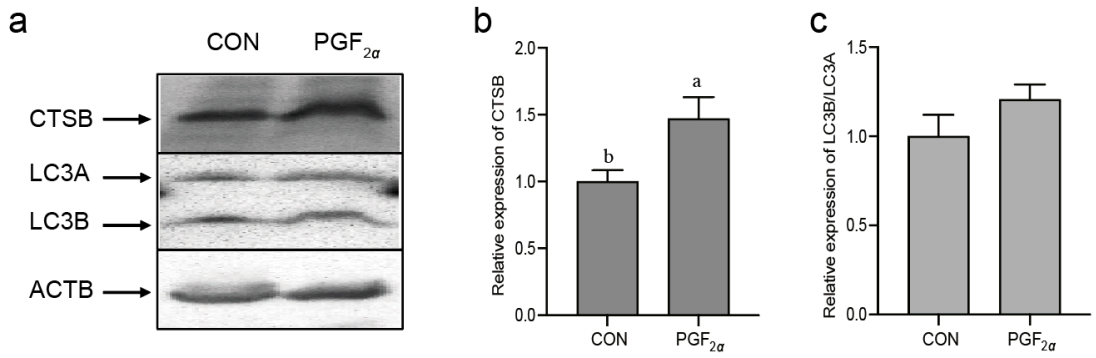


Figure 3-4. The expression levels of autophagy and apoptosis related proteins in bovine mid-CL treated with $PGF_{2\alpha}$ for 24 h

The expression levels of autophagy- and apoptosis-related proteins were detected using WB in mid-CL treated with $PGF_{2\alpha}$. a: WB analysis of the protein levels of LC3A, LC3B, and CTSB in mid-CL tissues, b-c: Relative amount of CTSB normalized to ACTB, and LC3B/LC3A ratio reflecting LC3 protein expression. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters in lowercase indicate significant differences between groups.

3.3.3 The expression levels of related circadian clock genes and proteins in bovine mid-CL

To figure out whether the CC system is regulated by $PGF_{2\alpha}$ in mid-CL, I investigated the expression of CC components NR1D1 and BMAL1 in $PGF_{2\alpha}$-induced luteal regression samples. As depicted in Figure 3-5, $PGF_{2\alpha}$ treatment downregulates

the *NR1D1* gene (P = 0.0604). Concurrently, *BMAL1* exhibited a similar trend of decrease without pronounced changes. Additionally, the WB analysis of protein expression further revealed that in the $PGF_{2\alpha}$-induced group, BMAL1 protein levels were downregulated (P = 0.0531) (Figure 3-6a, c), while the NR1D1 protein levels did not reach statistical significance, as shown in Figure 3-6.

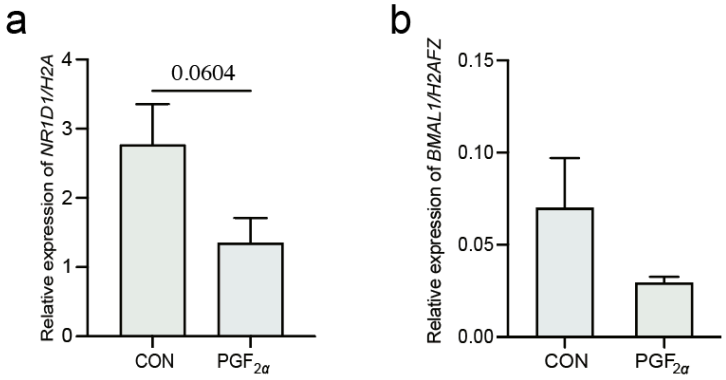


Figure 3-5. The expression levels of the CC system genes in bovine mid-CL treated with $PGF_{2\alpha}$ for 24 h

The expression levels of the CC system core factors *NR1D1* and *BMAL1* in bovine mid-CL treated with $PGF_{2\alpha}$ were analyzed by qPCR. a-b, *NR1D1*, *BMAL1*. The relative expression standard to *H2AFZ*. Data are expressed as mean ± SEM, and Student's t-test was used to analyze at least three replicated experiments.

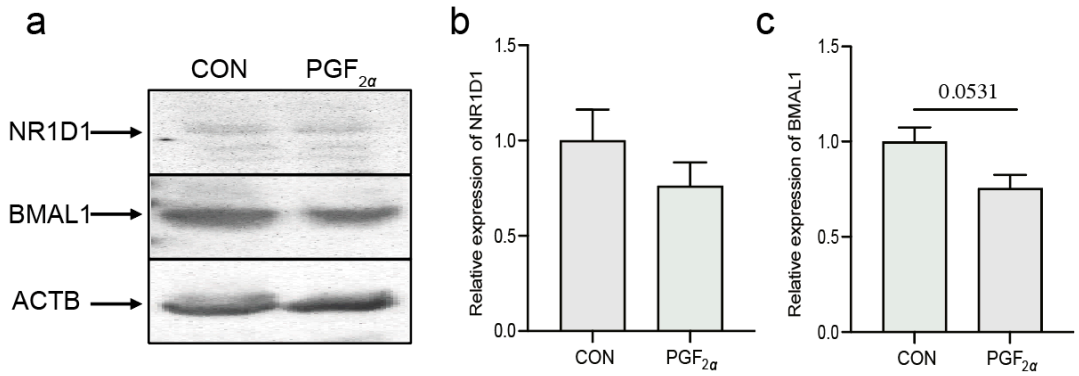


Figure 3-6. The expression levels of the NR1D1 and BMAL1 in bovine mid-CL treated with $PGF_{2\alpha}$ for 24 h

WB analysis of the protein levels of NR1D1 and BMAL1 in min-CL tissues (a). (b-c) Relative levels of NR1D1 and BMAL1 normalized to ACTB. Statistical significance was determined using one-way ANOVA. Each value represents the mean ± SEM in at least three independent experiments.

3.4 Discussion

In this chapter, I established a $PGF_{2\alpha}$-induced mid-CL regression model to dissect the synergistic mechanisms underlying P4 suppression, apoptosis activation, and the circadian rhythm disorder. $PG_{2\alpha}$ treatment significantly reduced mid-CL tissue and tissue culture medium P4 levels while downregulating *STAR*, the essential gene for steroidogenesis. These findings confirm that $PGF_{2\alpha}$ acts as a classic luteolysin by blocking cholesterol transport and metabolism, consistent with its known role in competitively inhibiting LH-mediated cAMP signaling and steroidogenic enzyme

activity [139,140]. Furthermore, during this process, the changes in autophagy (ATG5, LC3) and apoptosis (CTSB, CASPASE8) markers suggest that $PGF_{2\alpha}$ orchestrates luteal regression via dual lysosomal and caspase-dependent pathways. The marked upregulation of CTSB, which is a lysosomal cysteine protease, implicates it as a key executor of lysosome-dependent apoptosis, aligning with prior studies linking lysosomal membrane permeabilization to luteal cell demise [16,141,142]. This coordinated activation of apoptosis underscores the complexity cytolytic effects of $PGF_{2\alpha}$ in CL.

Crucially, this study provides the first evidence that the interference effect of $PGF_{2\alpha}$ on the CC system in luteal cells. Downregulation of NR1D1 and BMAL1 suggests that temporal regulation is involved in luteolysis. The CC system is closely related to cellular homeostasis, and its disorder may lead to the dysregulation of downstream cycle regulatory genes. As a core transcription factor of the circadian rhythm, the reduction of BMAL1 directly drives rhythmic *STAR* expression and likely impairs the synthesis of P4 [143–145]. NR1D1 repression may relieve inhibition of the inflammatory signaling pathway, accelerating apoptosis, and further weaken the metabolic and secretory functions of luteal cells [146,147]. This clock-steroid-apoptosis axis explains the synchronized decline of P4 synthesis and activation of cell death programs.

To sum up, in Chapter III, $PGF_{2\alpha}$ drives mid-CL regression from multiple dimensions such as metabolism, cell death, and clock regulation by directly inhibiting P4 synthesis, activating the CTSB-dependent apoptotic pathway, and interfering with

the circadian rhythm network. It is necessary for the future research needs to clarify the specific pathways by which $PGF_{2\alpha}$ regulates the CC system genes, and further explore the molecular mechanism of NR1D1/BMAL1 as the intersection node between $PGF_{2\alpha}$ signaling and the CC system, providing a new perspective for exploring the delay of luteal regression by targeting the CC system elements.

3.5 Conclusion

In this chapter, I employed $PGF_{2\alpha}$ to establish a bovine luteal regression model, revealing multifaceted regulatory mechanisms. $PGF_{2\alpha}$ induction not only reduced P4 synthesis and steroidogenic gene expression but also activated autophagic and apoptotic pathways, with CTSB emerging as a key executor of apoptosis. Concurrently, the CC system exhibited downregulation of NR1D1 and BMAL1, suggesting temporal coordination between circadian machinery and luteal regression. These findings reinforce the role of the CC in governing luteal developmental transitions—particularly during regression—and highlight its potential as a therapeutic target for modulating reproductive efficiency.

Chapter IV

Effect of the NR1D1 agonist and antagonist on the functional development in the bovine mid-CL tissue

4.1 Introduction

As mentioned in Chapter II, as a transient endocrine gland in the mammalian reproductive system, CL governs pregnancy establishment and reproductive efficiency through its precisely timed processes of formation, maintenance, and regression [148,149]. In livestock species like cattle, the lifecycle of CL (approximately 21 days) dictates the regularity of the estrus cycle, with the exact timing of post-ovulatory luteal regression serving as a critical checkpoint for pregnancy recognition. Emerging evidence suggests that the CC system may orchestrate sequential luteal functions by regulating hormone secretion, cell proliferation, and apoptosis [111,150,151]. However, the temporal regulatory mechanisms underlying CL development remain poorly understood, particularly the expression patterns and functional roles of CC genes in luteal tissues. This knowledge gap significantly limits our ability to manipulate reproductive cycles or address luteal phase defects in domestic animals. This chapter aims to explore the CC system gene and protein expression patterns across bovine mid-CL, providing foundational data for understanding this crucial biological timer in mammalian reproduction.

Notably, the core molecular components of the CC system, the *NR1D1* and *BMAL1*, play a significant role in modulating these effects, as evidenced by changes in steroid synthesis and P4 production in gene disorder models [152]. These are two key CC components with antagonistic functions: *BMAL1* promotes the transcription of clock-controlled genes when it forms a heterodimer with *CLOCK*, whereas NR1D1 represses BMAL1 expression through its interaction with the RORE/RevRE site in the *BMAL1* promoter [153,154]. Within the mammalian ovaries, accumulating studies have demonstrated that genetic ablation of clock genes impairs reproductive functions, especially BMAL1 and NR1D1 [74,86,89,107,151]. In addition, clock genes have been implicated in regulating ovarian granulosa cell function and follicular development. Nevertheless, it remains unclear whether luteal cells employ the CC system networks to coordinate temporal events such as P4 secretion and apoptosis in cattle.

As outlined in preceding chapters, this study categorizes the bovine CL into four developmental phases along a temporal axis (formation, maintenance, regression, and late regression) for exploration. Building on my demonstration of the CC system involvement in the development of CL and $PGF_{2\alpha}$-induced mid-CL. I developed a targeted disruption model using *NR1D1* modulators. GSK4112, a potent and selective NR1D1 agonist, was employed to enhance circadian repression pathways [155,156], while SR8278, a competitive NR1D1 antagonist [152,157], was utilized to disrupt normal circadian signaling. These compounds have been extensively validated in chronobiology research for their ability to specifically modulate clock-controlled gene networks without significant off-target effects at appropriate concentrations. Therefore,

it was employed to interrogate the CC system in mid-CL, enabling the mechanism of circadian-controlled pathways in luteal homeostasis and regression.

4.2 Materials & Methods

4.2.1 Collection of Bovine CL Tissues

The bovine CL tissue samples were prepared as described in Chapter II.

4.2.2 Determination of P4

The detection of P4 content was described in Chapter II.

4.2.3 Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA extraction and qPCR implementation were prepared as described in Chapter II.

4.2.4 Protein extraction and Western blotting

Total protein was prepared as described in Chapter II.

4.2.5 Effect of NR1D1 agonist and antagonist on mid-CL in *ex vivo* Models

GSK4112 acts as an agonist for NR1D1, which is often used as a drug to disrupt the CC system to treat various tissue and cell models [158–160]. In addition, an antagonist of NR1D1, SR8278 can reduce the expression level of NR1D1 by inhibiting its activity, thereby disrupting the stability of the CC system [161,162]. In this study, I designed the mid-CL treated with 20 μM GSK4112 (EMD Millipore Corp., Billerica, MA, USA) and 10 μM SR8278 (EMD Millipore Corp., Billerica, MA, USA) to establish a CC system disorder model. Similar to the $PGF_{2\alpha}$-inducted experiment, the mid-CL samples were divided into three groups. CL tissue pieces were cultured in CON (DMEM + 5% FBS), GSK4112 (DMEM + 5% FBS with 20 μM of GSK4112), and SR8278 (DMEM + 5% FBS with 10 μM of SR8278). Subsequently, three groups were cultured for 24 h in a humidified atmosphere of air with 5% CO_2 at 38.5°C. After that, the samples were collected and homogenized for the detection of gene and protein expression levels or stored at -80°C until the experiments.

4.2.6 Statistical analysis

All data are presented as mean ± SEM and represent at least three replicates. Statistically, significant differences were identified using one-way analysis of variance (ANOVA)-Tukey's multiple range test implemented in GraphPad Prism® 10 Software (LA Jolla, CA, USA). Statistical significance was set at $P < 0.05$. Different letters

represent significant differences.

4.3 Results

4.3.1 Expression levels of NR1D1 and BMAL1 genes and proteins in bovine mid-CL treated with GSK4112 and SR8278

Following $PGF_{2\alpha}$ treatment of mid-CL, I observed reduced P4 synthesis and altered expression of the CC system factors. To further investigate the crosstalk by modulating the CC system factor expression. Firstly, I examined the gene and protein levels of NR1D1 and BMAL1, the core regulators of the CC system. Results showed that GSK4112 (agonist) significantly upregulated *NR1D1* gene (Figure 4-1a) and protein (Figure 4-2a, c) expression levels, accompanied by a trend toward reduced BMAL1 protein (P = 0.079) (Figure 4-2b, d). This aligns with NR1D1's role as a negative feedback regulator of BMAL1. Conversely, a significant decline in NR1D1 protein (Figure 4-2a, c) expression was detected after treatment with SR8278 (antagonist) while increasing BMAL1 protein expression (Figure 4-2b, d). Notably, these pharmacological interventions did not show significant changes in *NR1D1* or *BMAL1* gene expression in Figure 4-1a, b.

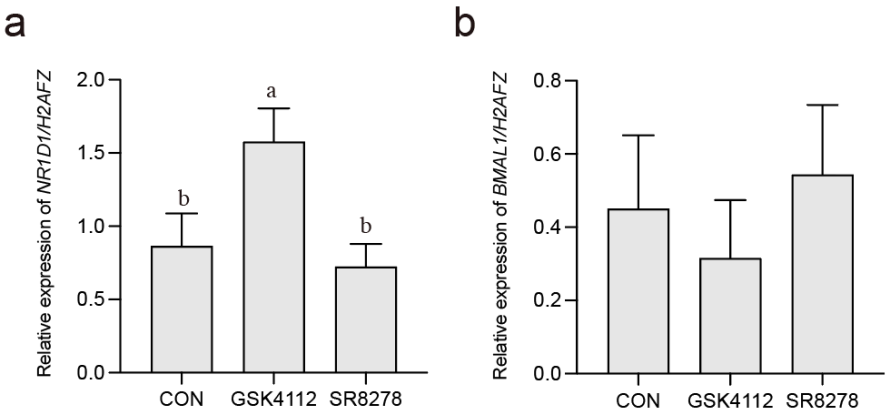
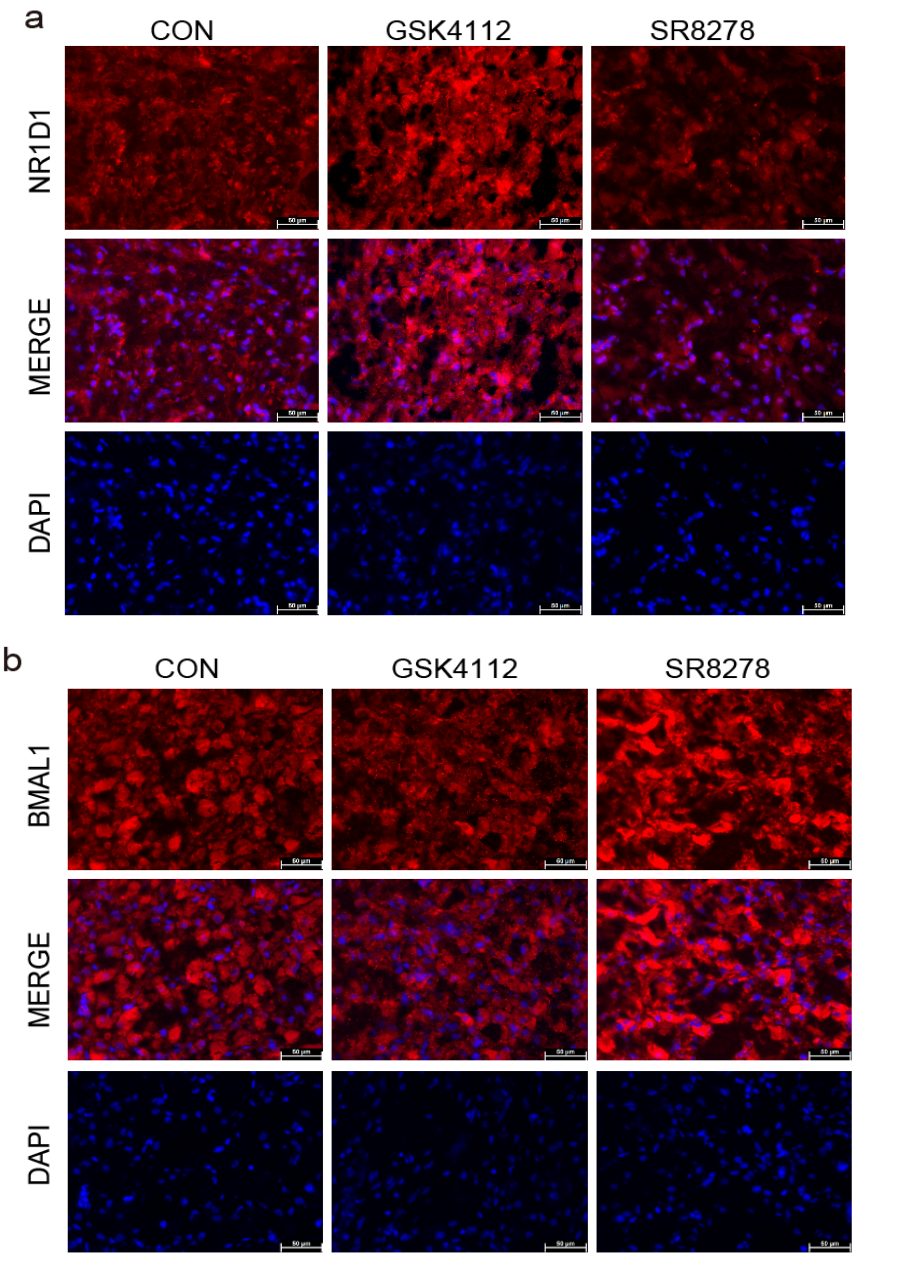


Figure 4-1. The expression of *NR1D1* and *BMAL1* after bovine mid-CL treatment with GSK4112 and SR8278 in *ex vivo.*

The expression levels of the *NR1D1* and *BMAL1* in mid-CL treated with GSK4112 and SR278 were analyzed by qPCR in *ex vivo*. a, *NR1D1*, b, *BMAL1*. The relative expression was normalized to *H2AFZ*. Data are expressed as mean ± SEM, and Student's t-test was used to analyze at least three replicated experiments. Value with Different letters in lowercase indicates significant differences.


a
CON
GSK4112
SR8278
NR1D1
MERGE
DAPI
b
CON
GSK4112
SR8278
BMAL1
MERGE
DAPI

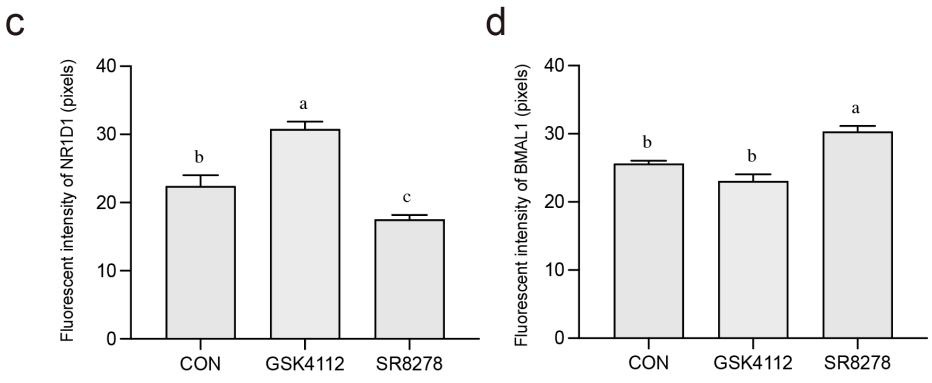

c
Fluorescent intensity of NR1D1 (pixels)
40
30
20
10
0
b
a
c
CON
GSK4112
SR8278
d
Fluorescent intensity of BMAL1 (pixels)
40
30
20
10
0
b
b
a
CON
GSK4112
SR8278

Figure 4-2. The expression levels of NR1D1 and BMAL1 proteins after bovine mid-CL treatment with GSK4112 and SR8278 in *ex vivo.*

The protein expression levels of the NR1D1 and BMAL1 in mid-CL treated with GSK4112 and SR278 were analyzed by IF staining in an *ex vivo* model. a, NR1D1, b, BMAL1, c-d, relative levels of NR1D1 and BMAL1 by calculating fluorescence intensity by ImageJ. Data are expressed as mean ± SEM, and Student's t-test was used to analyze at least three replicated experiments. Value with Different letters in lowercase indicates significant differences.

4.3.2 The secretion levels of P4 and the expression of related steroidogenic enzyme genes in bovine mid-CL treated with GSK4112 and SR8278

Future detection should monitor the function of the mid-CL following disruption in the CC system. I measured the content of P4 and expression of steroidogenic genes by ELISA and qPCR. As shown in Figure 4-3a, b, the GSK4112 and SR8278 treatments significantly reduced P4 levels compared to the CON group, yielding consistent results across both interventions. By contrast, steroidogenic gene expression showed minimal changes, except *CYP11A1* ($P = 0.0847$), which was downregulated following GSK4112 treatment (Figure 4-4). There is no significant expression after treatment with SR8278 in the steroidogenic gene (*STAR*, *CYP11A1*, *HSD3β*) expression (Figure 4-4a, b, c).

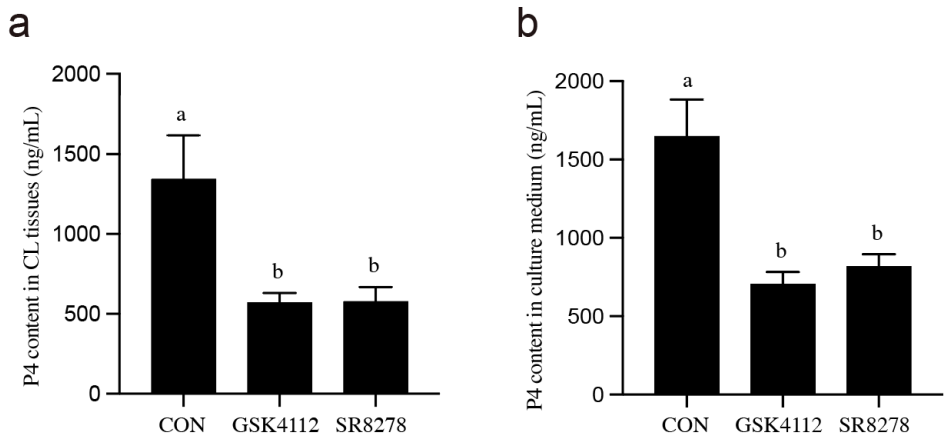


Figure 4-3. The secretion levels of P4 after bovine mid-CL treatment with GSK4112 and SR8278 in *ex vivo*.

The mid-CL treatment with GSK4112 and SR8278 in *ex vivo* was ground to form a homogenate, and the P4 content was detected using a Progesterone ELISA Kit. Different lowercase letters indicate significant differences between groups. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different letters indicate significant differences.

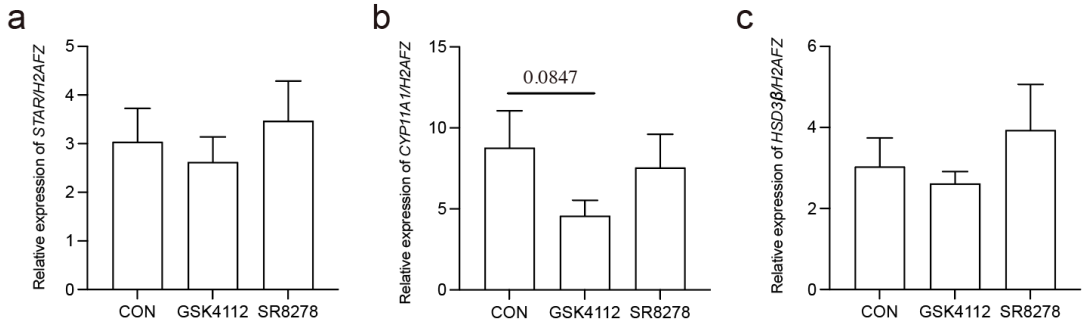


Figure 4-4. The expression levels of related steroidogenic enzyme genes after bovine mid-CL treatment with GSK4112 and SR8278 in *ex vivo*.

The expression levels of steroidogenic enzyme genes were detected using qPCR. a: *STAR*, b: *CYP11A1*, c: *HSD3β.* The relative expression was normalized to *H2AFZ*. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA.

4.3.3 The expression of related autophagy and apoptosis genes and proteins in bovine mid-CL treated with GSK4112 and SR8278

To further elucidate the downstream consequences of *NR1D1-BMAL1* axis disruption, I analyzed the expression of autophagy and apoptosis markers in genes and proteins. As shown in Figure 4-5, a significantly upregulated *CTSB* expression was observed in mid-CL tissues after treatment with GSK4112 (Figure 4-5d), whereas the expression levels of *LC3B*, *ATG5*, and *CASPASE8* remained unchanged compared with CON (Figure 4-5a, b, c). Unexpected results showed that compared with the treatment by GSK4112, the expressions of *ATG5* and *CTSB* were both reduced in the SR8278 group (Figure 4-5b, d). Consistent with the transcriptional results, Figure 4-6

demonstrates a corresponding increase in CTSB protein (P = 0.0812) (Figure 4-6b, d) and a significant elevation of ATG5 protein (Figure 4-6a, c). In contrast, SR8278 treatment did not elicit substantial observations in either autophagic or apoptotic markers compared to the CON group. Although both ATG5 and CTSB were significantly reduced compared with the GSK4112 group (Figure 4-6).

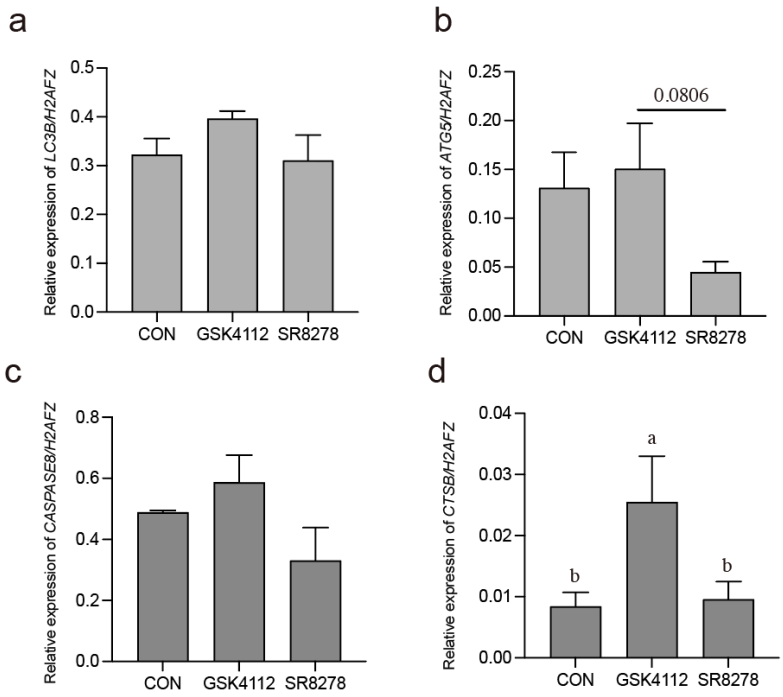


Figure 4-5. The expression levels of related autophagy and apoptosis genes after bovine mid-CL treatment with GSK4112 and SR8278 in *ex vivo*.

The expression levels of autophagy- and apoptosis-related genes were detected using qPCR in mid-CL treated with GSK4112 and SR8278. a: *LC3B*, b: *ATG5*, c: *CASPASE8*, d: *CTSB.* The relative expression was normalized to *H2AFZ*. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different lowercase letters indicate significant differences between groups.

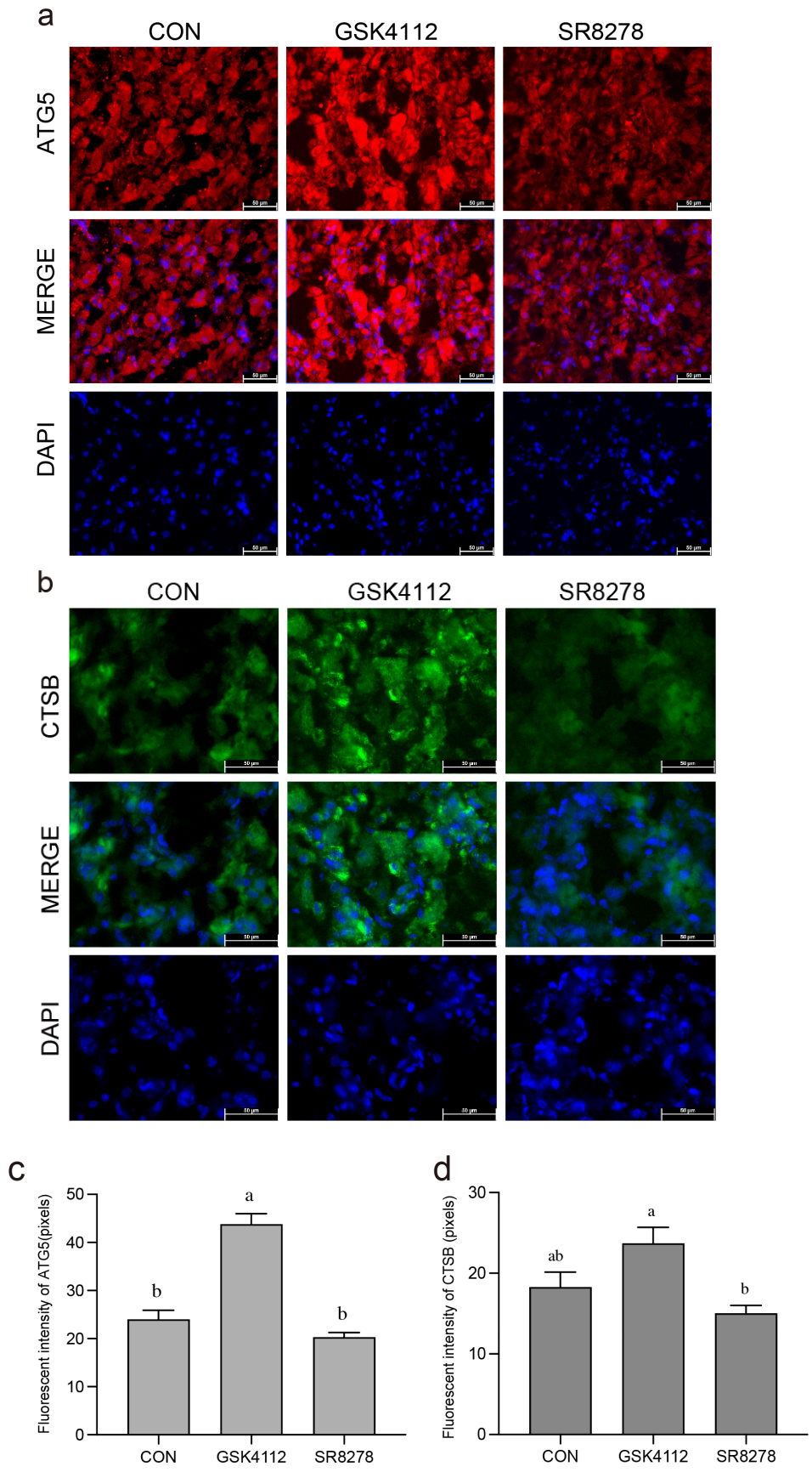

a
CON
GSK4112
SR8278
ATG5
MERGE
DAPI
b
CON
GSK4112
SR8278
CTSB
MERGE
DAPI
c
Fluorescent intensity of ATG5(pixels)
50
40
30
20
10
0
a
b
b
CON
GSK4112
SR8278
d
Fluorescent intensity of CTSB (pixels)
30
20
10
0
ab
a
b
CON
GSK4112
SR8278

Figure 4-6. The expression levels of ATG5 and CTSB after bovine mid-CL treatment with GSK4112 and SR8278 in *ex vivo*.

The expression levels of autophagy- and apoptosis-related proteins were detected using IF staining in mid-CL treated with GSK4112 and SR8278. a: ATG5, b: CTSB, c-d: the relative levels of ATG5 and CTSB by calculating fluorescence intensity. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different lowercase letters indicate significant differences between groups.

4.4 Discussion

In this chapter, GSK4112 and SR8278 were employed as mainly pharmacological tools to disrupt the CC system in mid-CL in an *ex vivo* model. By modulating these key circadian regulators, I investigated whether the CC dysfunction contributes to luteal regression via potential crosstalk with autophagy and apoptosis pathways. These findings demonstrate that CC system disruption plays a significant role in mid-CL phase regression, as evidenced by the consistent reduction in P4 production and downregulation of *CYP11A1* (a key enzyme in steroidogenesis) expression. Considering the function of $PGF_{2\alpha}$ in mid-CL induction in Chapter III, there may be a convergence with the CC system dysfunction to collectively drive mid-CL regression. Additionally, while GSK4112 treatment showed concurrent upregulation of apoptosis and autophagy markers (CTSB and ATG5), suggesting enhanced lysosomal

and autophagic activity, which may further impair luteal steroidogenesis by promoting cellular remodeling or degradation of steroidogenic machinery. These findings are consistent with the results of granulosa cells in sheep [163], mice [164], and bovine [165,166].

Furthermore, in this Chapter, the SR8278 treatment did not elicit effects in steroidogenic or autophagy- and apoptosis-related genes, despite similarly reducing P4 levels. This implies that NR1D1 inhibition may impair P4 production through mechanisms independent of transcriptional regulation, such as post-translational modifications, altered cholesterol availability, or mitochondrial dysfunction[167–170]. On the other hand, SR8278, as an NR1D1 antagonist, significantly upregulates the expression of BMAL1 by relieving the inhibition of its promoter. However, this transcriptional alteration failed to be effectively transmitted to the downstream pathways, or other compensation mechanisms buffered the direct effects. Furthermore, the CC system relies on the positive and negative feedback loops of the core transcription factors to maintain rhythmic oscillations. In this system, BMAL1, as the core positive regulatory factor, forms heterodimers with the CLOCK protein and drives the transcription of downstream target genes. The decrease in P4 may also result from the metabolic reprogramming caused by the upregulation of BMAL1 [171,172]. It leads to changes in cholesterol supply or mitochondrial function. The autophagy/apoptosis pathway is mainly regulated by local signals and thus is insensitive to disturbances in the core clock. Finally, other intracellular transcription factors, such as NF-κB or metabolic pathways, may be involved in compensation to maintain the homeostasis of

gene expression [173]. This buffering capacity highlights the system-level robustness of luteal cell regulation.

In summary, the divergent molecular responses between the two treatments suggest that NR1D1's role in luteal function is context-dependent, with both excessive activation and suppression disrupting P4 synthesis via distinct pathways. These data highlight the circadian regulator *NR1D1* as a critical modulator of luteal P4 production, with potential implications for fertility and reproductive disorders. On another hand, the CC system interacts with multiple regulatory and regressive pathways, though further investigation is required to elucidate whether these effects are mediated through autophagy induction, apoptotic activation, or an alternative mechanism. Importantly, I demonstrate that maintaining the CC homeostasis is essential for proper mid-CL function, revealing that targeting circadian pathways represents a promising new strategy for improving reproductive efficiency in cattle.

4.5 Conclusion

In this chapter, I established clock perturbation models using the agonist GSK4112 and antagonist SR8278 of *NR1D1*. Both pharmacological interventions significantly suppressed P4 synthesis, with GSK4112 treatment further downregulating the steroidogenic gene *CYP11A1* and upregulating the apoptotic factor CTSB. These findings demonstrate that the CC system can directly modulate luteal function, with NR1D1 activation mimicking the luteolytic effects of $PGF_{2\alpha}$. These results highlight NR1D1 as a potential therapeutic target for inducing luteal regression, offering a novel

protocol for reproductive management. While NR1D1 modulation effectively regulates luteolysis, further investigations are warranted to delineate the precise downstream signaling cascades involved.

Chapter V

Effect of the NR1D1 antagonist on the functional development in the $PGF_{2\alpha}$-induced mid-CL tissue

5.1 Introduction

As mentioned in Chapter III and Chapter IV of $PGF_{2\alpha}$ and the CC system in bovine CL, the mid-CL phase represents a critical juncture in the mammalian reproductive cycle, with its functional stability directly impacting pregnancy maintenance. In Chapter III, $PGF_{2\alpha}$-induced mid-CL inhibits P4 synthesis and drives luteal regression through the CTSB-led apoptosis pathway. In Chapter IV, Disruption of the CC system in mid-CL tissue using GSK4112 mimics $PGF_{2\alpha}$ effects, reducing P4 secretion and downregulating steroidogenic genes *CYP11A1* levels, while activating apoptotic and autophagic pathways. The antagonist of NR1D1 SR8278 is used as a CC system destruction model in Chapter IV. The secretion of P4 was also reduced, along with the expression level of CTSB in protein. Summarizing these findings from preceding Chapters, the results revealed that both promoting luteal regression after treatment with GSK4112 and $PGF_{2\alpha}$ alone at mid-CL in *ex vivo*. In contrast, SR8278 elicits distinct outcomes, diverging from the luteolytic patterns induced by GSK4112 and $PGF_{2\alpha}$. This knowledge gap underscores the significance of investigating combined $PGF_{2\alpha}$ and

SR8278 treatment, which offers unique insights into clock system-mediated luteal regression mechanisms.

Therefore, in this chapter, to delineate the crosstalk between circadian regulation and luteolytic pathways, I established a co-treatment system combining SR8278 (to disrupt circadian signaling) and $PGF_{2\alpha}$ (to induce luteolysis) in mid-CL, enabling simultaneous evaluation of their synergistic effects on luteal function.

5.2 Materials & Methods

5.2.1 Collection of Bovine CL Tissues

The bovine CL tissue samples were prepared as described in Chapter II.

5.2.2 Determination of P4

The detection of P4 content was described in Chapter II.

5.2.3 Total RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA extraction and qPCR implementation were prepared as described in Chapter II.

5.2.4 Protein extraction and Western blotting

Total protein was prepared as described in Chapter II.

5.2.5 Effect of NR1D1 antagonists on $PGF_{2\alpha}$-induced CL regression in an *ex vivo* Model

As an antagonist of NR1D1, SR8278 can reduce the expression level of NR1D1 by inhibiting its activity, thereby disrupting the stability of the CC system [161,162]. To further explore whether the CC system is involved in CL regression, I designed an *ex vivo* model to induce CL regression with $PGF_{2\alpha}$ following the previously described research [44,45,48] [38,137]. Specifically, CL tissue pieces obtained from mid-stage ovaries were divided into 4 groups, as shown in Figure 5-1: CON (DMEM + 5% FBS), PG (DMEM + 5% FBS with 1 μM $PGF_{2\alpha}$), PG+SR (DMEM + 5% FBS with 1 μM $PGF_{2\alpha}$ and 10 μM SR8278 (EMD Millipore Corp., Billerica, MA, USA), and PG + SR-6h (initial 6 h in DMEM + 5% FBS with 1 μM $PGF_{2\alpha}$, followed by addition of 10 μM SR8278). All four groups of CL tissues were cultured for 24 h in a humidified atmosphere of air with 5% CO_2 at 38.5°C. After the treatment, the CL samples were collected and homogenized for detection of gene and protein expression levels or stored at -80°C for subsequent analysis.

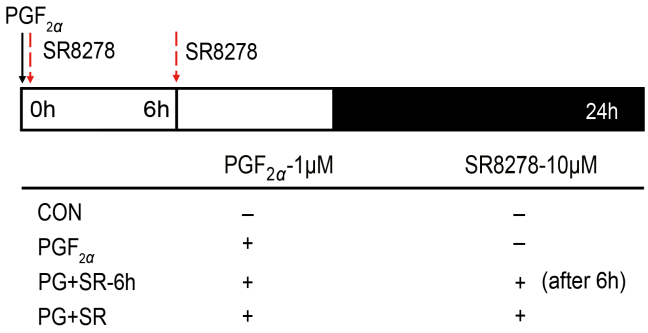


Figure 5-1. Method diagram of the Effects of an NR1D1 antagonist in an *ex vivo* $PGF_{2\alpha}$-induced mid-CL tissue

The mid-CL was cut into small pieces and divided into four groups: CON (DMEM + 5% FBS), PG (DMEM + 5% FBS with 1 μM $PGF_{2\alpha}$), PG+SR (DMEM + 5% FBS with 1 μM $PGF_{2\alpha}$ and 10 μM SR8278, and PG + SR-6h (initial 6 h in DMEM + 5% FBS with 1 μM $PGF_{2\alpha}$, followed by addition of 10 μM SR8278). Subsequently, all groups were continuously cultured in a humidified atmosphere of air with 5% CO_2 at 38.5°C for 24 h.

5.2.6 Statistical analysis

All data are presented as mean ± SEM and represent at least three replicates. Statistically, significant differences were identified using one-way analysis of variance (ANOVA)-Tukey's multiple range test implemented in GraphPad Prism® 10 Software (LA Jolla, CA, USA). Statistical significance was set at $P < 0.05$. Different letters indicated significant differences.

5.3 Results

5.3.1 The secretion levels of P4 and the steroidogenic genes after treatment with SR8278 on $PGF_{2\alpha}$-induced CL regression in an *ex vivo* Model

As previously reported, when mid-CL tissue pre-stimulated with $PGF_{2\alpha}$ was treated with SR8278, the P4 content significantly reversed in both tissue homogenates and culture media of the co-treatment group compared to the $PGF_{2\alpha}$-induced group alone (Figure 5-2a, b). This enhancement became more pronounced with prolonged exposure to SR8278, particularly evident in the culture media. Notably, P4 levels in the co-treatment group (PG+SR) exceeded those of the CON group (Figure 5-2a, b). Additionally, I investigated the expression of steroidogenesis-related genes. The results revealed that the expression of *STAR* was downregulated by $PGF_{2\alpha}$ compared to the CON group (P = 0.0975) (Figure 5-3a). Conversely, co-treatment with SR8278 effectively counteracted this suppressive effect, leading to an upward trend in expression. This restorative effect was more pronounced in the PG + SR group than in the PG + SR-6h group (P = 0.0985) (Figure 5-3a). In contrast, no significant differences were observed in the expression levels of *CYP11A1* and *HSD3β* across all groups (Figure 5-3b, c). These results suggest a potential role of *STAR* in mediating the stimulatory effect of SR8278 on P4 production in mid-CL tissue.

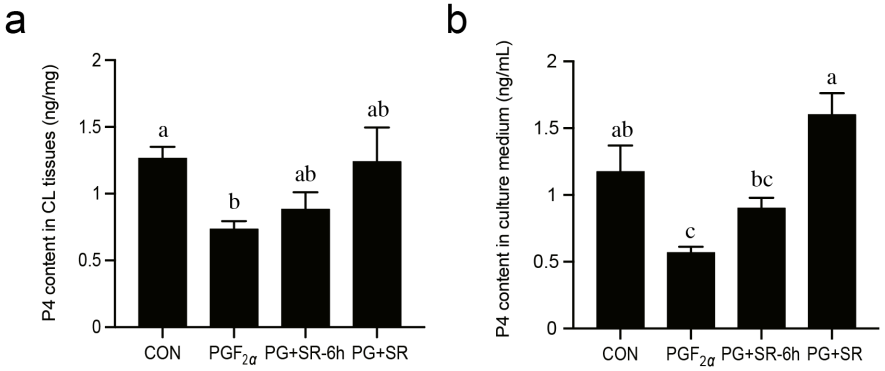


Figure 5-2. The content levels of P4 after treatment with SR8278 in *ex vivo* $PGF_{2\alpha}$-induced mid-CL tissue

a-b: P4 concentration in mid-CL tissues and the tissue culture medium after treatment with SR8278 in $PGF_{2\alpha}$-induced CL tissues was measured using a Progesterone ELISA Kit. The data are expressed as the mean ± SEM, and one-way ANOVA was used to analyze the data. Value with Different letters in lowercase indicates significant differences.

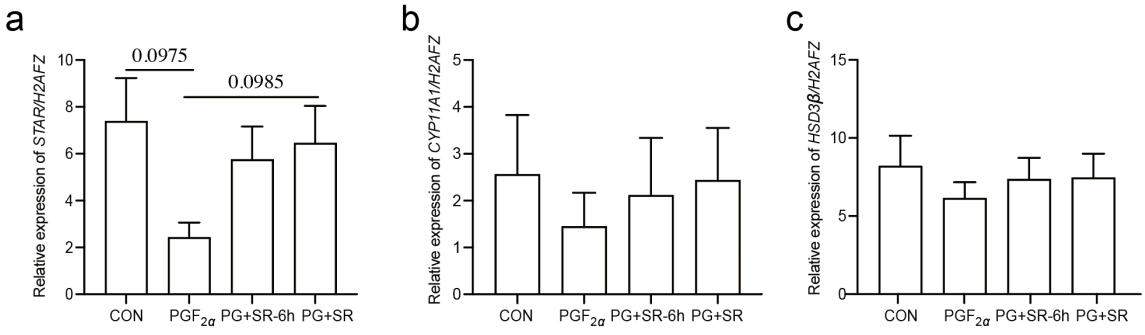


Figure 5-3. The expression levels of steroidogenic genes after treatment with SR8278 in $PGF_{2\alpha}$-induced mid-CL tissue

a-c: Gene expression levels of *STAR*, *CYP11A1*, and *HSD3β* were calculated using the qPCR assay. The relative expression levels are standardized to *H2AFZ*. The results are reported as the mean ± SEM, and one-way ANOVA was used to analyze at least three replicates for each experiment.

5.3.2 The expression levels of the autophagy and apoptosis genes after treatment with SR8278 on $PGF_{2\alpha}$-induced CL regression in an *ex vivo* Model

Further examined the expression of key factors in the autophagy and apoptosis pathways. Results indicated that the expression of the autophagy-related gene *ATG5* (P = 0.0867) (Figure 5-4b) increased in the group treated with both $PGF_{2\alpha}$ and SR8278 co-culture group (PG + SR). In contrast, the apoptotic factor *CTSB* showed distinctly different expression patterns (P = 0.0987) (Figure 5-4d) in the PG + SR group. Compared to the $PGF_{2\alpha}$ group, the addition of SR8278 effectively suppressed $PGF_{2\alpha}$-induced CTSB expression, and this suppression was more pronounced at the protein level (Figure 5-5a, b). Furthermore, the protein expressions of CTSB and LC3 were increased after treatment with $PGF_{2\alpha}$. However, when SR8278 was introduced, these two factors exhibited opposing trends (Figure 5-5). Specifically, in the group treated with both $PGF_{2\alpha}$ and SR8278 for 24 h (PG + SR group), CTSB expression decreased significantly (Figure 5-5a, b). Conversely, LC3 expression increased significantly in both the PG + SR-6h group and the PG + SR treated group (Figure 5-5a, c). Those findings suggest differential regulatory effects of SR8278 on autophagy and apoptosis pathways in luteal tissue.

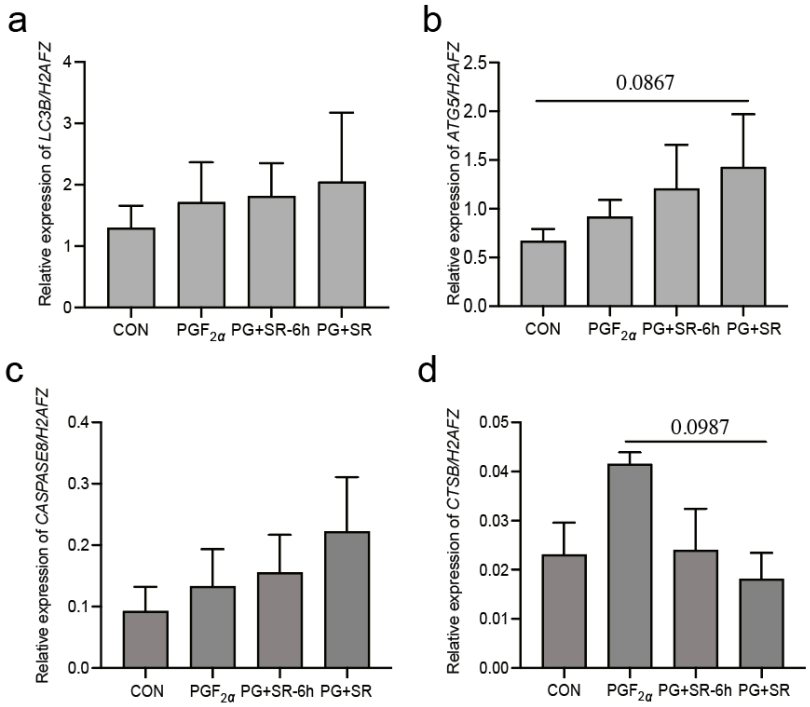


Figure 5-4. The expression levels of autophagy- and apoptosis-related genes after treatment with SR8278 in *ex vivo* $PGF_{2\alpha}$-induced mid-CL tissue

The expression levels of autophagy- and apoptosis-related genes were detected in mid-CL treated with SR8278 in an *ex vivo* $PGF_{2\alpha}$-induced model by qPCR. a: *LC3B*, b: *ATG5*, c: *CASPASE8*, d: *CTSB*, the relative levels of these genes normalized to *H2AFZ*. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA.

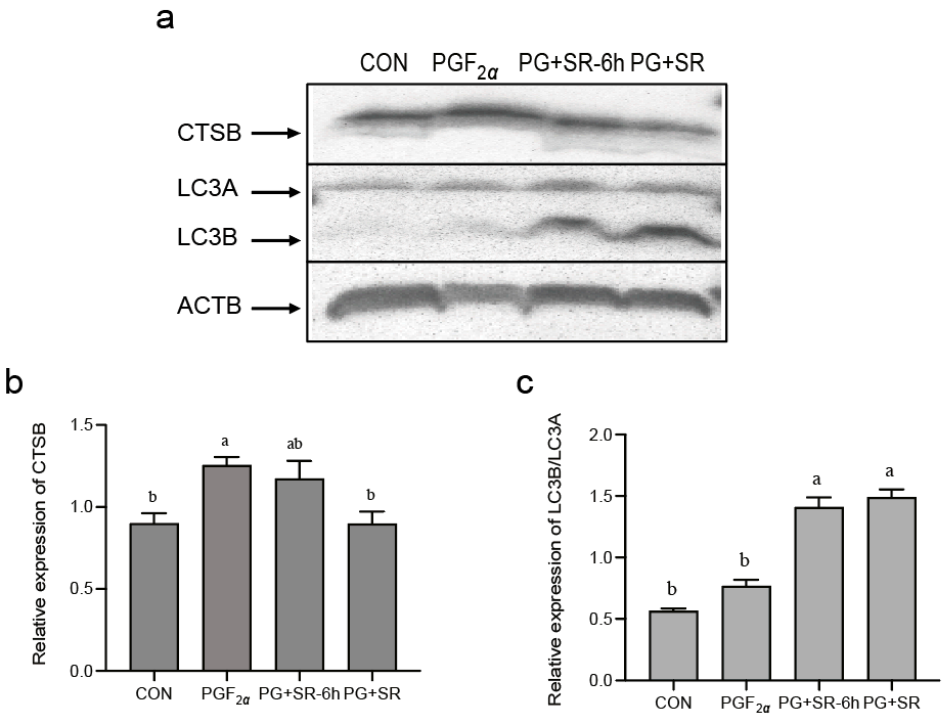


Figure 5-5. The expression levels of CTSB and LC3 after treatment with SR8278 in *ex vivo* $PGF_{2\alpha}$-induced mid-CL tissue

The expression levels of autophagy- and apoptosis-related proteins were detected in mid-CL treated with SR8278 in an *ex vivo* $PGF_{2\alpha}$-induced model by WB. a: CTSB and LC3 proteins, b: the relative levels of CTSB normalized to ACTB, c: the relative levels of LC3 calculated by LC3B/LC3A. All data represent means ± SEM from more than three independent experiments. Statistical significance was determined using one-way ANOVA. Different lowercase letters indicate significant differences between groups.

5.4 Discussion

Building upon the findings from previous Chapters, this study investigates the potential involvement of the CC system in regulating luteal regression. I established

four experimental groups: 1) CON group, 2) $PGF_{2\alpha}$ group (positive control), 3) PG +SR-6h (early induction group), and 4) PG +SR (simultaneous treatment group). The significant decrease in P4 levels in the $PGF_{2\alpha}$-treated group confirmed its well-documented luteolytic effect, as shown in Chapter III. Notably, supplementation with the NR1D1 antagonist SR8278 effectively restored P4 production, demonstrating that NR1D1 inhibition can counteract $PGF_{2\alpha}$-induced luteolysis.

These results revealed a particularly intriguing pattern of differential regulation by SR8278 on $PGF_{2\alpha}$-triggered apoptotic and autophagic pathways. While $PGF_{2\alpha}$ treatment markedly upregulated expression of the pro-apoptotic factor CTSB at both transcriptional and translational levels, SR8278 co-treatment produced a dose-dependent suppression of CTSB. This resistance to the apoptotic effect became more pronounced with extended treatment duration. Concurrently, SR8278 treatment further enhanced expression of autophagy-related markers ATG5 and LC3, suggesting a coordinated shift in cellular degradation pathways. This is a similar effect by the NF-κB pathway in lung injury in mice [174]. These findings support a novel dual regulatory mechanism whereby NR1D1 modulates luteal regression through two complementary pathways: (1) maintaining luteal cell viability by suppressing apoptosis (via CTSB downregulation), and (2) facilitating tissue remodeling by promoting autophagy (via ATG5/LC3 upregulation). The observation of Chapter IV shows that SR8278 single treatment did not significantly alter autophagy and apoptosis, suggests this differential regulation specifically counteracts $PGF_{2\alpha}$-induced luteolysis rather than acting through independent mechanisms.

In summary, in Chapter VI, the cumulative inhibitory effect of SR8278 on NR1D1 can rescue luteal dissolution caused by $PGF_{2\alpha}$. From a theoretical perspective, these findings provide compelling new evidence about how CC components participate in mammalian reproductive physiology. They expand our conceptual framework for understanding luteal regression from a systems biology perspective, where circadian factors interact with classical endocrine pathways. At the practical level, these insights open new possibilities for fertility management in livestock production.

5.5 Conclusion

In this Chapter, I developed a dual-intervention model targeting both luteolysis and the CC system by co-treating mid-CL with $PGF_{2\alpha}$ and the NR1D1 antagonist SR8278. The results revealed that SR8278 treatment significantly attenuated $PGF_{2\alpha}$-induced luteolysis, evidenced by restored P4 synthesis and upregulated expression of the steroidogenic gene *STAR*. Mechanistically, SR8278 activated autophagic pathways while suppressing CTSB-mediated apoptosis, suggesting a protective role for CC modulation in maintaining luteal function. These findings underscore the critical involvement of the CC system in regulating luteal development and demonstrate that pharmacological inhibition of CTSB can rescue $PGF_{2\alpha}$-induced luteal regression.

Chapter VI

General Discussion

Luteal regression is a key process in the estrus cycle of cattle, involving complex physiological mechanisms, including hormone secretion, apoptosis, and tissue remodeling [175]. In this study, I focused on the CC system associated with $PGF_{2\alpha}$-induced CL regression in bovine. In fresh CL tissues from bovine, these results demonstrated that the expression patterns of NR1D1 and BMAL1 genes and proteins are similar to those of steroidogenic genes *STAR*, *CYP11A1*, and *HSD3β*, with peaked expression observed in the mid to late stages of CL development, coinciding with maximal P4 secretion. IF showed that NR1D1 and BMAL1 were primarily detected in the cytoplasm, which contrasts with the conventional understanding that they are mainly localized in the nuclei. To confirm that this difference in immunolocalization was not due to variability in antibody reactivity, the same antibodies were used, revealing the nuclear localization of NR1D1 in both bovine granulosa cells (BGCs) and mouse embryonic fibroblasts (MEFs) cell monolayers, and the localization of BMAL1 in the cytoplasm of BGCs and in the nucleus of MEFs (Figure S1-1) not displayed in here).

Subsequently, the expression pattern was consistent in an *ex vivo* $PGF_{2\alpha}$-induced CL regression model. An agonist of NR1D1, GSK4112, was used to disrupt the expression of the CC system in *ex vivo*, the results pattern remained consistent, with

increased autophagy-related and apoptosis-related genes. And the single treatment with antagonist SR8278, a decrease level of P4 was observed. However, when SR8278 was combined with $PGF_{2\alpha}$ in mid-CL, this pattern was altered. Specifically, the expression levels of steroid synthesis-related genes were rescued while increasing the autophagy-related (LC3) and apoptosis-related (CTSB) factors expression levels. These findings demonstrated that SR8278 may mitigate luteal regression induced by $PGF_{2\alpha}$ in bovine mid-CL.

CC is a transcriptional system [176] that coordinates behavioral and physiological processes such as liver metabolism [177], sleep-wake cycle [76], and reproductive process in mammals [74]. This endogenous timekeeping system enables organisms to anticipate daily environmental changes and coordinate their internal processes accordingly. It's driven by core clock genes, especially the *BMAL1/CLOCK* heterodimer, constituting the central molecular diurnal oscillation circuit. This heterodimer binds with E-boxes in the promoter regions of its downstream genes and activates their transcription. Conversely, their proteins accumulate in the cytoplasm and then translocate back into the nucleus, where they inhibit the transcription of *CLOCK* and *BMAL1*, forming a negative feedback loop. In addition, the clock-controlled genes (CCGs) can inhibit *BMAL1* transcription and they can form a second negative feedback loop [178]. Notably, many CCGs, such as *Bmal1* and *E4bp4*, are under the control of NR1D1 and exhibit distinct expression patterns in contrast to NR1D1 [179,180]. As a molecular oscillator capable of resetting peripheral tissue, its expression patterns are frequently used in various studies. The study by Mazzoccoli et al. demonstrated that

NR1D1 is the only clock gene that maintains its oscillation time under both light-dark cycles (LD) and constant darkness (DD). Moreover, this study found that the expression phases of NR1D1 in different peripheral tissues (liver, kidney, spleen, and testis) of mice remain synchronized, whether in alternating LD conditions or constant DD [181]. NR1D1 also has tissue-specific functions; it recruits the NCoR-HDAC3 complex to repress the expression of *Bmal1* [182] and induces *Bmal1* expression in liver-specific deletion of the HDAC3 model in mice [153]. Beyond the CCGs, NR1D1 regulates the expression of genes involved in immune function, behavior, lipid and glucose homeostasis, muscle metabolism, hormone production, and more [183]. A study of breast cancer found that the NR1D1$^{-/-}$ mice model exhibited lower expression of type I IFNs, and the infiltration of CD8^{+} T cells and natural killer cells was suppressed in tumors. Conversely, pharmacological activation of SR9009, a ligand of NR1D1, enhanced type I IFN-mediated antitumor immunity by inhibiting tumor progression and lung metastasis [184]. A global deletion model of male NR1D1 showed significantly increased expression of the pro-inflammatory cytokines IL-1β and IL-6 [185]. NR1D1 regulates muscle sarcoplasmic reticulum calcium homeostasis by directly binding to the promoter to inhibit SERCA inhibitor myosin [186]. Additionally, GSK4112 treatment altered the rhythmic expression of autophagy genes in tilapia muscle [187]. There are some research has also shown that NR1D1 directly regulates autophagy genes, such as C/EBPβ, ATG5, and LC3 [188]. This study focused on the CC system in the bovine CL, an extremely important link affecting reproduction. Firstly, I measured the expression levels of genes and proteins in CC systems using fresh CL tissues in different stages.

The results are first reported in Chapter II. BMAL1 and NR1D1 are rhythmically expressed throughout the luteal development cycle. The protein analysis results demonstrate that both of them are localized and highly expressed within the nucleus and cytoplasm of the luteal cells (the accuracy was validated using MEF cells, not displayed in here). This phenomenon might be attributed to the intricate metabolic and endocrine functions that luteal cells undertake during their development. The factors within the CC system likely regulate various physiological functions of the luteal cells, such as hormone production and cell metabolism. They achieve this by interacting with metabolic enzymes and other molecules present in the cytoplasm, thereby orchestrating the complex regulatory network. Also, the same patterns are shown in steroid-synthesizing genes and P4. This finding is highly consistent with previous studies [38]. Furthermore, a hold hypothesis is that the CC systems regulate the development of the CL and mediate its regression. Therefore, the GSK4112 (an NR1D1 agonist) and SR8278 (an NR1D1 antagonist) were used to treat small CL pieces. The results provide strong support for this conjecture. Specifically, GSK4112 has a similar effect to $PGF_{2\alpha}$. When GSK4112 is used to treat CL tissues, P4 secretion and steroid synthesis genes are reduced. In contrast, when SR8278 is applied, this imagination is reversed. The data invite further investigation into the detailed mechanism by which the CC system acts on luteal regression.

In cattle, the estrous cycle typically lasts about 21 days, but it also can vary between 17 and 24 days [189] and divided into two main phases: the follicular phase and the luteal phase [190]. During the follicular phase, follicles grow and develop under

the influence of follicle-stimulating hormone (FSH) and LH. The dominant follicle eventually ovulates, leading to the formation of the CL. The luteal phase is characterized by the development and maintenance of the CL, which is formed after ovulation from the ruptured follicle and secreted P4 to inhibit further follicular development and prepare the uterus for pregnancy. If the pregnancy does not occur, the CL will degenerate, leading to the end of the estrous cycle and the onset of a new ovulation cycle again [8]. The regression of the CL is primarily regulated by $PGF_{2\alpha}$, which is released by the uterus. It causes luteolysis by reducing the synthesis of P4 and increasing the production of enzymes that break down the luteal tissue. As the levels of P4 decrease, the blood supply to the luteal tissue is reduced, leading to ischemia and necrosis. This process is facilitated by the action of $PGF_{2\alpha}$, which also increases the expression of genes involved in apoptosis and tissue remodeling. The regression of the CL is essential for the initiation of a new estrous cycle and the resumption of ovarian follicular activity. Therefore, it is easy to assume that the development and regression of CL in cattle have well-designed mechanisms and are effectively controlled [189]. A large of evidence displayed that the degenerative of CL is associated with autophagy and apoptosis, as well as steroid synthesis [191–193].

LC3 exists in two forms: LC3-I and LC3-II, LC3-I is the cytoplasmic form, while LC3-II is the lipidated form that associates with the autophagosome membrane. When the conversion of LC3-I to LC3-II is a hallmark of autophagy initiation and can use as a biomarker to detect autophagy [194,195]. ATG5 forms a complex with ATG12 through a conjugation process mediated by ATG7 and ATG10. The ATG12-ATG5

complex then interacts with ATG16L1 to facilitate the conjugation of ATG8 family proteins to phosphatidylethanolamine (PE) on the phagophore, which is essential for autophagosome formation [196,197]. The ATG8 family in mammals consists of at least 6 proteins (LC3A, B and C, GABARAP, GABARAPL1, and GABARAPL2/GATE-16) [60]. In addition, CASPASE8, also known as FLICE, MACH, or Mch5, is a member of the caspase family of proteases. It is an initiator caspase, encoded by the CASP8 gene and is a crucial enzyme in the apoptotic pathway, with its structure and function highly conserved across mammalian species. CTSB belongs to the lysosomal cysteine protease family, which can act as a procaspase activator and directly activate the activated caspase protein to induce cell apoptosis [198]. It is synthesized as an inactive pre-pro-enzyme in the endoplasmic reticulum and undergoes proteolytic cleavage to become active. CTSB is essential for the final steps of autophagy and has redundant functions with other cathepsins, such as CTSL, thus serving as a marker of apoptosis involved in autophagy and catabolism, leading to many pathological processes [199,200]. In the present study, I focus on these factors. The fresh sample's results demonstrate that the expression levels of both autophagy-related (*LC3B, ATG5*) and apoptosis-related (*CASPASE8, CTSB*) genes have an increasing expression as the CL approaches the luteolytic. This is similar to previous research, luteotrophic signaling was inhibited by LH/PKA/MTOR, while luteolytic signaling via $PGF_{2\alpha}$/AMPK activates a vital signaling mechanism involved in luteal cell regression [201]. Of course, numerous additional studies support this conclusion [16,68,71]. In an *ex vivo* model of $PGF_{2\alpha}$-induced a significant reduction in P4, steroid synthesis (*STAR*) expression levels, and

upregulation of apoptosis-related genes (*CTSB*). Moreover, the GSK4112-treated group also suggests that CC system disruption can enhance the expression levels of autophagy and apoptosis pathways genes in mid-CL. This finding is consistent with previous studies showing that CC genes regulate steroidogenesis and cell survival [137]. Interestingly, the expression of *CTSB* was decreased in the SR8278 plus $PGF_{2\alpha}$-treated group. The involvement of NR1D1 in autophagy and apoptosis is not entirely unexpected, given its previously reported roles in other physiological and pathological processes [157,202–204]. In contrast to expectations, following the application of SR8278, neither LC3 nor ATG5 was increased. This unexpected outcome could potentially be attributed to two possible mechanisms. Firstly, SR8278 might activate the activity of BMAL1, which in turn could trigger a cascade leading to the activation of autophagy [205]. Alternatively, SR8278 could activate the autophagy pathway by inhibiting NR1D1 [157]. Despite the different proposed pathways, the result is consistent in both scenarios, namely the upregulation of LC3 and ATG5. These findings align well with the results of previous research studies, reinforcing the validity of our observations and providing further insights into the complex regulatory mechanisms underlying autophagy in this context.

Understanding luteal regression is crucial for reproductive management and breeding practices in cattle. The CL serves as an excellent model for studying the regulation of cell survival and programmed cell death under physiological conditions. This knowledge helps in designing protocols for estrous synchronization, improving fertility rates, and reducing reproductive losses in cattle. The study has broader

implications for understanding similar processes in other tissues and species; it also can inform therapeutic approaches to manage reproductive disorders in both domestic animals and humans. However, the current study has some limitations. Firstly, the *ex vivo* models can't fully replicate the complex *in vivo* environment of the CL. Although this observation of high P4 and steroid-synthesizing gene expression in the middle and late stages of the CL and a successful establishment of the $PGF_{2\alpha}$-induced model. Secondly, regarding the choice of *ex vivo* model construction, all these models were built using the mid-CL small pieces while limiting possibilities for other periods. Thirdly, I didn't explore the detailed pathways of action, which may limit its applicability to broader studies. Future studies should consider using animal models for validating these findings, exploring the long-term effects of circadian disruption on luteal function, and elucidating the specific molecular pathways through which CC genes regulate luteal regression.

Chapter VII

General Conclusion

Overall, my research indicates that the CC system is dynamically expressed during luteal regression and participates in regulating the synthesis of P4 and the expression of steroid-related genes. After disrupting the clock model, both the inhibitory effect (SR8278) and the activating effect (GSK112) can reduce P4 secretion and accelerate mid-phase luteal regression. The disrupted clock model that inhibits NR1D1 through SR8278 can rescue the mid-stage luteal regression induced by $PGF_{2\alpha}$ through the autophagy apoptotic pathway. To my knowledge, this is the first study to demonstrate such a functional reversal of luteal regression via CC modulation in cattle. Therefore, this study may contribute to enhancing the production performance of cattle, helping to mitigate the risk of future food shortages, and supporting the Sustainable Development Goal of eliminating global hunger.

This study of luteal regression, especially in cattle, has a rich and extensive history replete with notable breakthroughs. Early research focused on identifying the morphological transformations associated with luteal regression, such as the decline in P4 synthesis and the eventual structural disintegration of the CL. As time progressed, scientific understanding of the underlying mechanisms has expanded to include autophagy and apoptosis as pivotal processes driving luteal cell death. The timing of CL regression is intricately regulated by the circadian rhythm, with studies showing

that disturbances in the internal body clock can disrupt this process. For instance, alterations in light exposure patterns or disruptions in the sleep-wake cycle have been shown to impact the timing of luteolysis in various animal models, highlighting the delicate balance of this biological process [206,207]. Against this backdrop, the present study endeavors to explore the intricate regulation between luteolysis and the CC system in ruminants. Although the CC system has been a subject of research in ovarian development for decades, the luteogenesis and the specific mechanisms that work in concert are still limited. this research findings clearly demonstrate that NR1D1, one of key components of the CC system, can significantly accelerate luteal regression by downregulating the expression of steroidogenic genes and P4 synthesis. This regulatory mechanism is closely intertwined with the induction of autophagy and apoptosis in luteal cells. Autophagy is a cellular process that helps in the degradation and recycling of cellular components, while apoptosis is a form of programmed cell death. Both processes are essential for the proper functioning of the luteal tissue and its eventual regression. The ability of NR1D1 to induce both autophagy and apoptosis suggests that this nuclear receptor may act as a crucial regulatory switch, precisely coordinating the timing and magnitude of luteal regression. Collectively, this study provides novel insights into the role of the CC system in luteal regression.

These findings not only enhance our understanding of reproductive physiology but also pave new avenues for developing therapeutic strategies to address luteal phase defects. Future research should focus on elucidating the specific molecular pathways through which NR1D1 exerts its effects on luteal cells, as well as exploring the potential

for developing NR1D1-based therapeutic strategies to modulate luteal function.

Taken together, these findings strongly suggest that the CC system, particularly NR1D1, is one of the potential regulators of CL regression by modulating steroidogenesis, autophagy, and apoptosis. The antagonistic relationship between NR1D1 and BMAL1 may serve as a regulatory switch, controlling the balance between luteotropic and luteolytic signals (Figure 7-1). These insights provide a foundation for future studies exploring the precise molecular pathways through which NR1D1 and BMAL1 interact to regulate luteal function. Understanding these mechanisms could enable the development of targeted interventions to optimize the reproductive performance in cattle, potentially increasing the annual global beef and milk output. It would help meet the growing demand for animal products and contribute to global food security, and address fertility-related disorders in humans. Future research should focus on *in vivo* validation of these findings, examining the long-term effects of CC disruption on reproductive functions, and identifying potential therapeutic interventions targeting the CC system in reproductive management for further improvement of reproductive efficiency in livestock production.

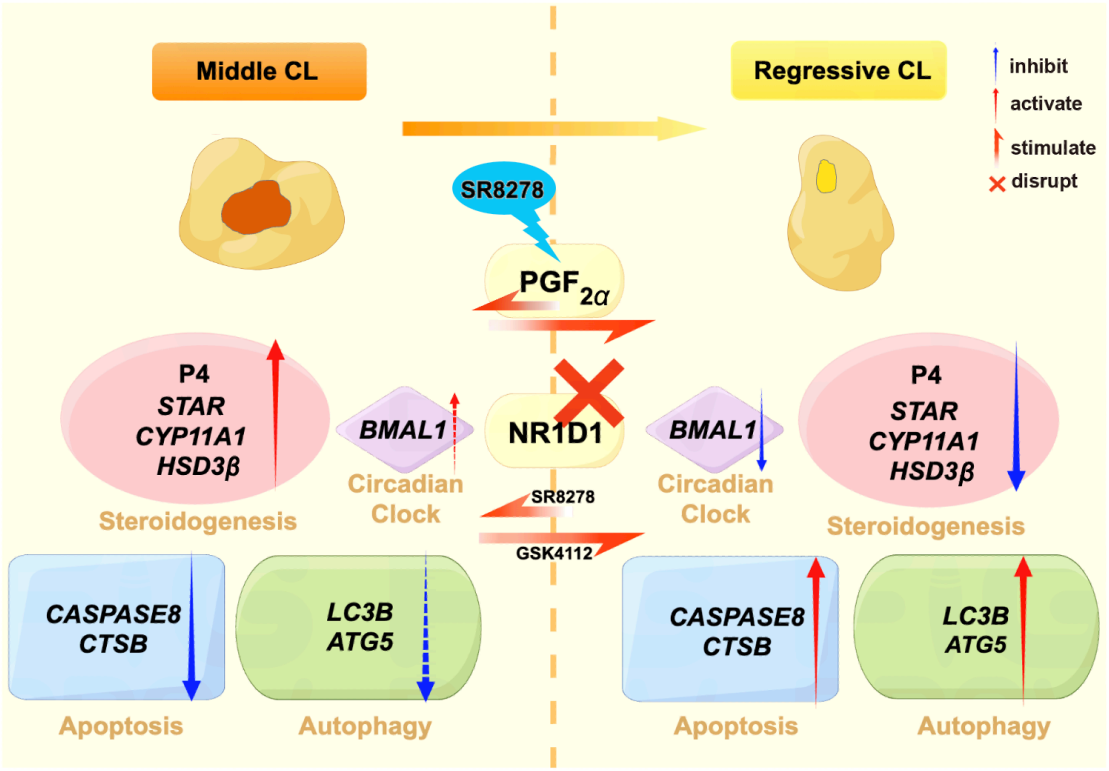


Figure 7-1 Working model of bovine CL induction and salvage *in vitro*.

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Supplementary list

Table 1. Primer sequences for the targeted genes in qPCR

Gene	Primer sequence	Product length/bp	Gene No.
STAR	F: TGGAAGTCCCTCAAGGACCA R: GCAAGTTGGCCTTCAACACC	178	NM_000349.3
CYP11A1	F: AGGCAGAGGGAGACATAAGCA R: GTGTCTTGGCAGGAATCAGGT	156	NM_176644.2
HSD3β	F: GTGAGCGTTTCTCAGTGCTC R: CTAGCACCCGGATTTCCTGC	136	NM_174343.3
CASPASE8	F: TCAGCAATCCGACGTGTGAA R: TAAAATCCCAGGGCCCTCAC	122	NM_001045970.2
CTSB	F: CTGATTGGGGTGACAATGGCTTC R: CTGTATTTAGGGGAAAATGCCCTG	163	NM_174031.2
LC3B	F: CGAGAGCAGCATCCTACCAA R: TGAGCTGTAAGCGCCTTCTT	151	NM001001169.1
ATG5	F: ATATAATGAAGCCGAAACCT R: TCGAGCTAAAGTTCAACCA	125	NM_001034579
BMAL1	F: TGCTAGGCCTTCATTGGTTTCT R: ACCTCAGATCCACCTTGGGA	126	NM_001191170.1
NR1D1	F: CGAGATCTTCACCTACGCCC R: GGAAGCCTGGCGTAAACTGT	190	NM_001078100.2

Gene	Primer sequence	Product length/bp	Gene No.
H2AFZ	F: AGAGCCGGTTTGCAGTTCCCG R: TACTCAGGATGGCTGCGCTGT	115	NM_002106.4

Table 2. The reagents used in the experiment

1 × PBS	**(g/500mL)**
NaCl (Wako)	4 g
KCl (Wako)	0.1 g
$Na_2HPO_4 \cdot 12H_2O$ (Wako)	1.4405 g
KH_2PO_4 (Wako)	0.1 g

After sterilization and store at room temperature.

4% PFA	**10 mL**
Paraformaldehyde	0.4 g
1x PBS (-)	10 mL

Heat in a beaker at approximately 60˚C, after dissolving, store at 4˚C

0.2% TritonX-100	**10 mL**
TritonX-100	20 μl
1x PBS (-)	10 mL

Store at 4 ˚C

TBS-T	**(g/1000mL)**
NaCl (Wako)	8 g
KCl (Wako)	0.2 g
Tris	3 g
0.1% Tween 20	1 mL

Store at room temperature.

5% FBS DMEM (Wako)	**500mL**
Streptomycin sulfate salt (Nacalai tesque)	0.03 g
Penicillin G Potassium Salt (Nacalai tesque)	0.05 g
FBS	25 mL

Store at 4 ˚C.

Antibodies are used for Western blotting

Primary antibodies: BMAL1 (#14020, 1:2,000, Cell Signaling Technology, Danvers, MA, USA); NR1D1 (#13418, 1:2,000, Cell Signaling Technology, Danvers, MA, USA); CTSB (ab58802, 1:500, Abcam, Cambridge, UK); LC3 (GTX82986, 1:1,000, Gene Tex, San Antonio, Texas). Secondly antibodies: ECL™ Anti-mouse IgG (NA931-100μL,1:25,000, Cytiva, Buckinghamshire, UK); ECL™ Anti-Rabbit IgG (NA934-100μL, 1:25,000, Cytiva, Buckinghamshire, UK).

Antibodies are used for Immunofluorescence staining

Primary antibodies: BMAL1 (BS65640, 1:200, Bioworld technology, Nanjing, China); NR1D1 (ab174309, 1:200, Abcam, Waltham, MA, USA); ATG5 (NB110-53818, 1:200, Novus Biologicals, Centennial, CO, USA); CTSB (1:500, ab58802, Abcam, Cambridge, UK). Secondly antibodies: Alpaca anti-Rabbit IgG Nano (VHH) Recombinant Secondary Antibody, Alexa Fluor™ 568 (1:800, SA5-10325, Thermo Fisher Scientific, Rockford, IL, USA); Alpaca Anti-mouse IgG1 Nano (VHH) Recombinant Secondary Antibody, Alexa Fluor™ 488 (1:800, A5-10328, Thermo Fisher Scientific, Rockford, IL, USA).

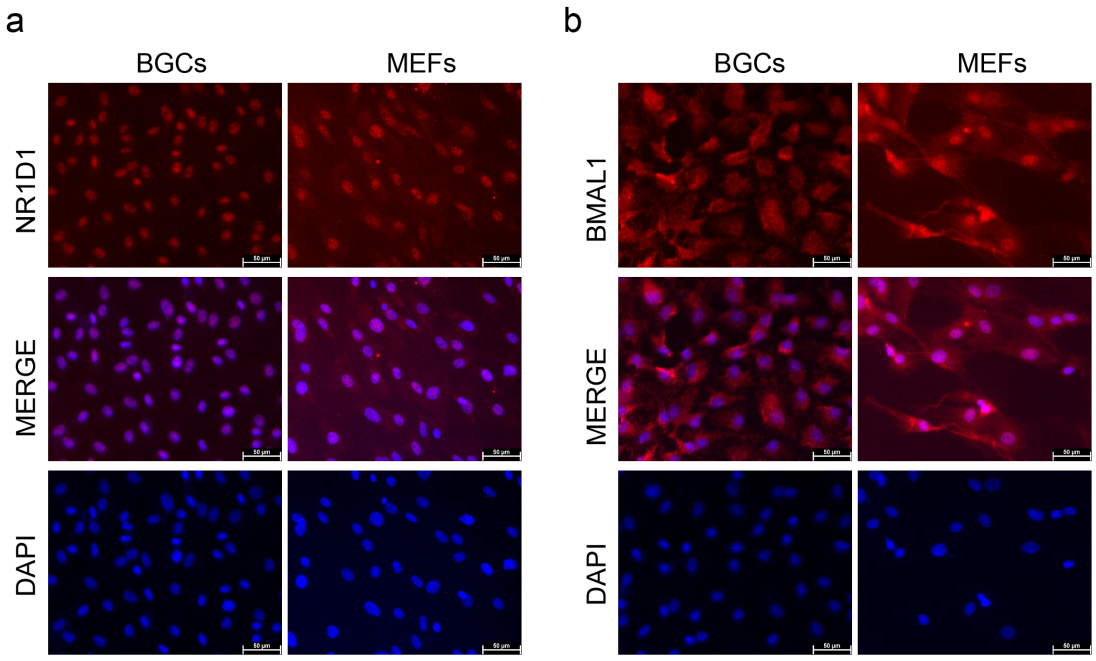

Figure S1-1. The reactivity of NR1D1 and BMAL1 antibodies were validated using MEFs and BGCs

(a) NR1D1 antibodies were detected by immunofluorescence staining using MEFs and BGCs. (b) BMAL1 antibodies were detected by immunofluorescence staining using MEFs and BGCs.

Obtain and culture of Mouse Embryonic Fibroblasts (MEFs) and Bovine Granulosa cells (BGCs).

MEFs: The pregnant mice were sacrificed by cervical dislocation. The embryos were carefully removed and washed with sterile PBS 3 times to eliminate bloodstains. The membranes and placenta surrounding the embryo were meticulously dissected away with tweezers and scissors, followed by rinsing three times with sterile PBS. Then, the embryo's head, internal organs (such as liver, heart, lungs, etc.), and limbs were carefully excised, leaving only the torso. Subsequently, sterile scissors were used to cut

the torso of the embryo into approximately 1-2 mm sized pieces as evenly as possible. The small pieces of tissue were evenly placed within the 60 mm petri dish, and cultured for 30 min in a humidified atmosphere of air with 5% CO_2 at 37°C until the tissue pieces were close to the bottom of the petri dish. After that, an appropriate amount of culture medium (DMEM + 10% FBS) was added. The culture medium was refreshed every 24 h, and the Mouse Embryonic Fibroblasts (MEFs) migrated from tissue pieces. On day 4, the MEFs were collected and seeded into 8-well plates. Immunofluorescence staining was performed when the MEFs grew to 60% density.

BGCs: Bovine ovaries were obtained from a local slaughterhouse and promptly transported to the laboratory. The ovaries were washed several times with a sterile saline solution. Follicular fluid was aspirated from follicles measuring 2-8 mm in diameter using a disposable 18-gauge needle attached to a 10-mL syringe. The follicular fluid was then mixed with the culture medium (DMEM + 5% FBS) at a ratio of 1:10 and incubated at 38.5 °C, 5% CO_2 in a humidified atmosphere of air. After about 24 h, the follicle membrane cell mass was gently detached from the bottom of the cell culture dish. The cells remaining at the bottom of the petri dish were Bovine Granulosa cells (BGCs). These BGCs were washed with PBS and then provided with a fresh culture medium for further culturing. Once the density of BGCs reached confluence, they were washed with PBS again and replated into 8-well plates. The cells were incubated at 38.5 °C in an atmosphere of 5% CO_2 in air. Immunofluorescence staining was carried out when the cells reached 70% confluence.

Immunofluorescence staining: the samples were fixed in 4% paraformaldehyde in PBS for 30 min, followed by three 5-min washes with PBS. Then, the cells were permeabilized with 0.25% (v/v) Triton-X 100. The samples were blocked with 20%

(v/v) Blocking One solution at room temperature (R.T.) for 1 h and washed with PBS. The slides were incubated with primary antibodies (NR1D1 and BMAL1) at 4°C overnight in a humidifying chamber to prevent drying. On the second day, after a 10-min equilibration at R.T., the primary antibodies were removed by PBS for 5 min and repeated 3 times. Subsequently, the second antibody was applied and incubated at R.T. for 1h in the dark. After incubation, the slides were washed 3 times with PBS. It was then stained with Hoechst33342 at R.T. for 15 min in the dark. Replace the Antifade mounting medium (extractant with Hoechst33342 solution) and the slides were coverslipped. Finally, the results were measured with LAS X on a DMi8 fluorescence microscope. Details about antibody information and reaction conditions are in the supplementary information.