

# Physiological crosstalk between the Mel1a and Mel1c pathways modulates melatonin-mediated, monochromatic light combination-induced bursa B-lymphocyte proliferation in chickens

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## Research

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# Abstract

**Background:** Our previous study demonstrated that a combination of green and blue light (G→B or B→G) can improve the antibody titer in chickens compared with single monochromatic light, and melatonin is a critical signaling in the coordination between external light stimulation and the cellular response in the body. This study further clarifies the role of melatonin receptors in monochromatic lights combinations-induced bursa B-lymphocyte proliferation in chickens.

**Results:** A total of 192 chickens were exposed to single monochromatic light (red [R], green [G], blue [B] or white [W] lights) or various monochromatic light combinations (B→G, G→B, R→B) from post-hatching day (P) 0 to P42. With consistency in the upregulation in melatonin level of plasma and melatonin receptors Mel1a, Mel1b and Mel1c expression of bursa, we observed increases in organ index, follicle area, lymphocyte density, B-lymphocyte proliferation, PCNA-positive cells and cyclin D1 expression in bursa of the G→B and B→G group compared with other light-treated groups. Melatonin bound to Mel1a and Mel1c and upregulated p-PKC, p-ERK and p-AKT expression, thereby activating PI3K/AKT and PKC/ERK signaling and inducing B-lymphocyte proliferation in vitro and in vivo.

**Conclusion:** These findings suggested that melatonin modulates a combination of green and blue light-induced B-lymphocyte proliferation in chickens via the Mel1a/PI3K/AKT and Mel1c/PKC/ERK pathways.

## Introduction

The poultry industry is currently undergoing a switch from traditional lighting sources to light-emitting diodes (LEDs). This switch will lead to benefits, such as decreased energy usage, increased avian performance, and improved avian welfare by reducing stress [1]. Birds are sensitive to light because of their highly developed visual systems. In addition to the intensity and photoperiod of light, the wavelength of light also plays an important role in influencing the behavior, growth and health of poultry [2]. Our previous laboratory studies showed that the sensitivity to light wavelengths in chickens is different in various developmental stages: green light enhances muscle growth [3], satellite cell mitotic activity [4] and meat quality properties [5] during the early stage (post-hatching (P) 0–P26), whereas blue light is more effective during the later stage (P27–P42) [3]. Based on this research, we observed that a combination of green and blue monochromatic light (G→B or B→G) resulted in better productive performance of chickens [6] and produced more antibodies to adapt to the outside environment [7]. The bursa of Fabricius is the central humoral immune organ that is unique to birds and plays an important role in B cell development and antibody production [8]. Our previous studies found that a combination of green and blue monochromatic light could promote peripheral blood T and B lymphocytes proliferation. Simultaneously, the levels of anti-Newcastle disease virus (NDV) and anti-bovine serum albumin (BSA) IgG in G–B group were elevated compared to single monochromatic lights [7]. Thus, the proliferation activity of B lymphocytes is closely related to the production of antibodies [9]. However, the effects of monochromatic lights combinations on B-lymphocyte proliferation and its intracellular mechanisms remain unclear.

Despite a large number of reports on the subject [7], the mechanisms underlying the effects of light wavelengths on the immune response still remain to be explored. Melatonin is a neuroendocrine hormone that regulates immune responses, mitochondrial functions, apoptosis, and prevents oxidative stress [10]. A large number of studies have demonstrated the presence of melatonin receptors in a variety of immune cells from various species [11], which explains the immunomodulatory capacity of melatonin administration both in vivo and in vitro. Our lab found that green light stimulated pinealocytes and retinal cells, increasing arylalkylamine N-acetyltransferase mRNA levels and melatonin secretion in chickens [12]. Moreover, we found that pinealectomy not only decreased the concentration of melatonin in plasma, but also decreased the thymus index and T-cell proliferation of chickens [13]. These findings suggest that melatonin may transmit external light signals to intracellular molecules to regulate the immune level of chickens. Thus, one question arises: does melatonin plays a role in promoting monochromatic lights combinations-induced B lymphocyte proliferation?

More than 15 different proteins have been proposed to bind melatonin ranging from receptors, enzymes, pore proteins, transporters and various other proteins [14]. G-protein coupled receptors are currently the best-characterized melatonin targets and are found in invertebrates and vertebrates. These receptors are classified into three membrane receptors called Mel1a, Mel1b, and Mel1c in avians [15]. The best-characterized role of melatonin receptors is on the synchronization of biological rhythms, but increasing evidence suggests that melatonin and its receptors regulate a much broader panel of physiological functions, including sleep, pain, and retinal, neuronal, and immune functions [16, 17]. Our previous studies showed that melatonin enhanced green light-induced T-lymphocyte proliferation in chickens via Mel1b and Mel1c receptors [18]. Overall, these results suggest that melatonin plays a key role in regulating cell proliferation and development. However, the downstream signaling pathway triggered by melatonin that promotes cell proliferation depends on the cell state and type [19, 20]. Thus, an investigation into whether melatonin promotes bursa B-lymphocyte proliferation through its membrane receptor and related intracellular signaling pathways is still required.

In this study, we raised chickens under different monochromatic lights combinations and explored the extent to which monochromatic lights combinations contributed to the development of bursa B-lymphocytes and assessed whether melatonin was involved in this process. Further, we postulated a mechanism for melatonin membrane receptor participation in this regulated process. Additionally, as the poultry industry transitions to antibiotic-free production, there is an urgent need to find economic solutions to improve the immune performance of poultry. If bursa structure, function, and humoral immunity level can be influenced by different monochromatic lights combinations in its developmental state, this approach can become a potentially valuable and economical approach to maintain body health and improve production efficiency in poultry.

## **Materials And Methods**

### **Animals and treatments**

All experimental procedures were approved by the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Approval No. CAU 20171114-2). A total of 192 post-hatching day P0 Arbor Acre male chickens (Beijing Hua du Breeding Co., Beijing, China) were used in this study. The chickens had ad libitum access to feed and water, and the diets were formulated to meet the nutrient recommendations for poultry (NRC,1994). The temperature in the chicken house was set at 32 °C for the first week, and then reduced to 30 °C in the second week, and the relative humidity was maintained at 60% for the entire period.

All chickens were randomly housed in four light-controlled rooms (n = 48), and each room contained six separate cells (eight birds per cell) at a density of 11.5 birds / m<sup>2</sup>, and each cell had a set of independent light sources. The birds were exposed to white light (WW, 400–700 nm), red light (RR, 660 nm), green light (GG, 560 nm), or blue light (BB, 480 nm), which was powered by an LED system. The light parameters are shown in Table 1. When the chickens were 26 d old, 24 chickens from the GG, 24 chickens from the BB, and 24 chickens from the RR groups were transferred to blue, green, or blue light, at 23:00, respectively. The remaining chickens were maintained under the original light color. Therefore, the four light-control groups (before P27) were changed into seven light groups until P42. The seven light groups were as follows: WW, RR, GG, BB, G→B, B→G, R→B.

Table 1  
Light parameters.

Items	Light treatments						
	WW	RR	GG	BB	G→B	B→G	R→B
Light wavelength[nm](1-26days)	400–700	660	560	480	560	480	660
Light wavelength[nm](27-42days)	400–700	660	560	480	480	560	480
Light intensity [W/m <sup>2</sup> ]	0.19	0.19	0.19	0.19	0.19	0.19	0.19
Photoperiod [Light : Dark]	23:1	23:1	23:1	23:1	23:1	23:1	23:1
WW, white light; RR, red light; GG, green light; BB, blue light; G→B, green light and blue light combination; B→G, blue light and green light combination; R→B, red light and blue light combination.							

## Sampling

At P42, six chickens were randomly selected from each light treatment group and body weights (g) were recorded. Blood samples were collected by veins and heparinized with 1,000 UI/mL heparin in avian saline, followed by decapitation. After centrifugation at 3000 × g for 15 min, the plasma was decanted and stored at -80 °C until melatonin measurement. The whole bursa of Fabricius was removed, and the bursa weight of each bird was measured. The organ index was as follows: organ index = bursa weight / body weight.

## Hematoxylin-eosin (H&E) and immunohistochemical staining

Paraffin-embedded tissue was cut into 5 µm-thick sections and then stained using H&E. At least 25 random fields in eight sections of each sample were photographed at 400 × magnification with a microscope (BX51, Olympus, Tokyo, Japan). The 10 largest bursa follicles per field and a total of 12000 bursa follicles (six chickens) per treatment were analyzed.

For immunohistochemical staining, the sections were incubated with primary antibodies (rabbit anti-PCNA, 1:500; Abcam, Cambridge, UK) overnight at 4 °C. Immunoreactivity was visualized by incubating the tissue sections in 0.01 M PBS containing 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.003% hydrogen peroxide for 10 min in the dark. Brown-stained cells indicated a positive reaction to PCNA, and positive cells were counted in 25 random fields from five cross-sections for each sample. The data were analyzed by measuring the integrated optical density (IOD) using Image-pro Plus software.

### **Lymphocyte proliferation assay**

Bursa tissues were aseptically removed, gently triturated, and filtered through a tissue sieve (200 mesh per 2.5 cm). Then, a single-cell suspension was prepared with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were then stimulated with LPS (25 µg/mL, Sigma, St. Louis, MO, USA) and different melatonin concentrations (0, 10, 50, 250, 1000, or 2000 pg/mL; Sigma, St. Louis, MO, USA). Then, the cells were incubated at 37 °C with 5% CO<sub>2</sub> for 44 h. The results are expressed as the stimulation index (SI), which was calculated for each sample by the OD value (570 nm) for cells with stimulation divided by the OD value (570 nm) for cells without stimulation.

Furthermore, to determine the mechanism of action of melatonin, the cell suspensions of the G→B group were prepared with either 10 µM of luzindole (a nonselective antagonist of both Mel1a and Mel1b; Santa Cruz Biotechnology Inc, Dallas, TX, USA), 0.1 µM 4-P-PDOT (a selective antagonist of Mel1b; Tocris Bioscience, Bristol, UK), 0.1 µM prazosin (a selective antagonist of Mel1c; Santa Cruz Biotechnology Inc, Dallas, TX, USA), 5 µM Go9863 (a pan-PKC inhibitor, T6313, Topscience, TX, USA), 10 µM PD98059 (a selective inhibitor of MEK-1 that is the upstream kinase of ERK1/2, Tocris Bioscience, MO, USA), 5 µM LY294002 (an inhibitor of PI 3-kinase inhibitor, L9908, Sigma, St. Louis, MO, USA), 0.5 µM HY102 (an AKT inhibitor, HY-10249A, MCE, Weehawken, USA), or 0.5 µM TWS119 (a GSK inhibitor, HY10590, MCE, Weehawken, USA) for 30 min before the addition of LPS (25 µg/mL, Sigma, St. Louis, MO, USA) and melatonin (250 pg/mL, Sigma, St. Louis, MO, USA). The suspensions were incubated at 37 °C with 5% CO<sub>2</sub> for 44 h, and the OD value was later determined. Additionally, the cells that were incubated with 1 µM of luzindole, 0.1 µM 4P-PDOT, or 0.1 µM of prazosin for 30 min before the addition of LPS (25 µg/mL, Sigma, St. Louis, MO, USA) and melatonin (250 pg/mL, Sigma, St. Louis, MO, USA) for 30 min were collected and used to detect the cAMP content.

### **Enzyme-linked immunosorbent assay**

The culture cells or plasma were collected and assayed using a competitive inhibition enzyme-linked immunosorbent assay kit (USCN Life Science INC., Wuhan, China) for melatonin and cAMP according to

the manufacturer's instructions. The data were measured using a microplate reader equipped with a 450 nm filter. Each sample was tested in triplicate.

### Real-time reverse transcription-polymerase chain reaction(qRT-PCR)

Total RNA (n = 6) was extracted with TRIzol reagent (CW0580A, CoWin Biotech Co., Inc., Beijing, China) according to the manufacturer's instructions. qRT-PCR was performed according to previously described methods [21]. All primers used in the present study are shown in Table 2. The experiments were repeated in triplicate.

Table 2  
Sequences of primers used for RT-PCR.

Gene	Product size	Primer sequences (5'–3')	Accession no.
<i>Mel1a</i>	333	F: CAATGGATGGAATCTGGG A R:GCTATGGGAAGTATGAAGTGG	NM_205362.1
<i>Mel1b</i>	259	F:TTTGCTGGGCACCTCTAAAC R: CGCTTGCTCTTCTGTCCATC	XM_417201.2
<i>Mel1c</i>	237	F:CACTATGGGAAACATTCACTGCC R:TATAGCAGCAGGTGTTCTTCAGG	NM_205361.1
<i>GAPDH</i>	124	F:ATCACAGCCACACAGAAGACG R:TGACTTTCCCCACAGCCTTA	NM_204305
<i>Mel1a</i> , melatonin receptor subtype 1a; <i>Mel1b</i> , melatonin receptor subtype 1b; <i>Mel1c</i> , melatonin receptor subtype 1c; <i>GAPDH</i> , glyceraldehyde-3-phosphate dehydrogenase.			

### Western blot analysis

Proteins were extracted from tissues and cells with RIPA lysis buffer (CoWin Biotech Co., Inc., Beijing, China) containing protease inhibitor (Invitrogen). The protein concentration was determined using a bicinchoninic acid (BCA) kit (Beyotime, P0012). Equal amounts of protein were loaded on SDS-polyacrylamide gel, transferred onto PVDF membranes, and blocked in 5% skimmed milk for 1 h. Subsequently, the membranes were incubated with the following specific antibodies: anti-Mel1a (1:1000; orb11085, Biorbyt, Cambridge, UK), anti-Mel1b (1:1000; ab203346, Abcam, Cambridge, UK), anti-Mel1c (1:500; sc-18574, Santa Cruz Biotechnology, Dallas, TX, USA), anti-phospho-PKC-PAN (pThr497) antibody (1:500, sc-13149, Santa Cruz Biotechnology, Dallas, TX, USA), anti-PKC antibody (1:200, sc-13149, Santa Cruz Biotechnology, Dallas, TX, USA), anti-phospho-ERK1/2 antibody or anti-ERK1/2 antibody (1:4000, M8159, M5670, Sigma, St. Louis, MO, USA), anti-phosphor-AKT (Ser473) antibody (1:500, #4060, CST, Boston, USA), anti-AKT antibody (1:500, #9272, CST, Boston, USA), anti-phosphor-GSK-3 $\beta$  antibody (1:500, PAB10055, Abnova, Taiwan, China), anti-GSK-3 $\beta$  antibody (1: 500, 22104-1-AP, Proteintech Group,

Inc, Wuhan, China), anti- $\beta$ -catenin antibody (1:1000, 51067-2-AP, Proteintech Group, Inc, Wuhan, China), anti-cyclinD1 antibody (1:200, abx100482, Abxexa, Cambridge, UK), or anti- $\beta$ -actin (1:4000; Co Win Biotech Co., Inc, Beijing, China) overnight at 4 °C. The membranes were washed with tris buffered saline tween (TBST) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse/ rabbit antibody (1:8000; Co Win Biotech Co., Inc. Beijing, China). The bands obtained in the blots were scanned and analyzed by measurement of the IOD using ImageJ software (version 4.0.2; Scion Corp., Frederick, MD, USA). The values of the target bands were normalized to the corresponding  $\beta$ -actin values. The results were obtained from three separate experiments.

### **Data analysis**

All data are presented as mean  $\pm$  standard error of the mean (SEM) and analyzed by a one-way ANOVA using SPSS 25.0 software (SPSS, Chicago, IL, USA). Results were considered statistically significant when the P-value was  $< 0.05$ . Correlation analysis, expressed as Pearson's coefficient, was performed to determine the possible linear relationship between B-lymphocyte stimulation index under different light colors and melatonin content in the plasma, as well as B lymphocyte-stimulation index under different light colors and protein expressions of Mel1a, Mel1b, and Mel1c, respectively.

## **Results**

### **Effect of monochromatic lights combinations on morphological change of bursa**

To investigate whether monochromatic lights combinations promoted bursa development, we examined the morphological changes in the bursa. The organ index is shown in Fig. 1a. At P42, the organ index of G $\rightarrow$ B was the highest and was significantly larger by 27.61–95.45% ( $P = 0.000–0.038$ ) than that of WW, RR, GG, BB, and R $\rightarrow$ B, but no significant difference was found between G $\rightarrow$ B and B $\rightarrow$ G ( $P = 0.075$ ). The H&E staining results showed that the bursa of the G $\rightarrow$ B had the largest follicle area and the highest lymphocyte density in the medulla compared to that of WW, RR, GG, BB, and R $\rightarrow$ B, respectively (Fig. 1b-j). However, there was no significant difference between G $\rightarrow$ B and B $\rightarrow$ G ( $P = 0.090–0.747$ ). These results indicated that a green and blue monochromatic light combination could better promote the bursa of Fabricius development than WW, RR, GG, BB, or R $\rightarrow$ B.

### **Effect of monochromatic lights combinations on PCNA, cyclin D1 expression, and B-lymphocyte proliferation in the bursa**

Next, we assessed whether a combination of monochromatic light could affect bursa B-lymphocyte proliferation in vivo and in vitro. In vivo, B-lymphocyte proliferation was detected by PCNA immunohistochemistry. The PCNA-positive cells were scattered in the bursa medulla and cortex. As shown in the Fig. 1k-r, the IOD of PCNA-positive cells was higher in G $\rightarrow$ B than in WW, RR, GG, BB, and R $\rightarrow$ B (31.72–170.44%,  $P = 0.000–0.025$ ). Similarly, the proliferation of bursa lymphocytes in response to LPS treatment was highest in the G $\rightarrow$ B group at P42 (Fig. 2a). There was no significant difference between the G $\rightarrow$ B and B $\rightarrow$ G ( $P = 0.353$ ), but G $\rightarrow$ B was higher than that of B $\rightarrow$ G by 1.29%. Additionally, we tested the protein level of cyclin D1, which plays an important role in the G1 phase progression of the

cell cycle in proliferating cells. As shown in Fig. 2b, cyclin D1 expression was higher in G→B than in WW, RR, GG, BB, and R→B (38.78–61.31%,  $P = 0.009–0.028$ ). However, there was no significant differences between G→B and B→G ( $P = 0.225$ ). These results showed that a green and blue monochromatic light combination could enhance B-lymphocyte proliferation in the chick bursa.

### **Effect of monochromatic lights combinations on plasma melatonin level**

The plasma melatonin levels of chickens after exposure to various monochromatic lights are shown in Fig. 2c. By 42 d, the chickens that were exposed to G→B showed significantly higher levels of circulatory melatonin compared to chickens reared under WW, RR, GG, and R→B ( $P = 0.005–0.045$ ). There was no significant difference between the G→B and B→G ( $P = 0.502$ ), but G→B was higher than that of B→G by 8.89%. Additionally, there was a strong correlation between the melatonin concentration in the plasma and the stimulation index of B lymphocytes ( $r = 0.9674$ ,  $P = 0.000$ ). These results suggested that melatonin plays an important role in the monochromatic lights combinations-induced B lymphocyte proliferation in response to LPS.

### **Effect of exogenous melatonin on B-lymphocyte proliferation in vitro**

To further verify whether exogenous melatonin promoted the proliferation of B lymphocytes in vitro, we used an MTT assay to determine the effect of 0 pg/mL to 2 000 pg/mL of melatonin on B-lymphocyte proliferation after 44 h of culture. As shown in Fig. 2d, when the concentration of exogenous melatonin was  $\leq 250$  pg/mL, there was a strong correlation to strengthen the statement of melatonin enhanced lymphocyte proliferation in a dose-dependent manner ( $r = 0.9994$ ,  $P = 0.022$ ). According to the results of the MTT assay, we chose 250 pg/mL of melatonin for the follow-up experiments. These results indicated that melatonin has the proliferation promotion effect on B lymphocyte.

### **Effects monochromatic lights combinations on melatonin receptor expression in the bursa**

The classic melatonin signaling was induced by binding to its membrane receptor (Mel1a, Mel1b and Mel1c), then activated melatonin membrane receptors initiated downstream intracellular signaling pathways. To investigate the effects of a combination of monochromatic light on the melatonin membrane receptors, we measured the expression levels of *Mel1a*, *Mel1b*, and *Mel1c* mRNA and protein. As shown in Fig. 3a-c, G→B significantly upregulated *Mel1a*, *Mel1b*, and *Mel1c* expression, while RR suppressed the *Mel1a*, *Mel1b*, and *Mel1c* expression at the mRNA levels. However, there were no differences found among G→B and B→G ( $P = 0.089–0.315$ ). Similar results were seen in western blot analysis (Fig. 3d-f), although no differences were found among G→B, and B→G ( $P = 0.182–0.473$ ). Additionally, a strong correlation between B-lymphocyte proliferation and the protein expression of Mel1a ( $r = 0.9111$ ,  $P = 0.0043$ ), Mel1b ( $r = 0.9307$ ,  $P = 0.0023$ ), and Mel1c ( $r = 0.8949$ ,  $P = 0.0065$ ) was noted. These findings implied that Mel1a, Mel1b, and Mel1c may be involved in a combination of monochromatic light-induced-B lymphocyte proliferation and bursal development, but they may play different roles in this process.

### **Effects of melatonin receptors on the combinations of monochromatic lights-induced bursa B-lymphocyte proliferation**

To determine the involvement of melatonin receptors on B-lymphocyte proliferation, primary cultures were pretreated with luzindole (a nonselective antagonist of both Mel1a and Mel1b), 4-P-PDOT (a selective antagonist of Mel1b), and prazosin (a selective antagonist of Mel1c). As shown in Fig. 5a and 5c, we found that pretreatment of B lymphocytes with luzindole or prazosin in response to LPS + melatonin significantly decreased the B lymphocyte stimulating index and cyclin D1 protein expression by 13.01–15.93% ( $P = 0.000–0.001$ ) and 22.93–31.29% ( $P = 0.000–0.007$ ) compared with the LPS + melatonin co-treated group, respectively. However, 4P-PDOT (0.1  $\mu\text{M}$ ), which was co-incubated with LPS and melatonin, showed no statistical significance when compared with the LPS + melatonin co-treated group ( $P = 0.120$ ). Thus, melatonin-induced B-lymphocyte proliferation was mediated by Mel1a and Mel1c, but not Mel1b.

### **Effects of a combination of monochromatic light-induced p-AKT, p-PKC, p-ERK, p-GSK-3 $\beta$ , and $\beta$ -catenin protein expression in the bursa**

To determine the molecular mechanisms underlying the effects of a combination of monochromatic light-induced B lymphocyte proliferation, we examined the expression of PI3K/AKT signaling and PKC/ERK signaling related proteins in chick bursa under WW, RR, GG, BB, G $\rightarrow$ B, B $\rightarrow$ G, and R $\rightarrow$ B. Western blot analysis revealed that the p-AKT, p-PKC, p-ERK, p-GSK-3 $\beta$ , and  $\beta$ -catenin protein were significantly upregulated in G $\rightarrow$ B (Fig. 4a-e), but no significant difference was found between G $\rightarrow$ B and B $\rightarrow$ G ( $P = 0.054–0.772$ ). These results indicated that AKT, GSK-3 $\beta$ , PKC, and ERK cascade may participate in a combination of monochromatic light-induced Mel1a-and Mel1c-mediated B-lymphocyte proliferation.

### **Gi coupled to Mel1a is involved in melatonin-mediated a combination of monochromatic light-induced B-lymphocyte proliferation**

Melatonin receptors are typically coupled to Gi, Gq or Gs proteins. To identify which specific G proteins coupled to Mel1a or Mel1c for the relay of melatonin to downstream activation in B lymphocytes, we isolated B lymphocytes from G $\rightarrow$ B chickens bursa and treated them with melatonin, LPS, and melatonin receptor antagonists either alone or together. Then, cAMP production was determined by ELISA. As shown in Fig. 5b, the melatonin + LPS co-treated group showed significantly decreased cAMP levels (36.10%) compared to that of the control group ( $P = 0.000$ ), and this response was prevented by luzindole and not affected by 4P-PDOT or prazosin. Taken together, these data indicated that Gi might couple to Mel1a and be involved in B-lymphocyte proliferation in G $\rightarrow$ B chickens. Notably, pretreatment of B cells with Mel1c antagonists (prazosin) before melatonin addition did not affect melatonin-induced inhibition of cAMP levels, indicating that Mel1c might not depend on Gi or Gs to activate B-lymphocyte proliferation.

### **Mel1a-activated PI3K/AKT through Gi involved in melatonin-mediated a combination of monochromatic light-induced B-lymphocyte proliferation**

To further confirm that the PI3K/AKT pathway was involved in melatonin-induced B lymphocyte proliferation, we treated cells with LY294002, a PI3K inhibitor, and HY102, an AKT inhibitor. We found that the treatment of isolated B lymphocytes with LPS + melatonin for 30 min markedly elevated the ratios of p-AKT/total-AKT. However, LY294002 and HY102 significantly abrogated the melatonin induced-

upregulation of the p-AKT/total-AKT ratio (Fig. 6a) and inhibited B-lymphocyte proliferation (Fig. 6f). Additionally, the observed melatonin-induced upregulation in the ratio of p-AKT/total-AKT was abrogated by luzindole and not affected by 4P-PDOT or prazosin (Fig. 5d). Taken together, these results indicated that melatonin activated PI3K/AKT through Mel1a in LPS-stimulated B-lymphocyte proliferation.

### **Mel1c-activated PKC/ERK in melatonin-mediated a combination of monochromatic light-induced B-lymphocyte proliferation**

Regulation of ERK activity by melatonin has been reported to play an important role in the proliferation, migration, and differentiation of a variety of cell types. Phospholipase C (PLC $\beta$ )/protein kinase C (PKC), which is a typical upstream transduction factor of ERK, can stimulate the ERK module by direct phosphorylation or by indirect C-Raf stimulation. We blocked the activity of PKC and ERK using the specific inhibitors Go9863 and PD98059 to determine whether the Mel1c-mediated B-lymphocyte proliferation is PKC/ERK-dependent or independent. The MTT and western blots assays showed that Go9863 or PD98059 significantly inhibited B-lymphocyte proliferation and decreased the ratio of p-ERK/total-ERK compared with the LPS + melatonin co-treated group (Fig. 6b, 6f). Additionally, melatonin-induced upregulation of p-PKC and p-ERK1/2 protein expression was abrogated by the Mel1c antagonist and not affected by 4P-PDOT or luzindole (Fig. 5e–5f). These data implied that Mel1c-activated PKC/ERK in melatonin-mediated a combination of monochromatic light-induced B-lymphocyte proliferation.

### **Mel1a/Gi/PI3K/AKT and Mel1c/PKC/ERK signaling pathways are involved in melatonin-mediated a combination of monochromatic light-induced B-lymphocyte proliferation**

To better understand the different intracellular signal dependency of B lymphocytes by Mel1a and Mel1c, we determined the downstream pathways involved in PI3K/AKT and PKC/ERK. It was previously reported that GSK-3 $\beta$ / $\beta$ -catenin pathways are important candidates as downstream mediators of AKT protein. As expected, the results showed that LPS + melatonin co-treatment had markedly elevated ratios of p-GSK-3 $\beta$ /total- GSK-3 $\beta$  ratio and the protein level of  $\beta$ -catenin by 69.33–69.62% compared with the control cells ( $P = 0.000$ ) (Fig. 6c-6d). Additionally, the phosphorylation of GSK-3 $\beta$  and  $\beta$ -catenin evoked by treatment with melatonin was markedly inhibited by treatment with luzindole, LY294002, or HY102 (Fig. 5g-5h, 6c-6d). Consistent with this, the promoting effect of melatonin on B-lymphocyte proliferation and cyclin D1 protein expression was significantly strengthened by the GSK inhibitor TWS119 (Fig. 6e-6f). Unexpectedly, prazosin, Go9863, and PD98059 all significantly inhibited the p-GSK-3 $\beta$ /total- GSK-3 $\beta$  ratio,  $\beta$ -catenin, and cyclin D1 protein expression compared with the LPS + melatonin co-treatment (Fig. 5g-5h, 6c-6e). These results provide strong evidence that Mel1a mediates the-PI3K/AKT signal pathway and the Mel1c-mediated-PKC/ERK signaling pathway has a cooperative action in promoting melatonin-induced B-lymphocyte proliferation.

## **Discussion**

### **Effects of monochromatic lights combinations on B-lymphocyte proliferation in the bursa**

As a key cell type mediating humoral adaptive immunity, B lymphocytes have first been described in chickens as antibody-producing cells [22]. Consistent with this essential function, B-lymphocytes development and maturation can be affected by several environmental factors, such as the caging environment [23], heat stress [24], and photoperiod. Here, we constructed a light-color conversion system throughout the growth of chickens to explore the effect of monochromatic lights combinations on B-lymphocyte proliferation and bursa development. We found that compared with other light treatments, G→B and B→G significantly increased the organ index, area of the bursa follicle, and density of bursa lymphocytes in the medulla. At present, there is a scoring scale to evaluate the development of the bursa of Fabricius. They found that organ index, histopathology scoring, and morphometric analysis of the total follicle area were associated with the extent of bursa development [25] and increased the index of immune organs or histologic changes, suggesting enhanced immune function and ability to resist various infections, diseases, and stress [26]. Based on these results, we demonstrated that G→B and B→G could better promote bursa development in chickens than WW, RR, GG, BB, or R→B. Our results were also supported by previous studies that used a meta-analysis to establish light spectral models of chickens and verify the shift to green-blue of combined LED lights could produce the optimized production performance [27]. However, the effects of light wavelengths on growth and development are also related to the species and photoperiod. In Yangzhou geese, white or red monochromatic lights, when imposing a long photoperiod of 11 h daily could result in greater egg-laying performance [28].

In the present study, we demonstrated that G→B and B→G could promote B-lymphocyte proliferation *in vivo* and increase PCNA and cyclin D1 protein expression. PCNA, which is an indicator for evaluating the state of cell proliferation, and cyclin D1, which is considered to be a proliferation marker. The upregulation of PCNA and cyclin D1 indicated an increase in cell proliferation. A similar observation has been documented that a combination of monochromatic light could effectively enhance the peripheral blood T-lymphocyte proliferation of chickens compared to single monochromatic lights [7]. These results revealed that a combination of green and blue monochromatic light may increase mitogenic activity and B-lymphocyte proliferation, which would result in the enhancement of B cell antibody production.

### **Effects of melatonin on a combination of monochromatic light-induced B-lymphocyte proliferation**

Along with increased B-lymphocyte proliferation, G→B increased the plasma melatonin concentration in chickens. Similar observations have been documented in the early stages of chickens, which have been reported to promote the secretion of melatonin by enhancing pinealocytes to express melatonin key synthetase AA-NAT mRNA, upregulating the expression of positive clock genes and downregulating the expression of negative clock genes [29]. Interestingly, Pearson's correlation analysis showed that melatonin was strongly positively correlated with B-lymphocyte proliferation. It was previously reported that the physiological levels of melatonin in chickens are in the 60–250 pg/mL (0.26~1.08 nmol/L) in the blood [30]. In the present study, our results showed that exogenous melatonin at a level of 250 pg/mL stimulated B-lymphocyte proliferation in the bursa. Consistent with our findings, Luo et al. found that melatonin has an activation effect on mice peripheral blood T/B cells in mice [31]. However, when the exogenous melatonin concentration > 250 pg/mL, the B lymphocyte stimulating index was decreased. In

fact, when the melatonin exceeds the physiological concentration, it will inhibit cell activity. Gao et al. found that treatment with melatonin at the dose from 0.125 mmol/L to 1 mmol/L significantly inhibited cell viability in SW620 and LOVO cells, respectively [32]. Wang et al. found melatonin at pharmacological concentrations (1 mmol/L) significantly suppressed cell proliferation and induced apoptosis in human breast cancer cells [33]. In addition, the melatonin main biologically active metabolite, AFMK can stimulate differentiation in human epidermis and promote epidermal barrier formation [34]. Milani et al. also found melatonin and its metabolites act as free radical scavengers could improve signs of skin aging evaluated clinically [35]. Thus, we inferred G→B not only increased the melatonin concentration but also lead to the accumulation of melatonin metabolites in plasma. Overall, these results suggested that melatonin and its metabolites play a critical role in a green and blue monochromatic light combination illumination-enhanced B-lymphocyte proliferation and promoted bursa development in chickens.

### **Role of melatonin receptors in melatonin-mediated a combination of monochromatic light-induced B-lymphocyte proliferation**

It is well known that melatonin exhibits its immunomodulatory effects via specific receptors [36]. In the present study, we demonstrated that three melatonin receptor subtypes (Mel1a, Mel1b, and Mel1c) were expressed in the bursa, but Mel1b protein expression was markedly lower than Mel1c and Mel1a expression. However, other researchers found that the green light-induced mRNA level of *Mel1a* was lower than that of *Mel1b* and *Mel1c* in the spleen of chickens, suggesting a different role of melatonin membrane receptors in immunomodulation may be related to different cellular situations and various cell types [37]. In vitro experiment, we observed that Mel1a and Mel1c receptors could be, at least partly, involved in a green and blue monochromatic light combination-induced B-lymphocyte proliferation in response to LPS. These findings suggested that the effects of melatonin on Jurkat T lymphocytes seem to be mediated by Mel1a [38], Ahmad et al. found the possible involvement of Mel1a in regulating splenocyte proliferation in the seasonally breeding tropical rodent [39] supported our speculation. Although Mel1a and Mel1b receptors have a 60% homology, these two melatonin receptors appeared to play different roles in mediating immunity. Jockers et al. reported that the Mel1a receptor mainly plays a role in the acquired immune response and the Mel1b receptor was the target for innate immune responses [40]. Additionally, recent research determined that Mel1b plays an important signaling role in mediating melatonin to promote bone marrow stem cell differentiation, bone formation [41]. Thus, we speculate that Mel1b may promote a green and blue combination-induced bursa structure and function development and maturation.

### **Intracellular signaling pathways of melatonin receptor-mediated a combination of monochromatic light-induced B-lymphocyte proliferation**

In this study, we found that melatonin induced a decrease in the intracellular cAMP level in response to LPS, which was scarcely influenced by luzindole and not blocked by 4P-PDOT or prazosin. The descent of cAMP in response to melatonin + LPS treatment might occur because of Gi coupling to Mel1a. This result is consistent with the previous report that Mel1a response is Gi-dependent in NS-1 cells [42], Chinese hamster ovary cells [43], and human embryonic kidney cells [44]. Over the years, the AKT/GSK-3 $\beta$ / $\beta$ -

catenin signaling is essential for B-cell survival and development in the bursa [45]. In our study, we found that G→B and B→G significantly promoted AKT, GSK-3β and β-catenin phosphorylation compared with WW, RR, GG, BB, and R→B. Melatonin-induced AKT and β-catenin activation was blocked by luzindole. The trends shown for AKT activation and β-catenin stabilization under monochromatic light stimulation were consistent with the index of B lymphocyte stimulation, implying the involvement of AKT/GSK-3β/β-catenin in monochromatic light that affects the B-lymphocyte proliferation process. Consistent with our results, in pluripotent stem cells, melatonin-induced neural differentiation involves the PI3K/AKT pathway and is blocked by luzindole [46]. However, the repertoire of signaling pathways modulated by melatonin receptors is highly cell type-specific. In breast cancer models, melatonin has been reported to involve Mel1a to display anti-tumoural properties through inhibition of AKT signaling [47]. These results suggested that Mel1a mediates a green and blue monochromatic light combination stimulated B-lymphocyte proliferation through the intracellular Gi/PI3K/AKT/GSK-3β /β-catenin signaling pathway.

In this study, we observed that G→B and B→G significantly promoted the protein expression of p-PKC and p-ERK protein, and Mel1c may mediate a green and blue monochromatic light combination stimulated B-lymphocyte proliferation through the intracellular PKC/ERK signaling pathway. Consistent with our findings, Ning et al. reported that melatonin promoted IGF-1 mRNA expression through Mel1c mediated- PKC/ERK pathway in chick liver cells [48]. Interestingly, ERK is considered a signaling hub with many different input and output pathways with multiple crosstalks [49]. In our study, pretreatment with Go9863 or PD98059 not only resulted in inhibition of ERK activation but also led to a decrease of GSK-3β (Ser 9) phosphorylation and downregulation of β-catenin in B lymphocytes. This result suggested that melatonin-induced β-catenin upregulation depends on the common regulation of the PI3K/AKT and PKC/ERK pathways. This suggestion was strengthened by results from a series of studies suggesting there is a direct interaction between ERK and GSK-3β in osteoblastic cells [50], HepG2 cells [51], and RAW264.7 cells [52]. Taken together, our findings suggest that there is a crosstalk between the PI3K/AKT and PKC/ERK signals to induce β-catenin upregulation in melatonin receptor-mediated a green and blue monochromatic light combination-induced B-lymphocyte proliferation.

## Conclusions

In summary, a combination of green and blue monochromatic light not only promoted bursa B-lymphocyte proliferation and development on bursa histological structures but also increased melatonin secretion in chickens. Melatonin exerted its proliferation-inducing effect on LPS-stimulated B lymphocytes by binding to the membrane receptors Mel1a and Mel1c with subsequent activation of crosstalk between Gi/PI3K/AKT and PKC/ERK signals, leading to downstream stimulation of GSK-3β and β-catenin (Fig. 7). Our results provide new perspectives for the understanding of differential cellular responses upon Mel1a- or Mel1c-dependent activation of the same pathway (GSK-3β/β-catenin) by the same ligand (melatonin).

## Abbreviations

WW: white light; RR: red light; GG: green light; BB: blue light; G→B: green light and blue light combination; B→G: blue light and green light combination; R→B: red light and blue light combination; Mel1a: melatonin receptor subtype 1a; Mel1b: melatonin receptor subtype 1b; Mel1c: melatonin receptor subtype 1c; Mel: melatonin; LPS: lipopolysaccharide; 4P-PDOT: 4-phenyl-2-propionamideotetralin; SI: stimulation index; p-AKT: phosphorylated AKT; p-PKC: phosphorylated PKC; p-ERK: phosphorylated ERK; p-GSK-3β: phosphorylated GSK-3β

## **Declarations**

### **Acknowledgements**

The authors thank to all members of the Neurobiology Lab, College of Veterinary Medicine, China Agricultural University.

### **Authors' contributions**

CYX and ZYJ contributed to the study design; CYX obtained funding; ZYJ performed the experiments; ZYJ, WZX, CJ and DYL analysed the data; ZYJ and CYX wrote the

manuscript. All authors reviewed the final manuscript.

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### **Availability of data and materials**

The dataset used and/or analyzed during the current study are available from the corresponding author on request.

### **Ethics approval**

Experimental procedures were approved by the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Approval No. CAU 20171114-2), and humane animal care and handling procedures were followed throughout the experiment.

### **Consent for publication**

Not applicable

### **Competing interests**

The authors declare that they have no competing interests.

## Conflicts of Interest:

The authors have declared no conflict of interest.

## References

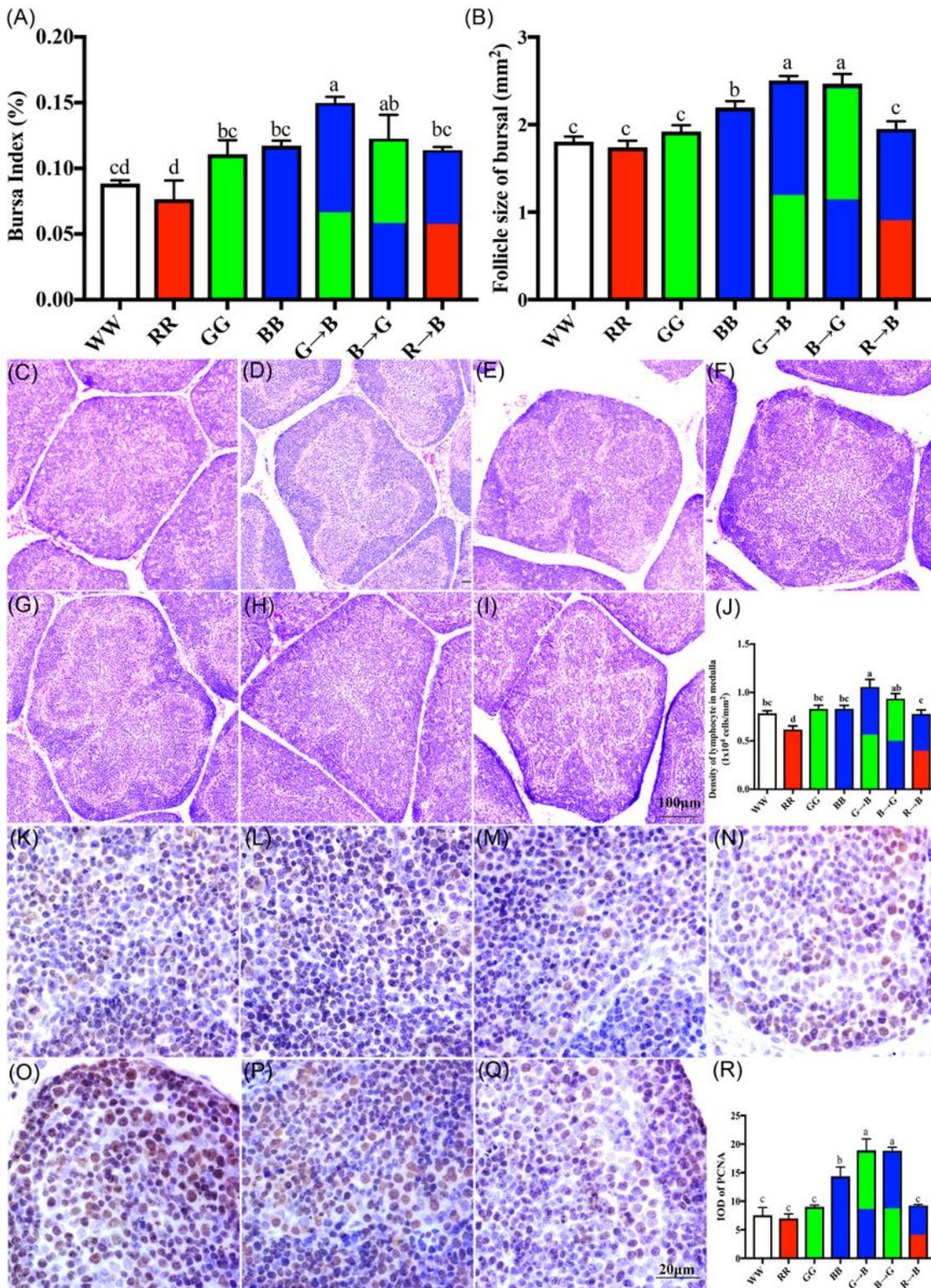
1. Huth JC, Archer GS. Comparison of two LED light bulbs to a dimmable CFL and their effects on broiler chicken growth, stress, and fear. *Poult Sci.* 2015;94: 2027-2036.
2. Akyuz HC, Onbasilar EE. Light wavelength on different poultry species. *Worlds Poult Sci J.* 2018;74:79-88.
3. Cao J, Liu W, Wang Z, Xie, D, Jia L, Chen Y. Green and blue monochromatic lights promote growth and development of broilers via stimulating testosterone secretion and myofiber growth. *J Appl Poult Res.* 2008;17:211-218.
4. Liu W, Wang Z, Chen Y. Effects of monochromatic light on developmental changes in satellite cell population of pectoral muscle in broilers during early posthatch period. *Anat Rec.* 2010;293:1315-1324.
5. Ke YY, Liu WJ, Wang ZX, Chen YX. Effects of monochromatic light on quality properties and antioxidation of meat in broilers. *Poult Sci.* 2011;90:2632-2367.
6. Cao J, Wang Z, Dong Y, Zhang Z, Li J, Li F, Chen Y. Effect of combinations of monochromatic lights on growth and productive performance of broilers. *Poult Sci.* 2012;91:3013-3018.
7. Zhang ZQ, Cao J, Wang ZX, Dong YL, Chen YX. Effect of a combination of green and blue monochromatic light on broiler immune response. *J Photochem Photobiol B.* 2014;138:118-123.
8. Liu XD, Zhou B, Cao RB, Feng XL, Li XF, Chen PY. Comparison of immunomodulatory functions of three peptides from the chicken bursa of Fabricius. *Regul Pept.* 2013;186:57-61.
9. Ko KH, Lee IK, Kim G, Gu MJ, Kim HY, Park BC, Park TS, Han SH, Yun CH. Changes in bursal B cells in chicken during embryonic development and early life after hatching. *Sci Rep.* 2018;8:16905.
10. Hardeland R, Cardinali DP, Srinivasan V, Spence DW, Brown GM, Pandi-Perumal SR. Melatonin—a pleiotropic, orchestrating regulator molecule. *Prog Neurobiol.* 2011;93:350-384.
11. Carrillo-Vico A, Lardone PJ, Alvarez-Sanchez N, Rodriguez-Rodriguez A, Guerrero JM. Melatonin: Buffering the immune system. *Int J Mol Sci.* 2013;14:8638-8683.
12. Jin EH, Jia LJ, Li J, Yang G, Wang ZX, Cao J, Chen YX. Effect of monochromatic light on melatonin secretion and arylalkylamine n-acetyltransferase mRNA expression in the retina and pineal gland of broilers. *Anat Rec.* 2011;294:1233-1241.
13. Xiong J, Wang Z, Cao J, Dong Y, Chen Y. Effect of the melatonin nuclear receptor ROR $\alpha$  on monochromatic light-induced T-lymphocyte proliferation in chicken thymus. *Immunol Lett.* 2019;213:21-29.
14. Liu L, Labani N, Cecon E, Jockers R. Melatonin target proteins: Too many or not enough? *Front Endocrinol.* 2019;10:791.

15. Skwarlo-Sonta K, Majewski P, Markowska M, Oblap R, Olszanska B. Bidirectional communication between the pineal gland and the immune system. *Can J Physiol Pharmacol.* 2003;81:342-349.
16. Posa L, De Gregorio D, Gobbi G, Comai S. Targeting melatonin MT2 receptors: A novel pharmacological avenue for inflammatory and neuropathic pain. *Curr Med Chem.* 2018;25:3866-3882.
17. Zisapel N. New perspectives on the role of melatonin in human sleep, circadian rhythms and their regulation. *Br J Pharmacol.* 2018;175:3190-3199.
18. Guo Q, Wang Z, Dong Y, Cao J, Chen Y. Physiological crosstalk between the AC/PKA and PLC/PKC pathways modulates melatonin-mediated, monochromatic-light-induced proliferation of T-lymphocytes in chickens. *Cell Tissue Res.* 2017;369:555-565.
19. Yu Q, Yu X, Zhong X, Ma Y, Wu Y, Bian T, Huang M, Zeng X. Melatonin modulates airway smooth muscle cell phenotype by targeting the STAT3/AKT/GSK-3beta pathway in experimental asthma. *Cell Tissue Res.* 2020;380:129-142.
20. Xu K, Wang J, Liu H, Zhao J, Lu W. Melatonin promotes the proliferation of chicken sertoli cells by activating the ERK/inhibin alpha subunit signaling pathway. *Molecules.* 2020;25:1230.
21. Bian J, Wang ZX, Dong YL, Cao J, Chen YX. Effect of pinealectomy on the circadian clock of the chick retina under different monochromatic lights. *Chronobiol Int.* 2019;36:548-563.
22. Glick B, Chang TS, Jaap RG. The bursa of fabricius and antibody production. *Poult Sci.* 1956;35:224-225.
23. Gurfein BT, Hasdemir B, Milush JM, Touma C, Palme R, Nixon DF, Darcel N, Hecht FM, Bhargava A. Enriched environment and stress exposure influence splenic B lymphocyte composition. *PloS one.* 2017;12: e0180771.
24. Aguanta BN, Fuller AL, Milfort MC, Williams SM, Rekaya R, Aggrey SE. Histologic effects of concurrent heat stress and coccidial infection on the lymphoid tissues of broiler chickens. *Avian Dis.* 2018;62:345-350.
25. Rosales AG, Villegas P, Lukert PD, Fletcher OJ, Mohamed MA, Brown J. Isolation, identification, and pathogenicity of two field strains of infectious bursal disease virus. *Avian Dis.* 1989;33:35-41.
26. Zheng L, Ma YE, Gu LY, Yuan D, Shi ML, Guo XY, Zhan XA. Growth performance, antioxidant status, and nonspecific immunity in broilers under different lighting regimens. *J Appl Poult Res.* 2013;22:798-807.
27. Yang YF, Pan CC, Zhong RH, Pan JM. The quantitative models for broiler chicken response to monochromatic, combined, and mixed light-emitting diode light: A meta-analysis. *Poult Sci.* 2018;97:1980-1989.
28. Zhu HX, Hu MD, Guo BB, Qu XL, Lei MM, Chen R, Chen Z, Shi ZD. Effect and molecular regulatory mechanism of monochromatic light colors on the egg-laying performance of Yangzhou geese. *Anim Reprod Sci.* 2019;204:131-139.
29. Ma SH, Wang ZX, Cao J, Dong YL, Chen YX. Effect of monochromatic light on circadian rhythm of clock genes in chick pinealocytes. *Photochem Photobiol.* 2018;94:1263-1272.

30. Jiang N, Wang Z, Cao J, Dong YL, Chen YX. Role of monochromatic light on daily variation of clock gene expression in the pineal gland of chick. *J Photochem Photobiol B*. 2016;164:57-64.
31. Luo J, Zhang Z, Sun H, Song J, Chen X, Huang J, Lin X, Zhou R. Effect of melatonin on T/B cell activation and immune regulation in pinealectomy mice. *Life Sci*. 2020;242:117191.
32. Gao Y, Xiao X, Zhang C, Yu W, Guo W, Zhang Z, Li Z, Feng X, Hao J, Zhang K, Xiao B, Chen M, Huang W, Xiong S, Wu X, Deng W. Melatonin synergizes the chemotherapeutic effect of 5-fluorouracil in colon cancer by suppressing PI3K/AKT and NF- $\kappa$ B/iNOS signaling pathways. *J Pineal Res*. 2017;62:e12380.
33. Wang J, Xiao X, Zhang Y, Shi D, Chen W, Fu L, Liu L, Xie F, Kang T, Huang W, Deng W. Simultaneous modulation of COX-2, p300, Akt, and Apaf-1 signaling by melatonin to inhibit proliferation and induce apoptosis in breast cancer cells. *J Pineal Res*. 2012;53:77-90.
34. Kim TK, Kleszczynski K, Janjetovic Z, Sweatman T, Lin Z, Li W, Reiter RJ, Fischer TW, Slominski AT. Metabolism of melatonin and biological activity of intermediates of melatonergic pathway in human skin cells. *FASEB J*. 2013;27:2742-2755.
35. Milani M, Puviani M. Anti-aging efficacy of melatonin-based cream: clinical and instrumental skin evaluation. *Cosmetics*. 2018;5:60.
36. Calvo JR, Gonzalez-Yanes C, Maldonado MD. The role of melatonin in the cells of the innate immunity: A review. *J Pineal Res*. 2013;55:103-120.
37. Acuna-Castroviejo D, Escames G, Venegas C, Diaz-Casado ME, Lima-Cabello E, Lopez LC, Rosales-Corral S, Tan DX, Reiter RJ. Extrapineal melatonin: Sources, regulation, and potential functions. *Cell Mol Life Sci*. 2014;71:2997-3025.
38. Lardone PJ, Rubio A, Cerrillo I, Gomez-Corvera A, Carrillo-Vico A, Sanchez-Hidalgo M, Guerrero JM, Fernandez-Riejos P, Sanchez-Margalet V, Molinero P. Blocking of melatonin synthesis and MT(1) receptor impairs the activation of Jurkat T cells. *Cell Mol Life Sci*. 2010;67:3163-3172.
39. Ahmad R, Haldar C, Gupta S. Melatonin membrane receptor type MT1 modulates cell-mediated immunity in the seasonally breeding tropical rodent *Funambulus pennanti*. *Neuroimmunomodulation*. 2012;19:50-59.
40. Jockers R, Delagrange P, Dubocovich ML, Markus RP, Renault N, Tosini G, Cecon E, Zlotos DP. Update on melatonin receptors: IUPHAR Review 20. *Br J Pharmacol*. 2016;173:2702-2725.
41. Wang B, Wen H, Smith W, Hao DJ, He BR, Kong LB. Regulation effects of melatonin on bone marrow mesenchymal stem cell differentiation. *J Cell Physiol*. 2019;234:1008-1015.
42. Chen M, Cecon E, Karamitri A, Gao W, Gerbier R, Ahmad R, Jockers R. Melatonin MT1 and MT2 receptor ERK signaling is differentially dependent on Gi/o and Gq/11 proteins. *J Pineal Res*. 2020;68:e12641.
43. Capsoni S, Viswanathan M, De Oliveira AM, Saavedra JM. Characterization of melatonin receptors and signal transduction system in rat arteries forming the circle of Willis. *Endocrinol*. 1994;135:373-378.

44. Browning C, Beresford I, Fraser N, Giles H. Pharmacological characterization of human recombinant melatonin MT(1) and MT(2) receptors. *Br J Pharmacol*. 2000;129:877-886.
45. Nuthalapati NK, Evans JD, Taylor RL, Branton SL, Nanduri B, Pharr GT. Transcriptomic analysis of early B-cell development in the chicken embryo. *Poult Sci*. 2019;98:5342-5354.
46. Shu T, Wu T, Pang M, Liu C, Wang X, Wang J, Liu B, Rong L. Effects and mechanisms of melatonin on neural differentiation of induced pluripotent stem cells. *Biochem Biophys Res Commun*. 2016;474:566-571.
47. Hill SM, Belancio VP, Dauchy RT, Xiang S, Brimer S, Mao L, Hauch A, Lundberg PW, Summers W, Yuan L, Frasch T, Blask DE. Melatonin: An inhibitor of breast cancer. *Endocr Relat Cancer*. 2015;22:R183-204.
48. Ning SJ, Wang Z, Cao J, Dong YL, Chen YX. Mel1c mediated monochromatic light-stimulated IGF-I synthesis through the intracellular G(alpha)q/PKC/ERK signaling pathway. *Int J Mol Sci*. 2019;20:1682.
49. Wainstein E, Seger R. The dynamic subcellular localization of ERK: Mechanisms of translocation and role in various organelles. *Curr Opin Cell Biol*. 2016;39:15-20.
50. Park KH, Kang JW, Lee EM, Kim JS, Rhee YH, Kim M, Jeong SJ, Park YG, Kim SH. Melatonin promotes osteoblastic differentiation through the BMP/ERK/Wnt signaling pathways. *J Pineal Res*. 2011;51:187-194.
51. Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, Bartholomeusz G, Li Y, Pan Y, Li Z, Bargou RC, Qin J, Lai CC, Tsai FJ, Tsai CH, Hung MC. ERK associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. *Mol Cell*. 2005;19:159-170.
52. Gong K, Zhou F, Huang H, Gong Y, Zhang L. Suppression of GSK3beta by ERK mediates lipopolysaccharide induced cell migration in macrophage through beta-catenin signaling. *Protein Cell*. 2012;3:762-768.

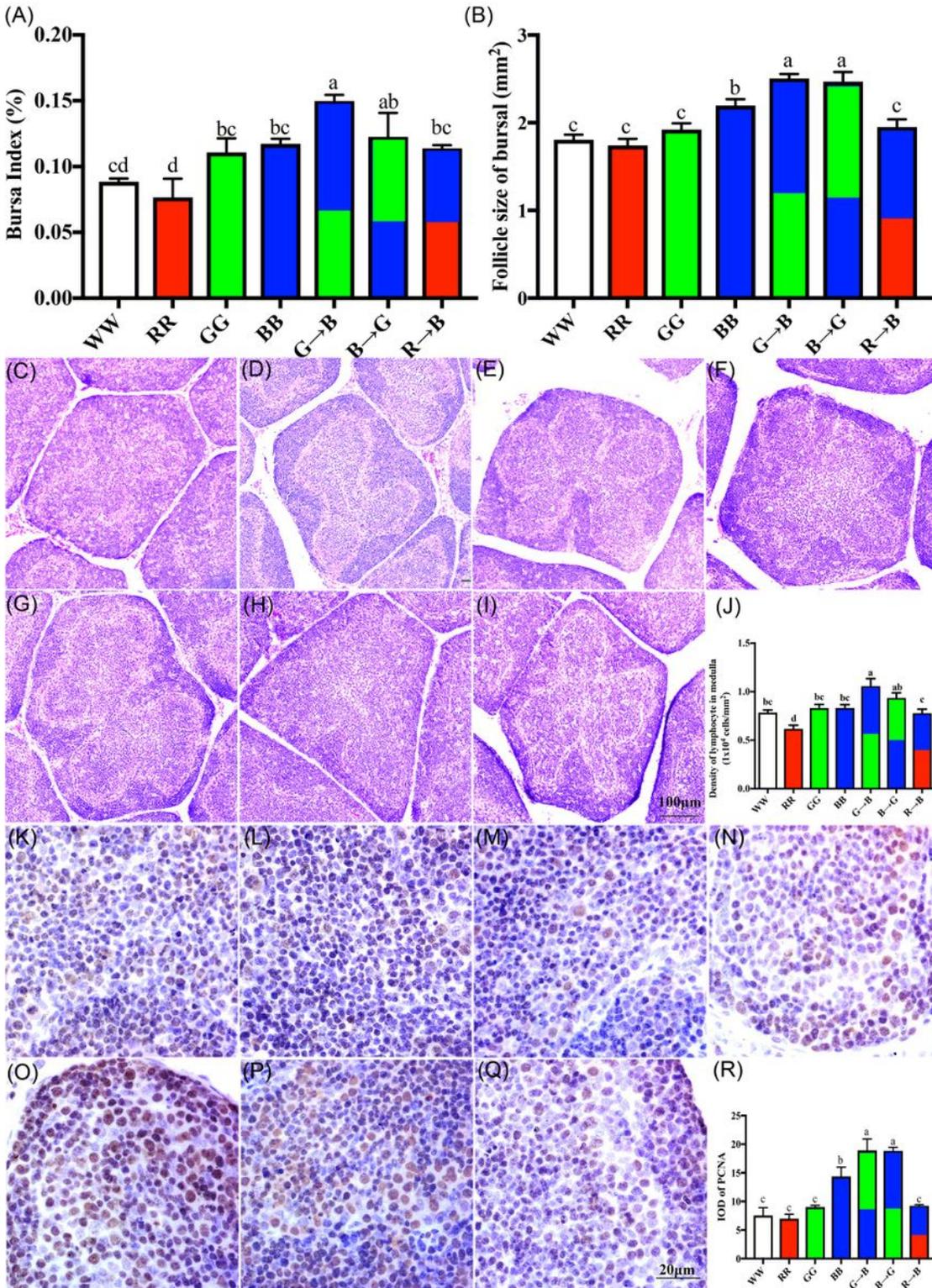
## Figures



**Figure 1**

Effects of combinations of monochromatic lights on bursa index (a), area of bursa follicle (b-i), the density of bursa lymphocyte in the medulla (j) (Scale bar = 100  $\mu$ m), immunohistochemical staining of PCNA in the bursa (k-r) (Scale bar = 50 $\mu$ m). WW: white light; RR: red light; GG: green light; BB: blue light; G→B: green light and blue light combination; B→G: blue light and green light combination; R→B: red light

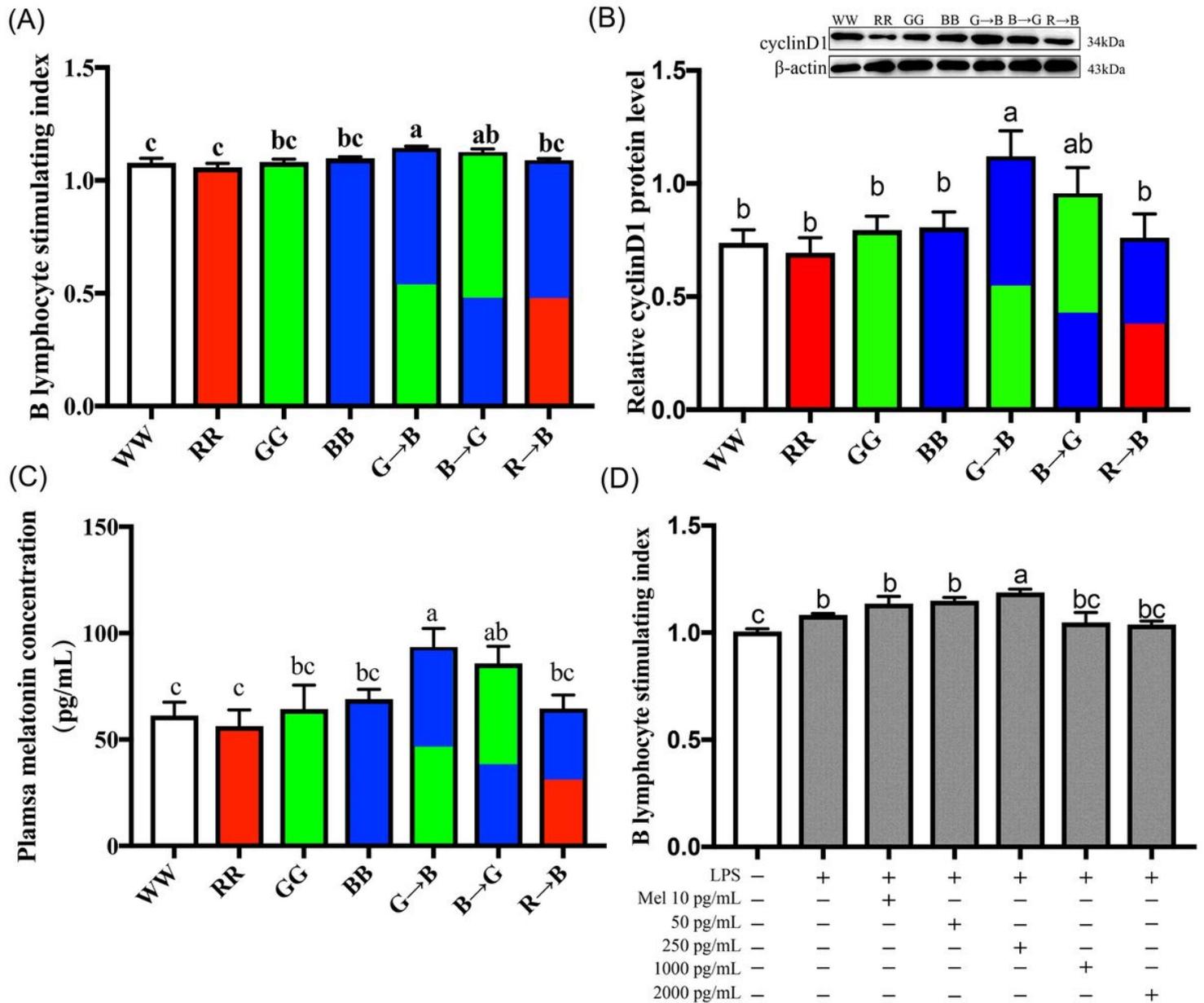
and blue light combination. The results are presented as means  $\pm$  SEM. Different letters (a-b) indicate significant differences between the treatments at the same age ( $P < 0.05$ ).



**Figure 1**

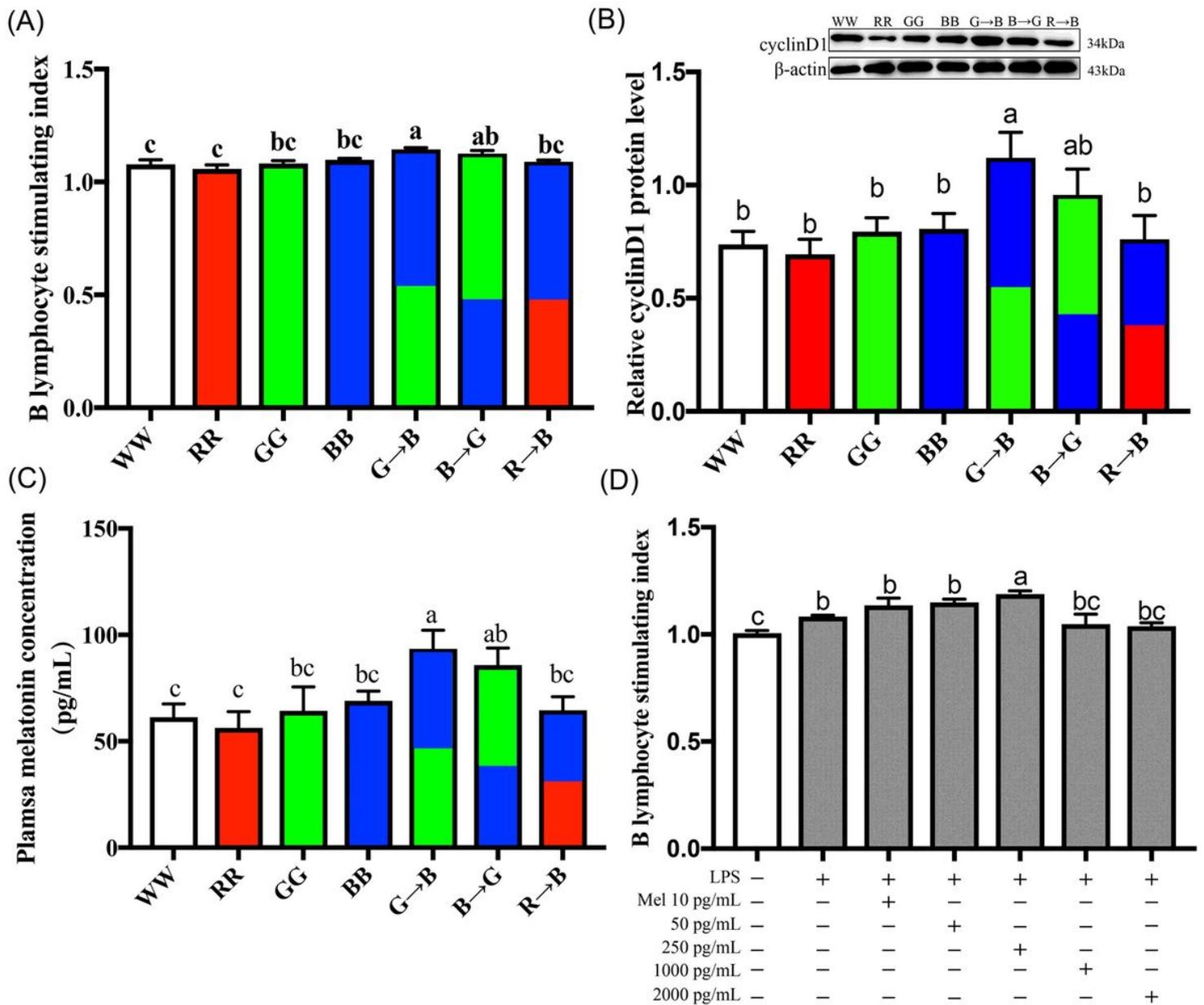
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G→B: green light and blue light combination; B→G: blue light and green light combination; R→B: red light and blue light combination. The results are presented as means ± SEM. Different letters (a-b) indicate significant differences between the treatments at the same age ( $P < 0.05$ ).



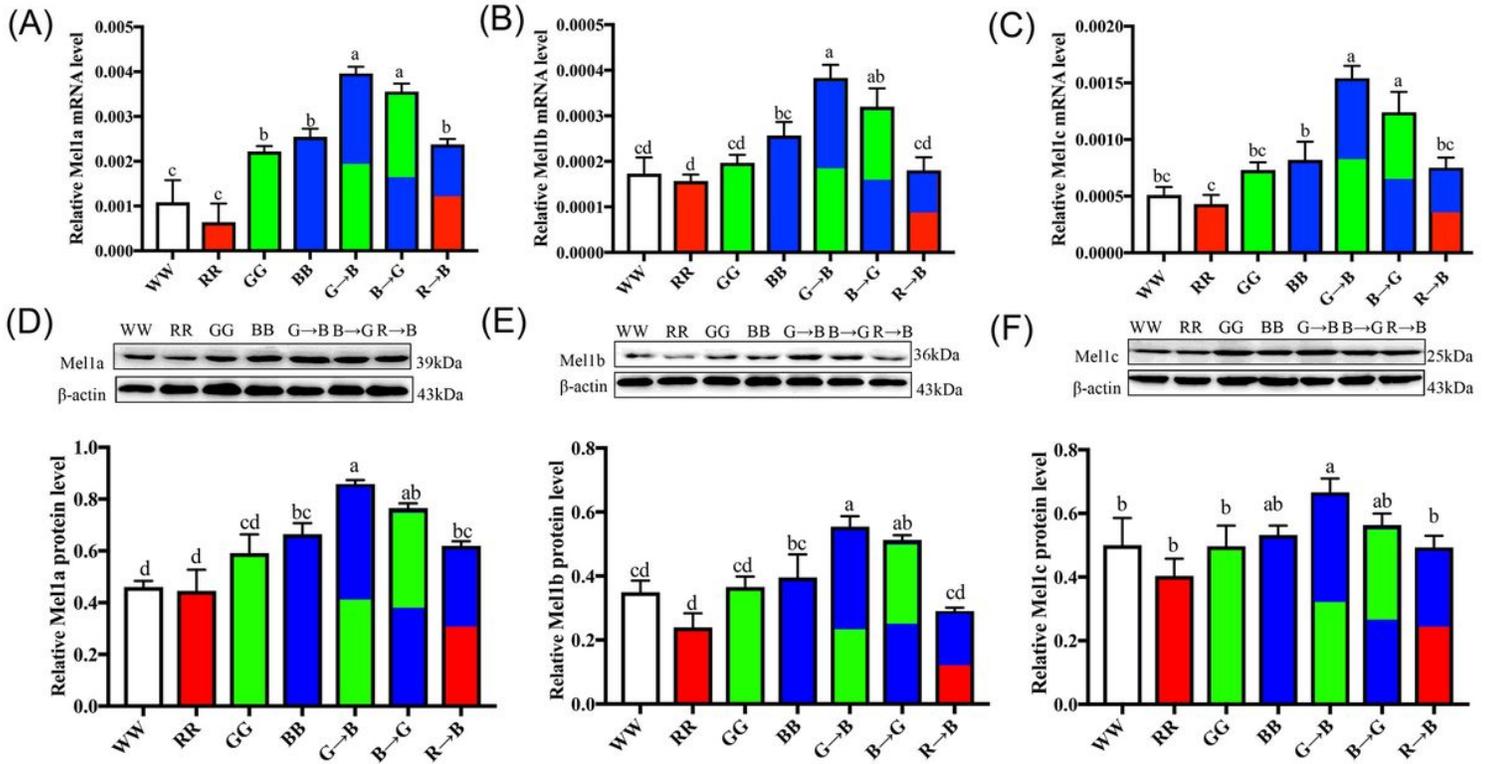
**Figure 2**

Effects of combinations of monochromatic lights on bursa B-lymphocyte proliferation in response to LPS (a), cyclin D1 protein expression (b), plasma melatonin concentration (c), exogenous melatonin on B-lymphocyte proliferation in the bursa in response to LPS in the G→B group (d). WW, white light; RR, red light; GG, green light; BB, blue light; G→B, green light and blue light combination; B→G, blue light and green light combination; R→B, red light and blue light combination. The results are presented as the means ± SEM. Different letters (a-c) indicate significant differences between the treatments at the same age ( $P < 0.05$ ).



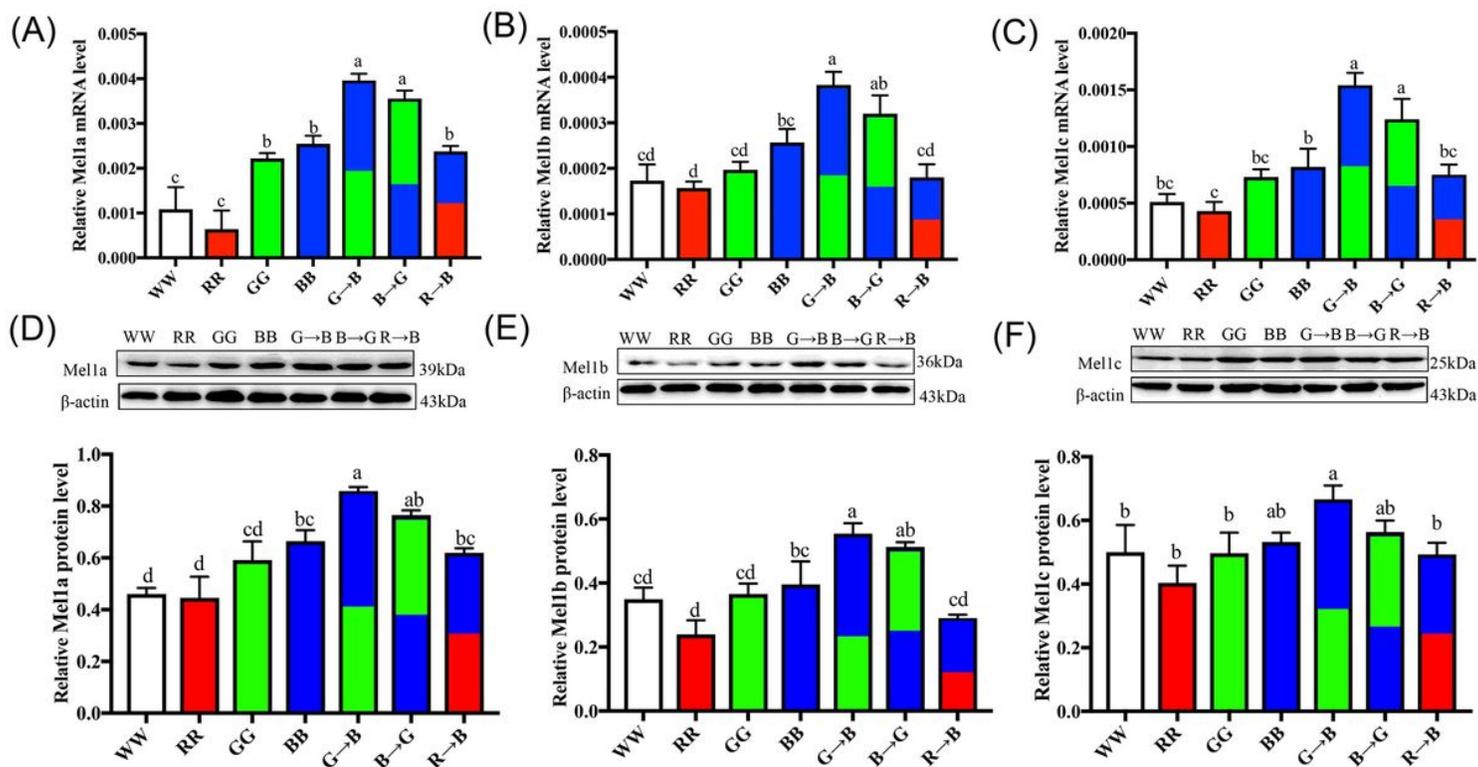
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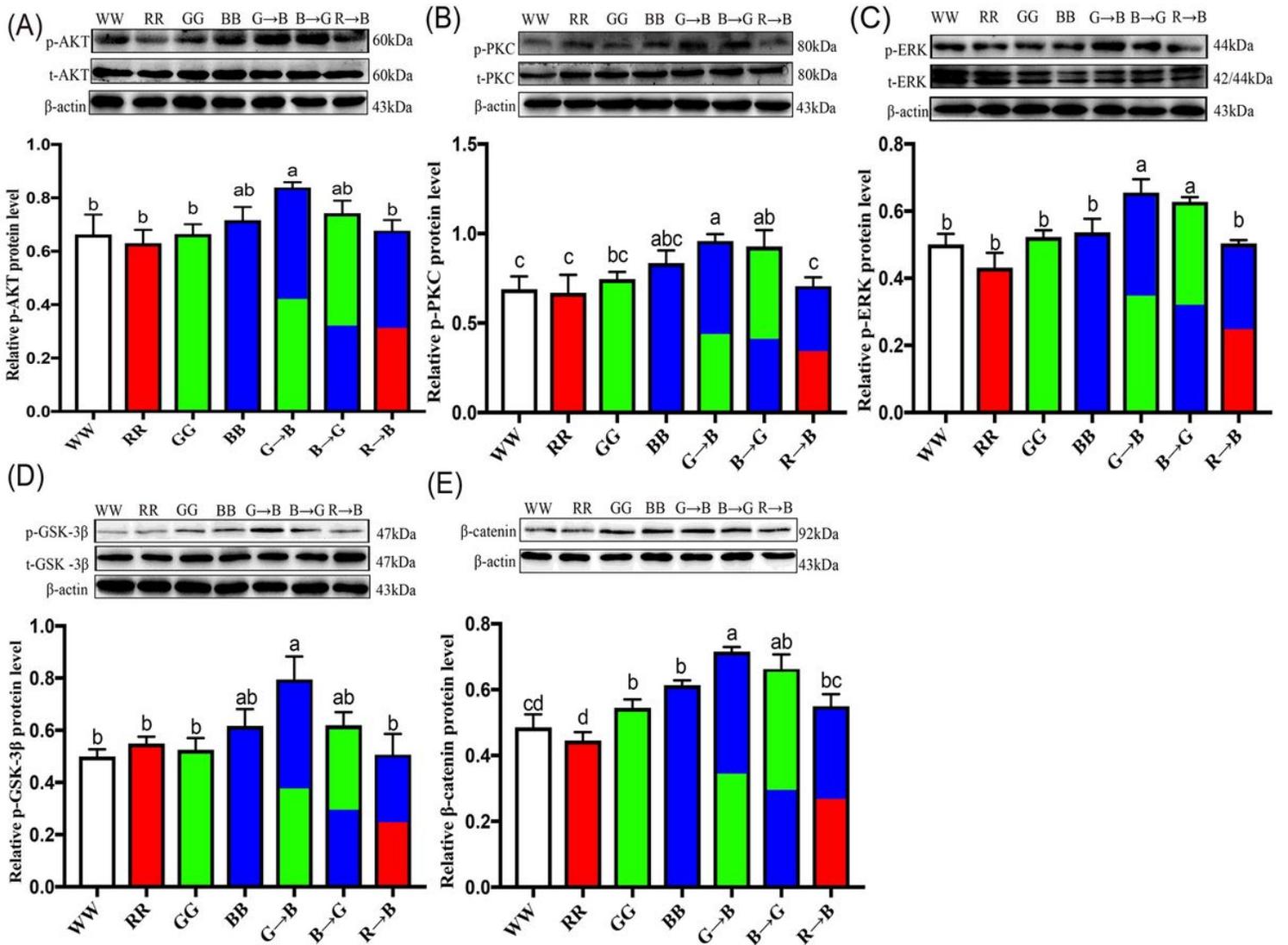
**Figure 3**

Effects of combinations of monochromatic lights on Mel1a mRNA expression (a); Mel1b mRNA expression (b); Mel1c mRNA expression (c); Mel1a protein expression (d); Mel1b protein expression (e); Mel1c protein expression (f) in bursa at P42. Mel1a, melatonin receptor subtype 1a; Mel1b, melatonin receptor subtype 1b; Mel1c, melatonin receptor subtype 1c. WW, white light; RR, red light; GG, green light; BB, blue light; G→B, green light and blue light combination; B→G, blue light and green light combination; R→B, red light and blue light combination. The results are presented as the means  $\pm$  SEM. Different letters (a-d) indicate significant differences between the treatments at the same age ( $P < 0.05$ ).



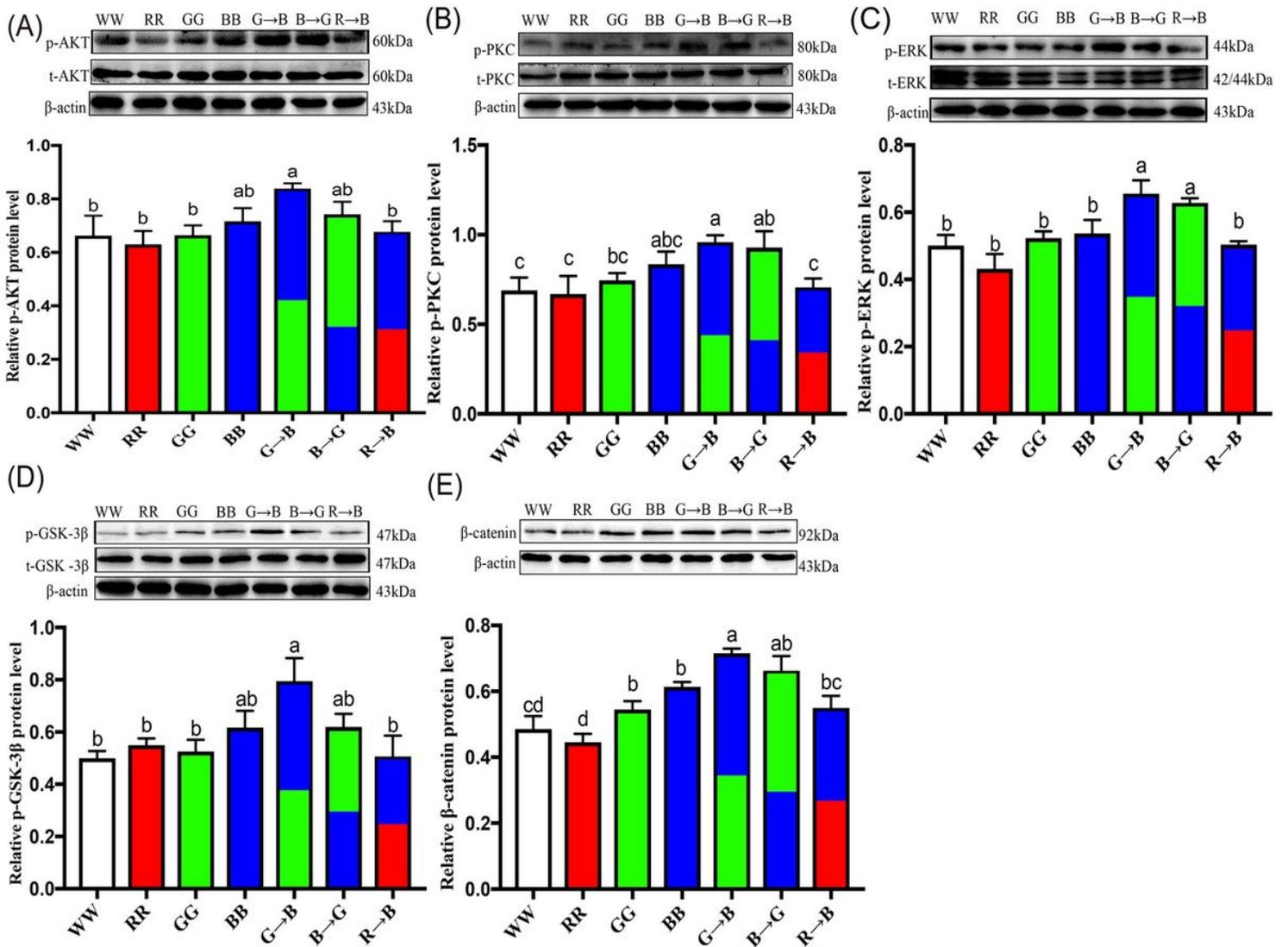
**Figure 3**

Effects of combinations of monochromatic lights on Mel1a mRNA expression (a); Mel1b mRNA expression (b); Mel1c mRNA expression (c); Mel1a protein expression (d); Mel1b protein expression (e); Mel1c protein expression (f) in bursa at P42. Mel1a, melatonin receptor subtype 1a; Mel1b, melatonin receptor subtype 1b; Mel1c, melatonin receptor subtype 1c. WW, white light; RR, red light; GG, green light; BB, blue light; G→B, green light and blue light combination; B→G, blue light and green light combination; R→B, red light and blue light combination. The results are presented as the means  $\pm$  SEM. Different letters (a-d) indicate significant differences between the treatments at the same age ( $P < 0.05$ ).



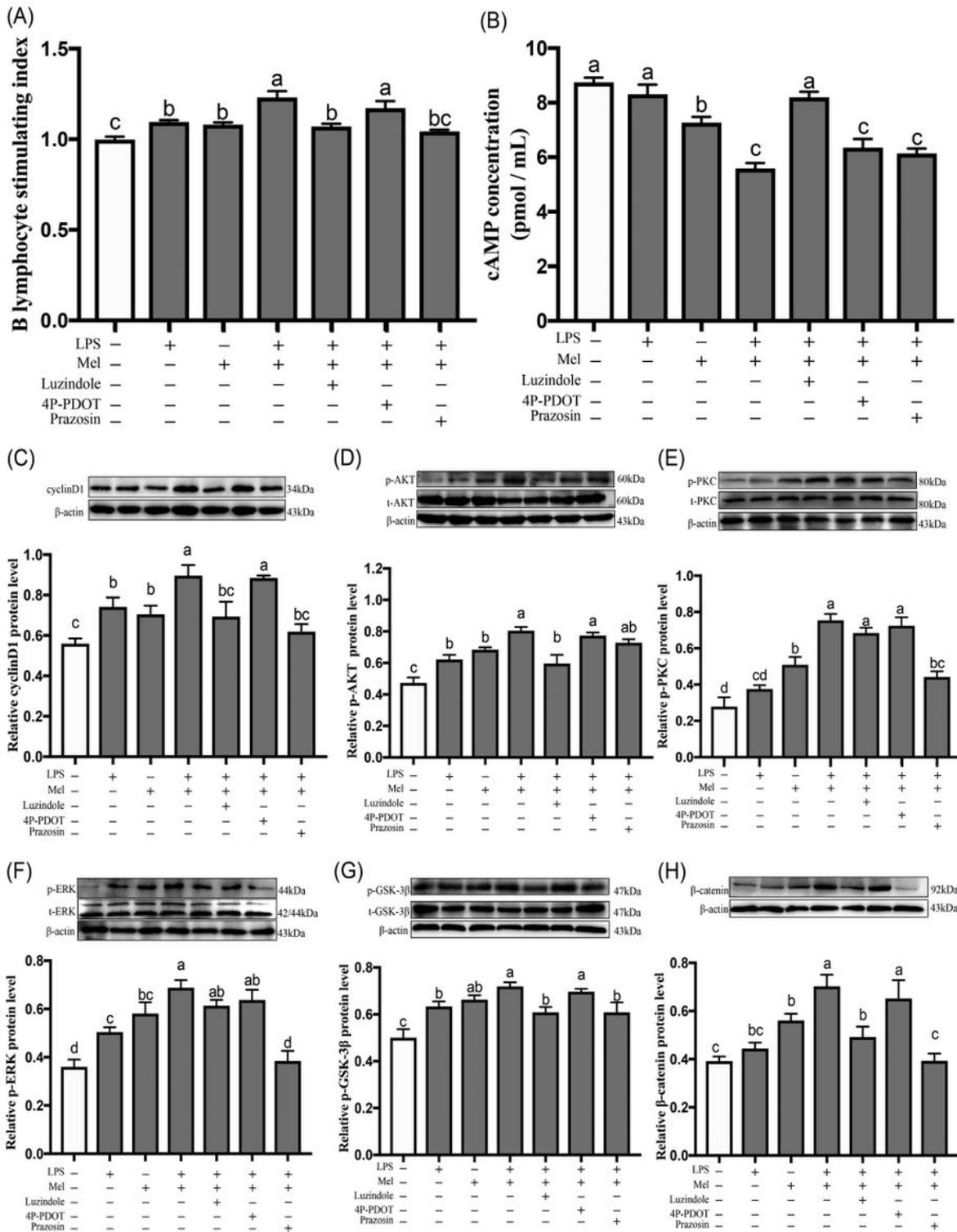
**Figure 4**

Effects of combinations of monochromatic lights on p-AKT/total-AKT ratio (a) p-PKC/total-PKC ratio (b), p-ERK/total-ERK ratio (c), p-GSK-3β/total- GSK-3β ratio (d), β-catenin protein level (e) in bursa at P42. WW, white light; RR, red light; GG, green light; BB, blue light; G→B, green light and blue light combination; B→G, blue light and green light combination; R→B, red light and blue light combination. The results are presented as the means ± SEM. Different letters (a-d) indicate significant differences between the treatments at the same age ( $P < 0.05$ ). p-AKT, phosphorylated AKT; p-PKC, phosphorylated PKC; p-ERK, phosphorylated ERK; p-GSK-3β, phosphorylated GSK-3β.



**Figure 4**

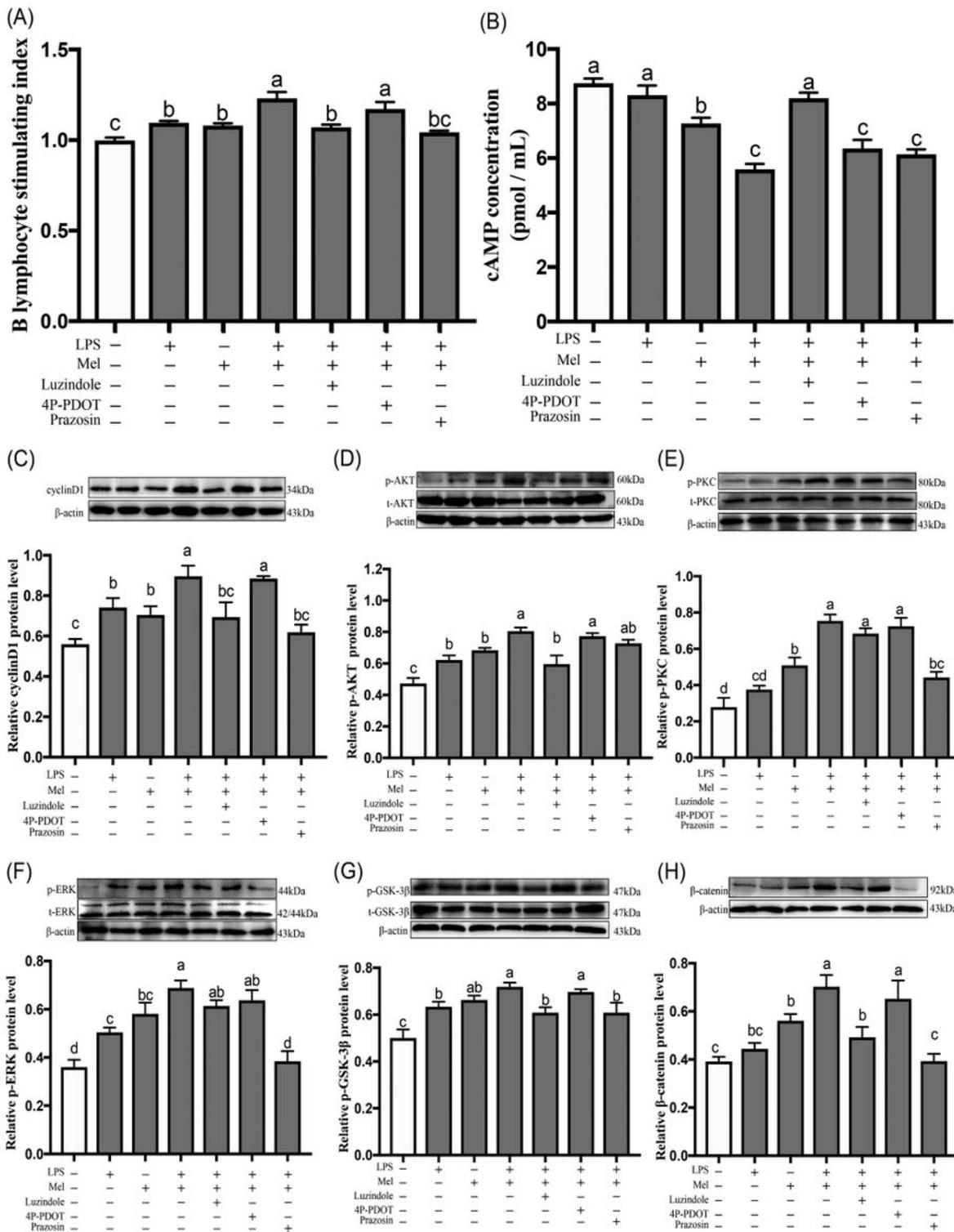
Effects of combinations of monochromatic lights on p-AKT/total-AKT ratio (a) p-PKC/total-PKC ratio (b), p-ERK/total-ERK ratio (c), p-GSK-3β/total- GSK-3β ratio (d), β-catenin protein level (e) in bursa at P42. WW, white light; RR, red light; GG, green light; BB, blue light; G→B, green light and blue light combination; B→G, blue light and green light combination; R→B, red light and blue light combination. The results are presented as the means ± SEM. Different letters (a-d) indicate significant differences between the treatments at the same age ( $P < 0.05$ ). p-AKT, phosphorylated AKT; p-PKC, phosphorylated PKC; p-ERK, phosphorylated ERK; p-GSK-3β, phosphorylated GSK-3β.



**Figure 5**

Effects of different melatonin receptor antagonists on B-lymphocyte proliferation (a), level of intracellular cAMP (b), cyclinD1 protein (c), p-AKT/total-AKT ratio (d) p-PKC/total-PKC ratio (e), p-ERK/total-ERK ratio (f), p-GSK-3β/total-GSK-3β ratio (g), β-catenin protein level (h) in the G→B group. Luzindole is a nonselective Mel1a/Mel1b antagonist; 4P-PDOT is a selective Mel1b antagonist; Prazosin is a selective Mel1c antagonist. Values with no common letters are significantly different ( $P < 0.05$ ) from each other.

4P-PDOT, 4-phenyl-2-propionamideotetralin; LPS, lipopolysaccharide; Mel, melatonin; SI, stimulation index. p-AKT, phosphorylated AKT; p-PKC, phosphorylated PKC; p-ERK, phosphorylated ERK; p-GSK-3 $\beta$ , phosphorylated GSK-3 $\beta$ .



**Figure 5**

Effects of different melatonin receptor antagonists on B-lymphocyte proliferation (a), level of intracellular cAMP (b), cyclinD1 protein (c), p-AKT/total-AKT ratio (d) p-PKC/total-PKC ratio (e), p-ERK/total-ERK ratio

(f), p-GSK-3 $\beta$ /total-GSK-3 $\beta$  ratio (g),  $\beta$ -catenin protein level (h) in the G $\rightarrow$ B group. Luzindole is a nonselective Mel1a/Mel1b antagonist; 4P-PDOT is a selective Mel1b antagonist; Prazosin is a selective Mel1c antagonist. Values with no common letters are significantly different ( $P < 0.05$ ) from each other. 4P-PDOT, 4-phenyl-2-propionamideotetralin; LPS, lipopolysaccharide; Mel, melatonin; SI, stimulation index. p-AKT, phosphorylated AKT; p-PKC, phosphorylated PKC; p-ERK, phosphorylated ERK; p-GSK-3 $\beta$ , phosphorylated GSK-3 $\beta$ .

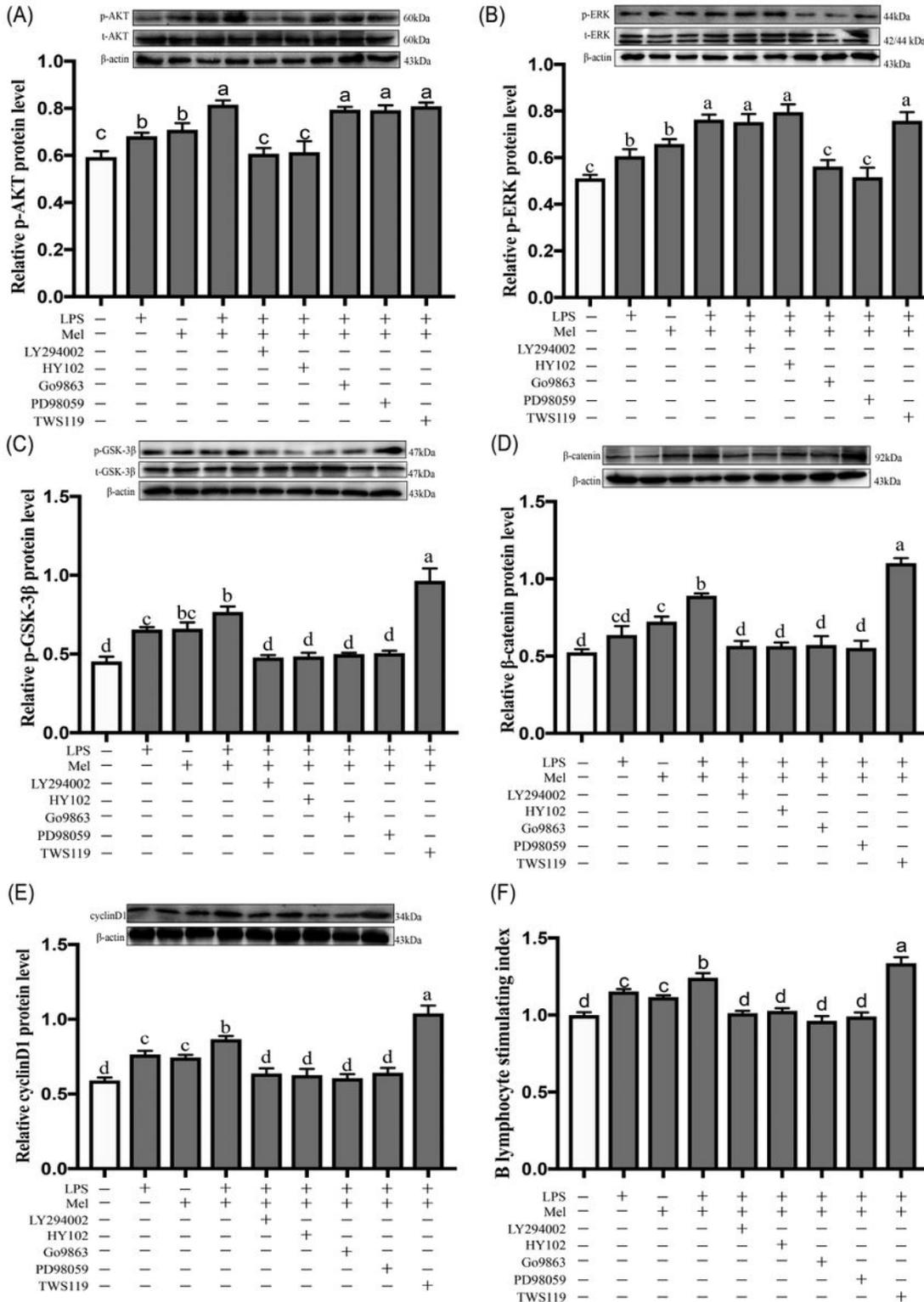
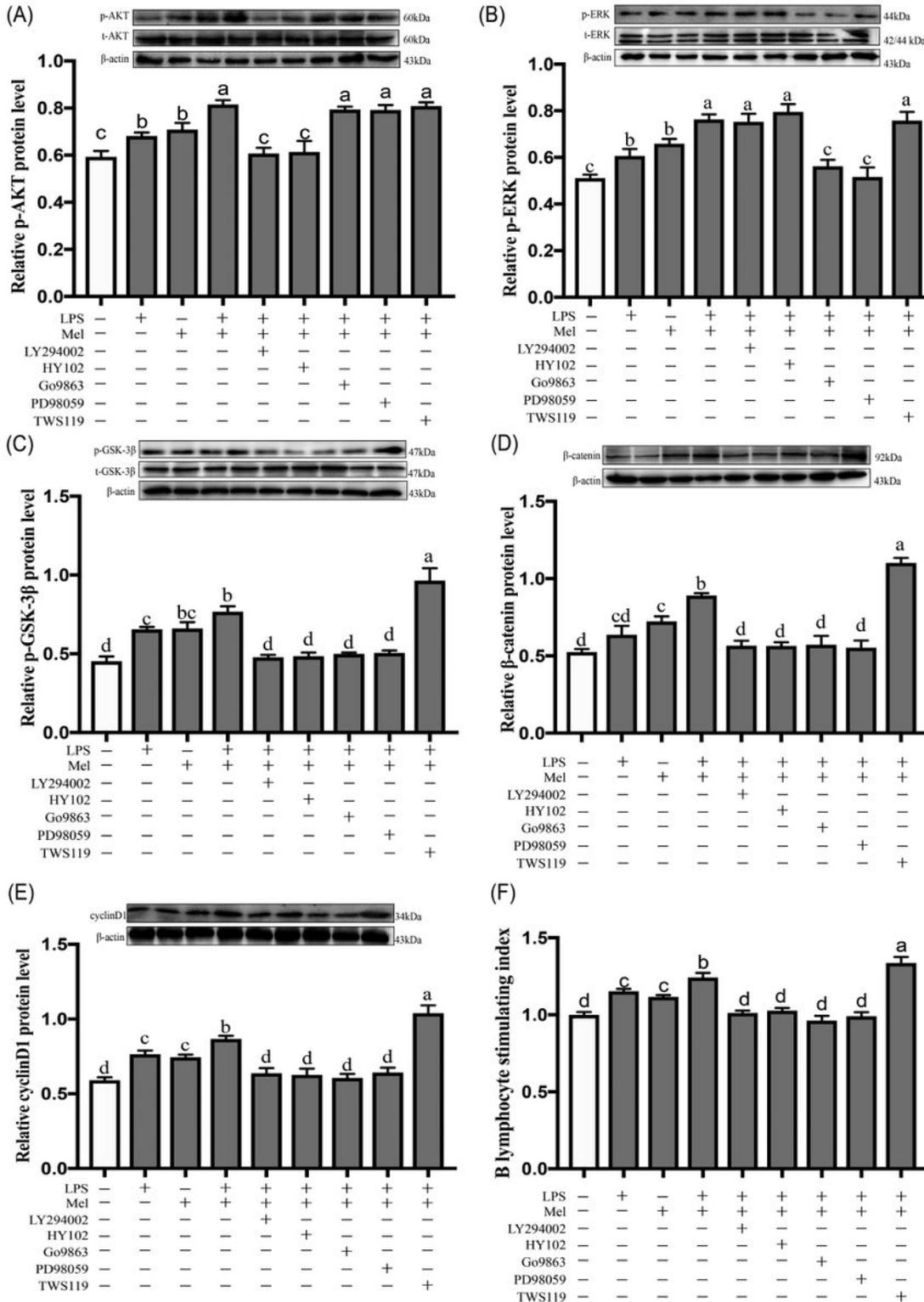


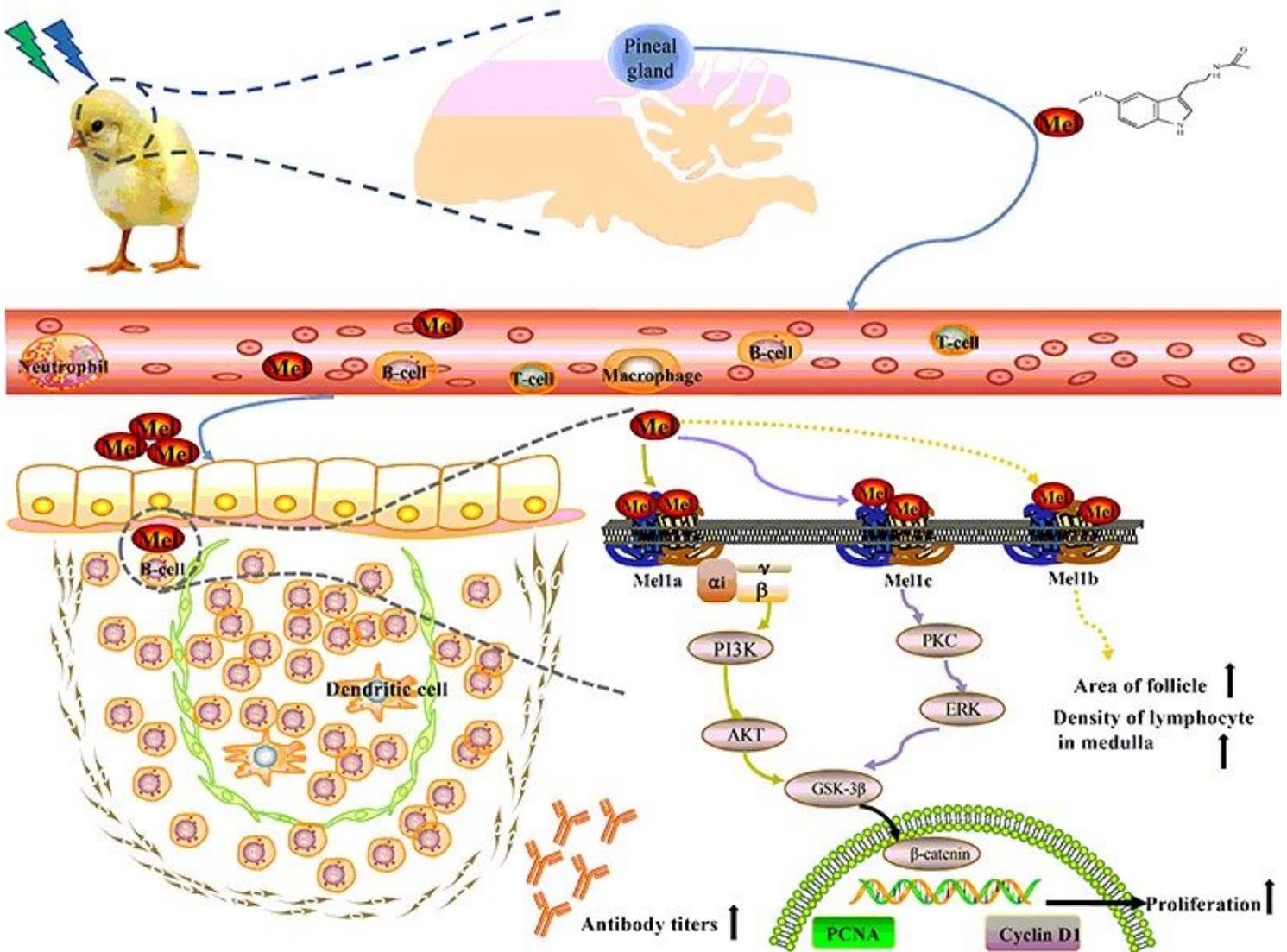
Figure 6

Effects of PI3K inhibitor, AKT inhibitor, PKC inhibitor, ERK1/2 inhibitor and GSK-3 $\beta$  inhibitor on p-AKT/total-AKT ratio (a), p-ERK/total-ERK ratio (b), p-GSK-3 $\beta$ /total-GSK-3 $\beta$  ratio (c),  $\beta$ -catenin protein level (d), cyclinD1 protein level (e), B-lymphocyte proliferation stimulating index (f). LY294002 is a PI3 kinase inhibitor; HY102 is an AKT inhibitor; Go9863 is a PKC inhibitor; PD98059 is an ERK inhibitor; TWS119 is a GSK inhibitor. Values with no common letters are significantly different ( $P < 0.05$ ) from each other. p-AKT, phosphorylated AKT; p-PKC, phosphorylated PKC; p-ERK1/2, phosphorylated ERK1/2; p-GSK-3 $\beta$ , phosphorylated GSK-3 $\beta$ .



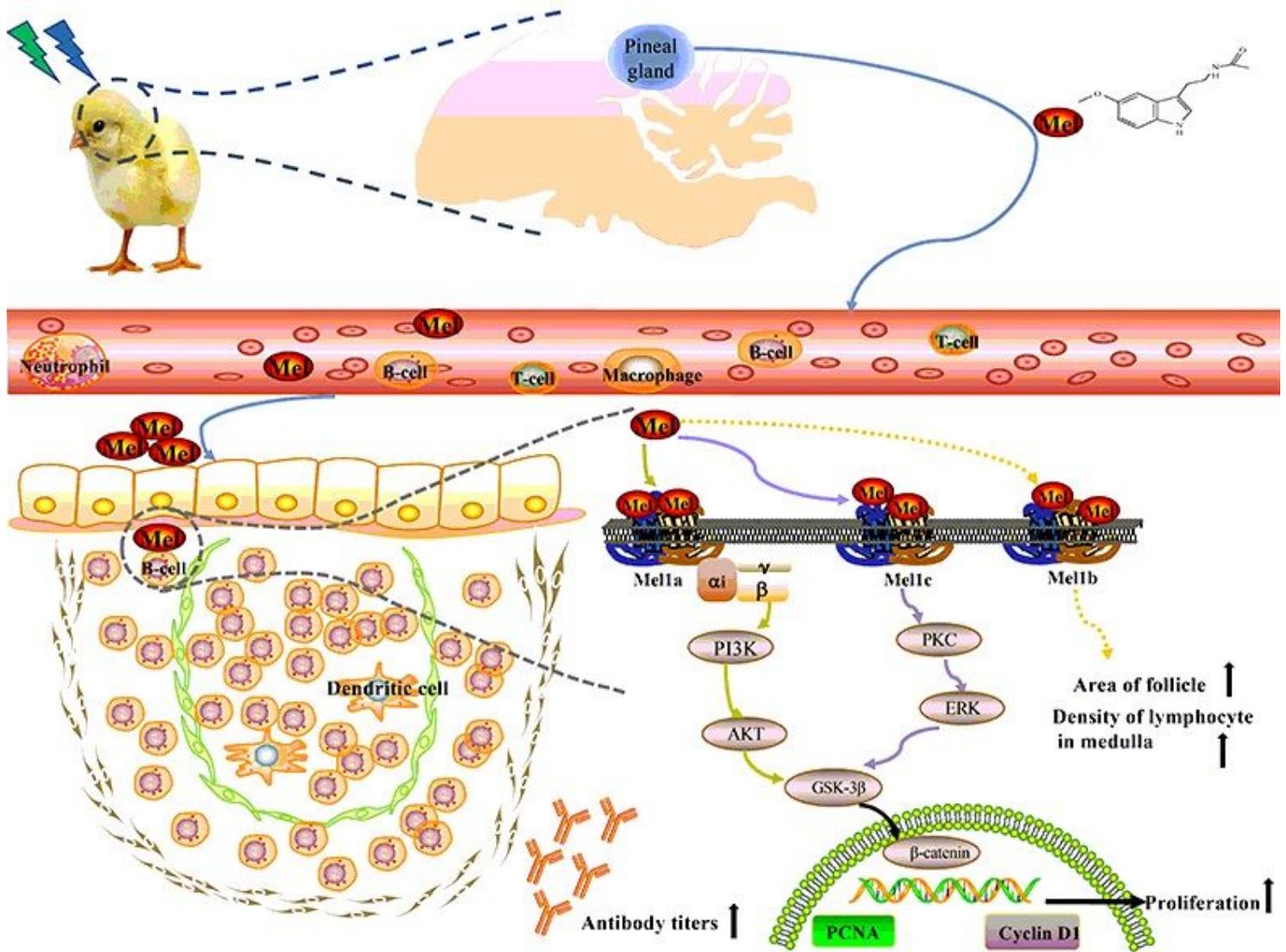
**Figure 6**

Effects of PI3K inhibitor, AKT inhibitor, PKC inhibitor, ERK1/2 inhibitor and GSK-3 $\beta$  inhibitor on p-AKT/total-AKT ratio (a), p-ERK/total-ERK ratio (b), p-GSK-3 $\beta$ /total-GSK-3 $\beta$  ratio (c),  $\beta$ -catenin protein level (d), cyclinD1 protein level (e), B-lymphocyte proliferation stimulating index (f). LY294002 is a PI3 kinase inhibitor; HY102 is an AKT inhibitor; Go9863 is a PKC inhibitor; PD98059 is an ERK inhibitor; TWS119 is a GSK inhibitor. Values with no common letters are significantly different ( $P < 0.05$ ) from each other. p-AKT, phosphorylated AKT; p-PKC, phosphorylated PKC; p-ERK1/2, phosphorylated ERK1/2; p-GSK-3 $\beta$ , phosphorylated GSK-3 $\beta$ .



**Figure 7**

A combination of green and blue monochromatic lights (G $\rightarrow$ B) promoted bursa B-lymphocyte proliferation in the chickens by increasing melatonin secretion. Melatonin bound to its Mel1a and Mel1c with subsequent activation of crosstalk between Gi/PI3K/AKT and PKC/ERK signals to induce B-lymphocyte proliferation.



**Figure 7**

A combination of green and blue monochromatic lights (G→B) promoted bursa B-lymphocyte proliferation in the chickens by increasing melatonin secretion. Melatonin bound to its Mel1a and Mel1c with subsequent activation of crosstalk between Gi/PI3K/AKT and PKC/ERK signals to induce B-lymphocyte proliferation.