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THE ROLE OF PHOTOPERIOD REGIMENS ON REGULATING THE IMMUNE RESPONSE IN MALE BROILERS

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SUMMARY

Day length changes markedly across the year. In many mammals and birds, these photoperiodic variations are associated with physiological modulation including immune function. However, the influence of this environmental variable on poultry physiology is less clear, and the potential underlying mechanisms are unknown. To address this issue, the effects of different photoperiod regimens on the immune function and the mechanism that regulates the immune response in male broilers were examined. A total number of 450 one-day-old male broilers were divided randomly into three equal groups. The broilers were exposed to continuous light, 23 h light: 1 h darkness (23L: 1D) in the first group; non-intermittent restricted light, 12 h light: 12 h darkness (12L: 12D) in the second group; and intermittent light, 2 h light: 2 h darkness (2L: 2D) in the third group. At 6 weeks of age, blood samples were collected to measure peripheral lymphocyte activation and antibody titer against bovine serum albumin (BSA). At the same age, broilers were slaughtered and the spleens were removed to measure splenocyte Interleukin-2 (IL-2) and the phosphorylation of Protein Kinase B (PKB also called Akt). The results indicated that intermittent light regimen induced phosphorylation of Akt and significantly stimulated the peripheral T and B-lymphocyte proliferation compared to the other two regimens. Furthermore, the secretion of Splenocyte IL-2 and anti-body production against BSA were significantly enhanced by intermittent light regimen in comparison with the other two groups. In contrast, no significant differences were observed between constant light regimen and non-intermittent restricted light regimen. Taken together, these data suggest that intermittent photoperiod regimen modulates positively the immune response by up-regulating the phosphorylation protein kinase Akt.

Keywords: Photoperiod regimens, broilers, immune response, Akt

INTRODUCTION

Light-dark cycle is an important exogenous factor for controlling many physiological and behavioral processes in birds. Broiler lighting schedules can be characterized in a number of ways, including the number of hours of darkness and how many periods of darkness are included in each 24-hour cycle. Research has shown that darkness is as important to growth and health of broilers as light (Classen *et al*., 1991). Furthermore, Classen *et al*. (2004) also compared lighting programs with 12-hour darkness per each 24-hour period provided in 1, 6, or 12 hour intervals. Their study indicated that growth rate and body weight in early stages were significantly reduced by longer periods of darkness, but gain from 14 to 35 days, as well as final body weight, were not affected by lighting programs.

On the other hand, from the physiological aspect, the impact of photoperiod on the immune response is poorly understood. In mammals, a short photoperiod could enhance both cellular and humoral responses of the immune system compared with a long photoperiod (Nelson and Blom, 1994, Demas and Nelson, 1996, and Demas *et al*., 1996). In birds, Bentley *et al*. (1998) suggested that immune function was suppressed in adult starlings photo-stimulated with long days (18L: 6D). Both the cellular and humoral immune responses were greater when birds were placed in daily light-dark cycle treatments as compared with constant light (Moore and Siopes, 2000). Onbaşilar *et al.* (2007), Kliger *et al*. (2000), and Abbas *et al*. (2007) reported that broilers housed in intermittent lighting had higher antibody titers of anti-Newcastle disease virus compared with continuous lighting. These results suggested that photo-stimulation plays an important role in affecting the immune response. However, little information has been published to date regarding the mechanism that up-regulates the immune function in response to photoperiod regimens.

Protein Kinase B (PKB), also known as Akt, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration (Sarbassov *et al*., 2005). Kitaura *et al*. (2000) reported that Akt regulates the production and secretion of IL-2 and TNF-α in stimulated mast cells. Altogether, these results revealed a novel function of Akt in transcriptional activation of cytokine genes that contributes to the production of cytokines. Furthermore, Kane *et al*. (2001) showed that Akt provides the CD28 co-stimulatory signal for up-regulation of IL-2 and IFN-γ but not $T_{helper\ 2}$ (T_{h2}) cytokines. Nelson, (2004) showed that IL-2 drove activated B cell differentiation toward plasma cell, finally differentiated cells of the B-cell, independently of its proliferation and survival functions resulted in increasing the antibodies

production. Thus, Akt is strongly offered as a key player in regulating the immune performance in response to photoperiod regimens. In this study, the effects of various photoperiod regimens on immune response, including antibody production, peripheral blood lymphocyte proliferation, and serum splenocyte interleukin-2 (IL-2) level was addressed. In addition, the mechanism that up-regulate the immune function in response to photoperiod regimens in male broilers was examined.

MATERIAL AND METHODS

Animal Management

A total of 450 one-days-old Arbor Acre male broiler chicks (chicks were sexed in commercial hatching facility using the Japanese method of vent sexing) were used in the current study and were kept for 6 weeks. The chicks were housed on a deep litter floor brooder. Feed and water were supplied *ad libtium*. The diets were formulated to meet the nutrient recommendation for poultry (NRC, 1994). The temperature in the chicken house was set at 33°C for the first 7 days and was reduced by 3°C each consecutive week until reaching 24°C with RH 50% ± 5%. All birds were vaccinated at 7 days of age against Newcastle disease with B1 strain vaccine, and were vaccinated at 21 days of age with Lasota strain vaccine, and against infection bursal disease at 15 days of age.

Experimental design

The broiler chicks were divided randomly into three equal groups with 3 replicates of 50 chicks each. All groups received 24 hour lighting for the first 72 hour. On the fourth day, the first group was exposed to continuous light, 23 hour light and 1 hour darkness (23L: 1D). The second group received non-intermittent restricted light, 12 hour light and 12 hour darkness (12L:12D), while the third group was exposed to intermittent light, 2 hour light and 2 hour darkness (2L:2D), until the end of the experiment.

Tissue samples and analysis

At six weeks of age, nine chickens from each photoperiod treatment (three chickens from each replicate) were chosen at random and slaughtered. The spleens were removed, processed by removing the capsule and expressing the tissue through a 60-mesh screen and then 300-mrsh with 8ml RPMI 1640media. Four ml from the suspension was used to measure the splenocyte Interlukin-2 (IL-2), while the other 4 ml was used to measure total and phosopho-Akt.

Western blot analysis

The suspension samples were used to measure total and phosphorylation levels of Akt through western blot analysis. Western blot was conducted as described (Chen *et al.*, 2005). In brief, proteins were extracted in RIPA buffer [0.15 M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 50 mM Tris-HCl; pH 7.4, protease inhibitor cocktail comprising 2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 10 mM Na4P2O7, and 2 mM Na3VO4]. Proteins extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After incubation with a blocking buffer (0.05 M Tris-base, 0.15 M NaCl, 0.05% Tween 20, 10% chicken serum, and 1.5% BSA) for 1 hour, the membranes were incubated with the primary antibody at room temperature for 1 hour and followed by four (15-min rounds) washings (in 0.05 M Tris-base, 0.15 M NaCl, 0.1% Tween 20, pH 8.3). The membranes were then incubated with the secondary antibody for 30 min and followed by four rounds of washing (15 min each). A rabbit anti-multispecies Akt, and phospho-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). A horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology) was used to identify the bands reactive to the primary antibodies through an enhanced chemiluminescence method.

Splenocyte Interleukin-2 (IL-2) bioactivity assay:

Determination of IL-2 bioactivity Splenocytes were suspended in RPMI 1640 media, and adjusted to a cell density of 10^7cell/ml. A 1-ml cell suspension was co-incubated with Con A (final concentration, 45 lg/ml) in 24-well plate and incubated at 41ºC with 5%CO2 for 24 hour. After 24 hour, supernatants were harvested for the IL-2 assay. Fifty microlitres of target cell suspensions, 50 µl of IL-2 sample supernatant (prepared by dilution of the supernatants 1:10 by using RPMI 1640 culture medium), and 50 ll RPML 1640 were added to a 96-well plate and incubated at 41ºC with 5% CO2 for 30 hour. At the end of incubation, Mossman's colorimetric assay (1983) with MTT salts was used for the measurement of cell bioactivity (see the procedure of proliferation of splenocytes). The absorbance of each sample was read on an automated ELISA reader at 570 nm (Bio-rad).

Blood Sampling and Analyses

At six weeks of age, nine blood samples were collected from each group, three samples from each replicate, into 2-mL tubes, and the serum harvested from the blood samples was stored at −20°C until analysis. A second 5-mL sample was taken from the same birds for the lymphocyte proliferation study. All blood measurements were analyzed in triplicates.

Proliferation Assay for T and B Lymphocytes

Proliferation of peripheral blood lymphocytes was determined by using a previously described method (Kliger *et al.*, 2000) with some modifications. Briefly, blood samples collected from nine broilers, which were exposed to each of the above photoperiod regimens, were assayed for lympho-proliferation in response to mitogen stimulation. The blood was mixed 1:1 with wash

medium, RPMI 1640 with L-glutamine4 supplemented with penicillin (100 units/mL)/streptomycin (100 μg/mL). The cell suspensions were then layered onto histopaque 10774 and were centrifuged at 220 × *g* for 30 min to separate the leukocytes. The white blood cell layer was isolated and washed twice. Leukocytes were adjusted to 1×10^7 viable cells/mL in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS). By using trypan blue exclusion, cell viability was determined to be ≥95% for suspensions of white blood cells. Leukocytes were plated in triplicate cultures at 5×10^5 lymphocytes/well in 96-wells, round-bottom plates. Each well contained leukocytes in 50 μL of medium. Fifty microliters of concanavalian (Con A) was added at a final concentration of 12.5 μg/mL in the well, or 50 μL of pokeweed mitogen (PWM) was added at a final concentration of 25 μg/mL in each well. Fifty microliters of RPMI 1640 with 10% FBS was also added to the cell culture to serve as a control. Cells were then incubated at 37º C in a humidified atmosphere of 5% CO2 for 48 hour. One microcurie of 3H-thymidine in 50 μL of medium was added to each culture. Cells were again incubated at 37º C under 5% CO2 for 18 h to allow for 3H-thymidine uptake. Cells were harvested onto glass-fiber filters using a cell harvester. The filters were placed in scintillation vials with scintillation fluid and were counted using a scintillation counter. Counts per minute were determined, and triplicate cultures were averaged; counts from cultures with no mitogens were subtracted.

Antibodies titer in serum

Total antibody serum was determined by a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottomed 96-well plates (Nunc Maxisorp; VWR International, Mississauga, Ontario, Canada) were coated with unconjugated rabbit anti-chicken IgG heavy plus light chains (Cedarlane, Hornby, Ontario, Canada) (2 µg/ml) and incubated at 4°C overnight. Plates were then washed and blocked for 1 hour at 37°C with PBS-Tween 20 containing 0.25% gelatin (HiPure liquid gelatin; Norland Products Inc., New Brunswick, N.J.). Serum diluted 1:100 were then added, and plates were incubated for 1 hour at 37°C. Subsequently, rabbit anti-chicken IgG heavy plus light chains conjugated with horseradish peroxidase (Cedarlane, Hornby, Ontario, Canada) (diluted 1:20,000 in blocking buffer) was added and plates were incubated for 1 hour at 37°C. The substrate, ABTS [2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); Mandel Scientific, Guelph, Ontario, Canada], was then added and plates were incubated for 30 min at room temperature in the dark. The absorbance was measured at 450 nm using a microplate reader (Bio-Tec, Winooski, VT).

Statistical analysis

Data were reported as means ± SE and the comparison between groups were performed using one-way ANOVA with SPSS10.0 (SPSS Inc., Chicago,II.). The significance of difference among different groups was evaluated by Duncan (1955) test. Differences were considered statistically significant at the level of $P < 0.05$.

RESULTS AND DISCUSSION

Akt phosphorylation

Continuous light regimen or non-intermittent restricted light regimen did not affect Akt phosphorylation in male broiler chickens. Akt is activated in chicks exposed to intermittent light regimen (Figure 1). The current result may be due to over-production of melatonin. Abbas *et al*. (2007) showed that intermittent photoperiod regimen induced over production of melatonin. Koh (2011) reported that melatonin exerts protective effects against damage by enhancing the Akt signal pathway, thus regulating apoptotic cell death.

The Phosphoinositide-3 kinase (PI3K)/Akt pathway is a major regulator of cell survival pathway (Manning and Cantely, 2007, and Kong *et al*., 2008). Jiang *et al*. (2012) showed that melatonin effectively activated Akt in Müller cells in a time-dependent manner. Similar findings have been reported in other cell types (Lee *et al*., 2006). Activation of PI3K/AKT pathway is a fundamental requirement for cell-cycle progression and T-cell proliferation (Wang *et al*., 2012). Mechanisms by which melatonin activates Akt will have to be determined in future studies. Furthermore, shifting the balance of pathways to favor Akt activation by increasing the expression levels of Akt led to an almost complete block of receptor-induced apoptosis, further supporting the idea that Akt activation sends a survival signal to B cells following Ag receptor. Interestingly, at this high Akt expression level, the activity of Akt was PI 3-kinase independent, suggesting that localization to the plasma membrane is not necessary for activation in this case. High levels of Akt protein were also necessary to observe constitutive Akt phosphorylation/activation in DT40 transfectants, and it is possible that Akt levels need to exceed a certain threshold level for this constitutive phosphorylation to occur (Li *et al*., 1998 and Pogue *et al*., 2000).

Cell-mediated immune response

Exposure to intermittent light regimen significantly induced stimulation of both peripheral T-lymphocytes proliferation in response to con-A mitogen and B-lymphocytes proliferation in response to PWA mitogen compared to other light schedule groups (Figure 2). On the other hand, the differences in peripheral T- and B-lymphocyte proliferation between non-intermittent restricted light group and continuous light group were not significant. These results are supported by

Maestroni (1998), Kliger *et al*. (2000) and Abass *et al*. (2007 and 2008) who reported that increasing light-dark cycle stimulate lymphocyte proliferation. The current results could be due to secreting high level of melatonin in response to intermittent light (Maestoni, 1998; Kliger *et al.*, 2000 and Abass *et al*., 2007). Kliger *et al*. (2000) and Gehad *et al*. (2008) reported that using melatonin, in vitro, enhanced peripheral and splenic lymphocyte proliferation. Furthermore, Champney *et al*. (1997) suggested that melatonin can disproportionately alter the number of peripheral and splenic lymphocyte proliferation, or it can modify the intrinsic mitogen activity of each lymphocyte. Even though the positive impact of intermittent photoperiod regimen on melatonin secretion is well reported, the mechanism by which melatonin induces peripheral lymphocyte proliferation is poorly understood. This study strongly suggests that protein kinase Akt pathway plays a key role in regulating this mechanism. The current study showed that intermittent photoperiod regimen activates protein kinase Akt (Figure 1). Kong *et al*. (2008) reported that melatonin induces Akt phosphorylation through melatonin receptor- and PI3K-dependent pathway. Weichhart and Saemann (2010) showed that protein kinase Akt promotes the differentiation of T_{h1}, which leads to enhancing the secretion of IL-2 and IFNγ (Garcia- Maeriño *et al*., 1997). This explanation may clarify the result reached by Maestroni (1998) who reported that melatonin can possibly stimulate lymphocyte proliferation by stimulating T_{h1}. Another explanation was offered by Kuhlwein and Irwin (2001), who suggested that improving the lymphocyte proliferation in response to administration of physiological doses of melatonin can possibly be due to suppressing the production of the inhibitory cytokine interlukin-10 (IL_10).

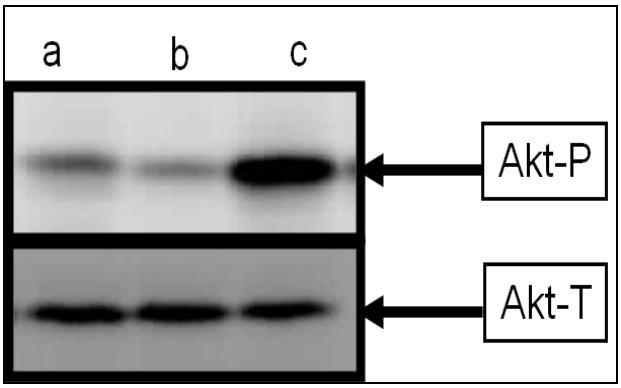

Fig. 1. The effect of photoperiod regimens on the phosphorylation of Akt in male broiler chicks at 6 weeks of age. a. Broiler chicks exposed to continuous light (23L: 1D), b. Broiler chicks exposed to non-intermittent restricted light (12L: 12D), c. Broiler chicks exposed to intermittent light (2L: 2D)

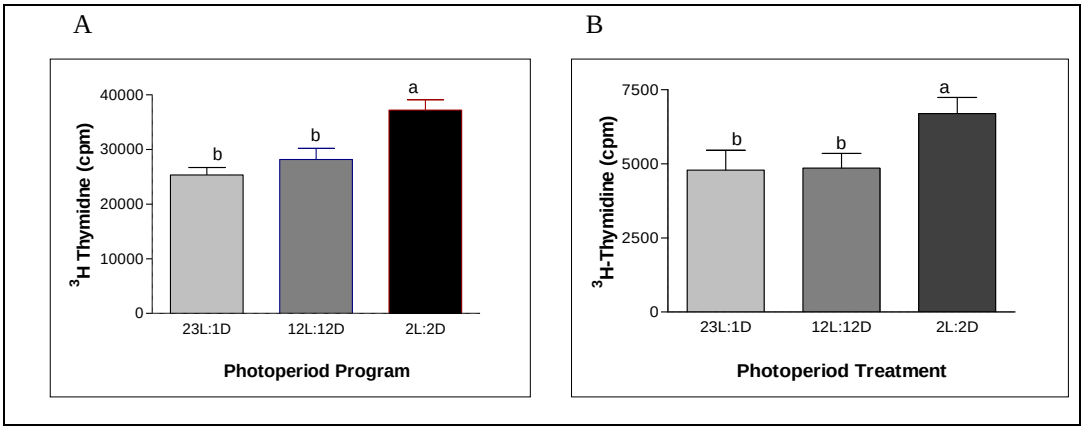

Fig. 2. The effect of photoperiod regimens on peripheral leukocytes proliferation stimulated by concanavalian A (A) or pokeweed mitogen (B) in male broilers at 6 weeks of age. Bars are means ± SE. Bars with different letters are significantly different (P< 0.05). For each light regimen, n = 9

Splenocyte Interleukin-2 (IL-2)

The concentration of splenocyte IL-2 in male broilers was detected in response to three photoperiod regimens (Figure 3). In the present study, intermittent light regimen induced significant increase in IL-2 concentration compared to the other two regimens. On the other hand, there was no significant difference in the concentration of IL-2 between continuous light group and non-intermittent restricted light group. Lahiri and Haldar (2009) analyzed the effect of natural solar insolation and artificial photoperiodic conditions on melatonin MT1 receptor expression of a tropical

rodent. They reported that melatonin mediates reproductive and circadian responses and regulates the production of a large number of cytokines, including interleukin-2 (IL-2), via activating Akt (Kong *et al*., 2008), and promoting T_{h1}-cells (Weichhart and Saemann, 2010). Furthermore, Zhou *et al*. (2010) showed that melatonin could up-regulate the Th1-type cytokines and down-regulate the T_{h2}-type cytokines. This indicates that melatonin could modulate the disturbance of cytokine network in the SLE mice by regulating the imbalance of T_{h1}/T_{h2}. Therefore, melatonin may be suggested as a molecular messenger of photoperiodic signals that regulate IL-2 production.

Humoral immune response

The differences in the antibody production in non-intermittent restricted light group or continuous light group were not significant ($P \geq 0.05$). However, the highest antibody production value was obtained in the group that received intermittent light regimen (Figure 4). Enhancing the antibody production for chicks exposed to intermittent regimen may be due to increasing melatonin levels in darkness, (Abbas *et al*., 2007). The effect of melatonin on the immune function may be directed via melatonin receptors located on the immune tissues located in white blood cells (Calvo *et al*., 1995), or may be indirect by acting through other endocrine hormones most notably thyroid hormone (Poon *et al*., 1994).

Intermittent photoperiod regimen promoted IL-2 production (Figure 3). Another explanation is offered by Shelef and Calame (2005) who showed that IL-2 stimulated B-cell proliferation and the differentiation of B-cells into plasma cells resulted in increasing antibody production. Moreover, Raghavendra *et al*. (2001) found that chronic administration of melatonin activates T-helper$_2$, which induces B-cell activation and increases the antibody production.

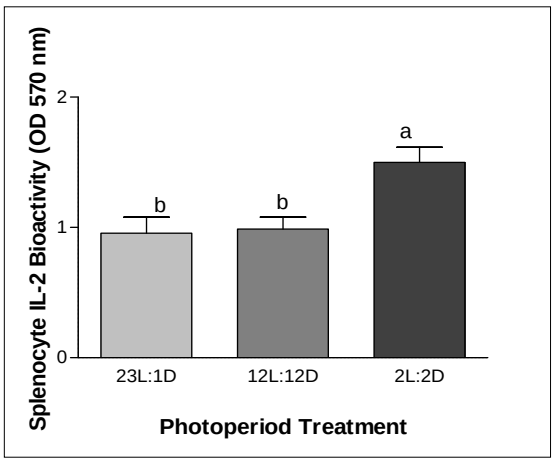

Fig. 3. Histogram demonstrating the effect of photoperiod regimens on splenocyte interleukin-2 (IL-2) in male broilers at 6 weeks of age. Bars are means ± SE. Bars with different letters are significantly different (P< 0.05). For each light regimen, n = 9

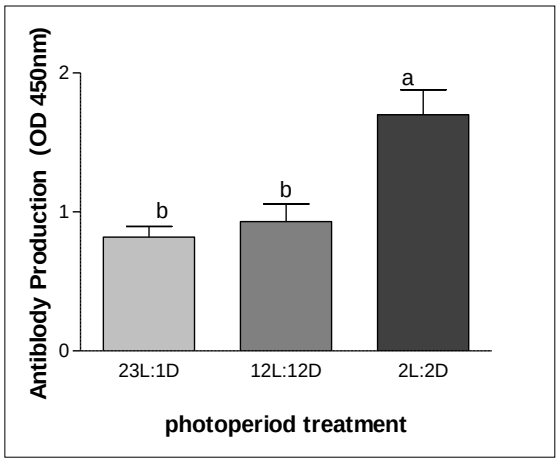

Fig. 4. Total antibody levels in sera of three groups of photoperiod regimens in male broilers at 6 weeks of age. The figure shows ELISA optical densities means for total antibodies. Error bars represent standard error of the means of optical densities. Means between photoperiod regimens with different letters are significantly different (P<0.05). For each light regimen, n = 9

GENERAL DISCUSSION

There is a major concern over the potential that intermittent photoperiod regimen can positively influence the immune response in broilers. Melatonin synthesis is synchronized to the prevailing daily light: dark cycle and exhibits a marked circadian rhythm, with peak synthesis and secretion occurring at night. In mammals as well as in chickens, both neuroendocrine and immune systems help an organism to cope with changing environmental demands. The immune system of chickens, especially lymphocyte proliferation, can be enhanced by controlling photoperiods through stimulating melatonin secretion. Over production of melatonin activates protein kinase Akt, which promotes T_{h1} cells to increase the secretion of IL-2 cytokine. IL-2 stimulates lymphocyte proliferation and enhances B cell differentiation toward plasma cell, finally differentiates cells of the B-cell, independently of its proliferation and survival functions leading to increase the antibodies production (Figure 5).

CONCLUSION

The present study showed that the intermittent light regimen, indirectly through melatonin, activates protein kinas Akt and stimulates peripheral lymphocytes proliferation, increases antibody titers, and promotes Splenocyte IL-2 in male broilers when compared with continuous or non-intermittent restricted light regimens. These results indicate the important role that photoperiod could play in affecting the immune response.

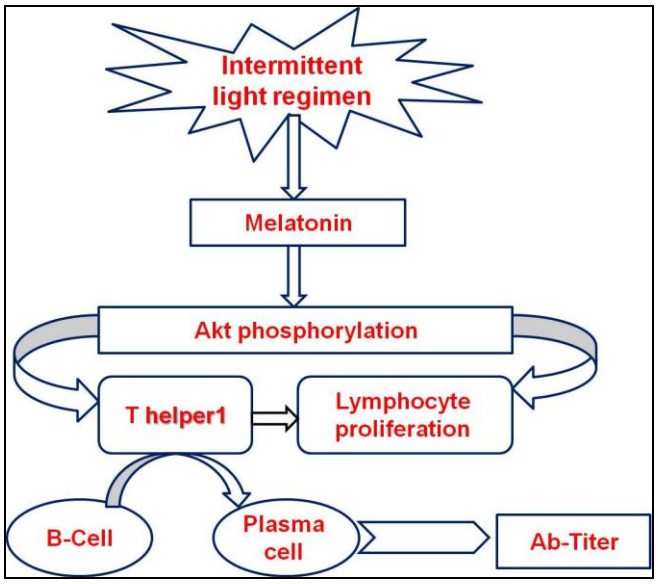

Fig 5. Diagram summarizes the Impacts of intermittent photoperiod regimen on the immune function and the mechanism that regulates the immune response in male broilers

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دور نظم الإضاءة قى تنظيم الإستجابة المناعية لذكور بدارى التسمين

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يحدث تغير فى طول اليوم بشكل ملحوظ على مدار العام. وفى العديد من الثدييات والطيور يكون التباين فى فترات الإضاءة متزامناً مع التغيرات الفسيولوجية بما فى ذلك الوظائف المناعية. ومع ذلك فإن تأثير المتغير البيئى على فسيولوجيا الدواجن أقل وضوحاً والآليات المحتمل إستخدامها غير معروفة. ولمعالجة هذا الموضوع فقد تم بحث تأثير نظم الإضاءة المختلفة على الإستجابة المناعية والآلية التى تنظم الإستجابة المناعية فى ذكور بدارى التسمين ، حيث تم تقسيم عدد 450 من ذكور كتاكيت التسمين على عمر يوم واحد إلى ثلاث مجموعات متساوية العدد ، وتعرضت المجموعة الأولى إلى برنامج إضاءة مستمرة (23 ساعة إضاءة : 1 ساعة إظلام) بينما تعرضت المجموعة الثانية إلى برنامج إضاءة غير متقطع محدد (12 ساعة إضاءة : 12 ساعة إظلام) أما المجموعة الثالثة فتعرضت إلى برنامج إضاءة متقطع (2 ساعة إضاءة : 2 ساعة إظلام). وعلى عمر 6 أسابيع تم جمع عينات الدم لتقدير نشاط الخلايا الليمفاوية ومستوى الأجسام المضادة ضد الألبيومين المستخلص من السيرم البقرى (BSA). وعلى نفس العمر تم ذبح بدارى التسمين وفصل الطحال لتقدير مستوى السيتوكين (IL-2) ومستوى نشاط البروتين كينيز B والذى يطلق عليه أيضاً Akt. ولقد أشارت النتائج إلى أن برنامج الإضاءة المتقطع أحدث نشاطاً معنوياً فى Akt وقام بتنشيط الخلايا الليمفاوية بنوعيها (B & T) بالمقارنة بمجموعتى الإضاءة الآخرتين. وعلاوة على ذلك فإن مستوى إفراز IL-2 فى الطحال ومستوى الأجسام المضادة ضد BSA تحسن بشكل معنوى بالمقارنة ببرنامجى الإضاءة الآخريين. وفى المقابل لم يلاحظ أى فروق معنوية بين برنامج الإضاءة المستمرة وبرنامج الإضاءة غير المتقطع المحدد فى المقاييس سابقة الذكر. وتشير هذه النتائج مجمعة إلى أن برنامج الإضاءة المتقطع ينظم إيجابياً الإستجابة المناعية من خلال التحكم فى تنظيم مستوى نشاط بروتين Akt.

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