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The Effect of Different Post-Hatching Periods on TLR4 and VEGF Expression Patterns in Broiler Ileum

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Abstract

This study is aimed to evaluate the relationship between VEGF and TLR4 expression in the ileum during broiler post-hatching development. The material for the study was taken from the ileum tissue of 7, 21, and 42 day-old broilers. In tissue sections VEGF and TLR4 expression were demonstrated by Streptavidin-biotin complex immunohistochemistry method. On the 7th day following hatching, TLR4 protein expression was not seen in crypt epithelial cells. At day 21, crypt epithelial cells began to stain and gave a more intense immunoreaction at day 42. In VEGF-stained sections, the ileum villus epithelial cells, crypt, and smooth muscle tissue showed brown intracytoplasmic response. The expression of the VEGF protein in the upper villus epithelial cells started to increase on the 7th day, and it stained intensely, especially on the 42nd day. In addition, it was observed that the staining intensity of the tunica muscularis layer was the same on the 7th and 21st days, and increased on the 42nd day. It was remarkable that goblet cells gave negative results in both immunostaining. In summary it seen that TLR4 and VEGF expression were found to be increase in this study from the 7th to the 42nd day following hatching. Thus, it was concluded that angiogenesis mechanisms and the development of innate and adaptive defense systems continue throughout the post-hatching period.

Key Words: Ileum, post-hatching, TLR4, VEGF.

Broiler İleumunda Kuluçka Sonrası Farklı Dönemlerin TLR4 ve VEGF Ekspresyon Paternleri Üzerine Etkisi

Öz

Bu çalışmanın amacı, etlik piliçlerin kuluçka sonrası gelişimi sırasında ileumda VEGF ve TLR4 ekspresyonu arasındaki ilişkiyi değerlendirmektir. Çalışma materyali 7, 21 ve 42 günlük piliçlerin ileum dokusundan alındı. Doku kesitlerinde VEGF ve TLR4 ekspresyonu, Streptavidin-biotin kompleksi immünohistokimya yöntemi ile gösterildi. Yumurtadan çıkmayı takip eden 7. günde, kript epitel hücrelerinde TLR4 protein ekspresyonu yoktu. 21. günde kript epitel hücreleri boyanmaya başladı ve 42. günde daha yoğun bir immünreaksiyon verdi. VEGF ile boyanmış bölümlerde, ileum villus epitel hücreleri, kript ve düz kas dokusu kahverengi intrasitoplazmik tepki gösterdi. VEGF proteininin üst villus epitel hücrelerinde ekspresyonu 7. günde artmaya başladı ve özellikle 42. günde yoğun boyandı. Ayrıca tunika muskularis tabakasının boyanma yoğunluğunun 7. ve 21. günlerde aynı olduğu, 42. günde arttığı gözlendi. Goblet hücrelerinin her iki immün boyamada da negatif sonuç vermesi dikkat çekiciydi. Özetle bu çalışmada kuluçkadan sonraki 7. günden 42. güne kadar TLR4 ve VEGF ekspresyonunun arttığı görülmüştür. Böylece anjiyogenez mekanizmalarının ve doğuştan gelen ve adaptif savunma sistemlerinin gelişiminin kuluçka sonrası dönem boyunca devam ettiği sonucuna varılmıştır.

Anahtar Kelimeler: İleum, kuluçka sonrası dönem, TLR4, VEGF.

INTRODUCTION

The intestinal epithelial lining forms the luminal surface of the external environments of the small and large intestines. To mediate the balance of the many functions of the digestive system, the intestinal epithelium provides a channel to proteins and small molecule metabolites while acting as a mucosal barrier against microorganisms (1). The small intestine is thought to maintain homeostasis by replacing epithelial cells, replenishing its surface with new cells, and maintaining the overall number of epithelial cells at a constant level. This ongoing renewal process improves the small intestine's absorption and defense mechanisms (2). According to studies, epithelial cells respond to signals from both the apical and basal compartments and act as the mucosal immune system's defensive front line (3). Additionally, epithelial cells have the ability to create proinflammatory cytokines and serve as antigen-presenting cells (4).

Toll-like receptors (TLRs), which are found on the surfaces of epithelial cells, act as sensors for pathogens that are infiltrating the body. TLR activation triggers the host's signa-

ling cascades as a defense strategy against invaders. The resulting host reaction limits foreign invasion and leads to adaptive immunity (5). TLRs stimulate the production and release of pro-and anti-inflammatory cytokines, which contributes significantly to immune activity in maintaining intestinal homeostasis (6). Studies have demonstrated the expression of TLR4 in fetal, neonatal, and adult intestinal epithelial cells. It has been known that TLR signals play an influential role in healing the damaged intestinal epithelium. Moreover, TLR4 plays a vital role in maintaining the delicate balance between tolerogenic and inflammatory properties of the gut microbiota by regulating innate immunity (7).

Vascular endothelial growth factor (VEGF) is a protein that plays a role in angiogenesis, lymphopoiesis, and lymphangiogenesis, as well as resistance to oxidative stress, lipid metabolism regulation (8). Furthermore, VEGF can contribute to inflammation and coagulation, as well as increase the expression of cell adhesion molecules, intercellular adhesion molecule 1, and vascular cell adhesion molecules (9). In preparation for the switch from parenteral to enteral nutrition, it is known that increased intestinal VEGF protein expression in late pregnancy stimulates the formation of villous microvascular and lymphatic systems (10).

Changes in TLR4 and VEGF expression in the ileum may shed light on immunity and developmental stages of cells in the intestinal system. Although there are studies on TLR4 and VEGF expression during postnatal developmental periods in mammals, there is a lack of information on this subject in poultry ileum tissue. For this purpose, we aimed to investigate TLR4 and VEGF expression in the broiler ileum during the post-hatching period.

MATERIAL AND METHODS

Broiler eggs were incubated in a forced draft poultry incubator at 50-60% relative humidity at 35°C and incubated under appropriate conditions. (Eggs were supplied from Beypiliç A.Ş., Bolu, Turkey). Three groups were formed, each with six animals. 7, 21, and 42-day post-hatching. After the animals were sacrificed under anesthesia, the ileum was sampled for immunohistochemical investigation. Samples of tissue were preserved in a 10% formaldehyde solution for 24 hours. The tissues were kept in 70, 80, and 96 percent alcohol for 1 hour each after being preserved in a running water bath for 24 hours to get rid of the formalin. Then, three one-hour applications of xylol and pure alcohol were made. The tissue samples were then embedded using paraplast.

Immunohistochemistry

Using the Streptavidin biotin complex method, five-micrometer ileum sections were stained immunohistochemically using mouse monoclonal VEGF (1/300 dilution, Santa Cruz Biotechnology, sc7269) and mouse monoclonal antibody TLR4 (1/300 dilution, Santa Cruz Biotechnology, sc-293072) primary antibodies (11). The secondary antibody was Histostain Plus (Zymed kit: 85-6743, United States). Sections were heated in a microwave oven at 700 W for proteolysis in a citrate buffer (pH: 6) solution after deparaffinization. To stop endogenous peroxidase activity, the tissues were incubated in a 3 percent hydrogen peroxide solution. To prevent nonspecific protein binding in sections, serum from the kit was instilled after washing with phosphate buffer solution (PBS). The primary antibody was applied, and the samples were kept at $+4^0$C overnight. On the tissues of the negative control group, only PBS solution was used. After washing, sections were instilled with biotinylated secondary antibody and incubated at streptavidin-horseradish peroxidase complex. The final stage involved using 3, 3'-diaminobenzidine (DAP) as a chromogen and covering the slides with entellan after hematoxylin counterstaining.

Immunohistochemical Examination

The intensity of positive staining in immunohistochemical examination was evaluated semiquantitatively using a standard four-point scoring scale for intensity being scored as -: no staining, +: minimal, ++: mild to moderate, +++: severe stained (12). A Nikon digital-sight imaging system was used with a Nikon Eclipse 50i microscope to take histological pictures. The immunopositivity was scored from 0 to 3 semiquantitatively (13) as follows. Histoscore was derived from the intensity (0: negative (-), +1: minimal (+), +2: mild to moderate (++), +3: severe (+++)) of staining immune-reactivity.

Statistical Analysis

IBM SPSS Statistics Version 22.0 statistical software program was used for all statistical analyses. Comparisons between groups were made with the independent Student's t-test for parametric data. Results were presented as mean ± SEM (standard error of mean) and statistical significance was accepted at $p < 0.05$.

RESULTS

TLR4 Immunolocalization

Intestinal epithelial cells and smooth muscle cells forming the tunica muscularis were found to exhibit intracytoplasmic immunopositive staining in all examined samples. On the 7th day after post-hatching, it was observed that the TLR4 reaction, which was moderate in the villus epithelial cells, disappeared towards the basal part of the intestinal villi, and there was no immunoreactivity in the goblet cells. The epithelial cells lining the villus intestinalis were exhibit an increase in the number of stained cells and staining intensity starting on the 7th day after hatching. TLR4 protein expression was not seen in crypt epithelial cells on the 7th-day post-hatching. The crypt epithelial cells began to stain on day 21, and immunoreaction markedly increased on day 42. It was observed that the tunica muscularis gave a positive reaction to all groups. In addition, when the differences between the days after post-hatching were examined, it was determined that TLR4 expression intensity, which was the lowest on the 7th day, increased throughout the development and was the highest on the 42nd day (Figure 1). The immunohistochemistry findings are analyzed and summarized in Table 1.

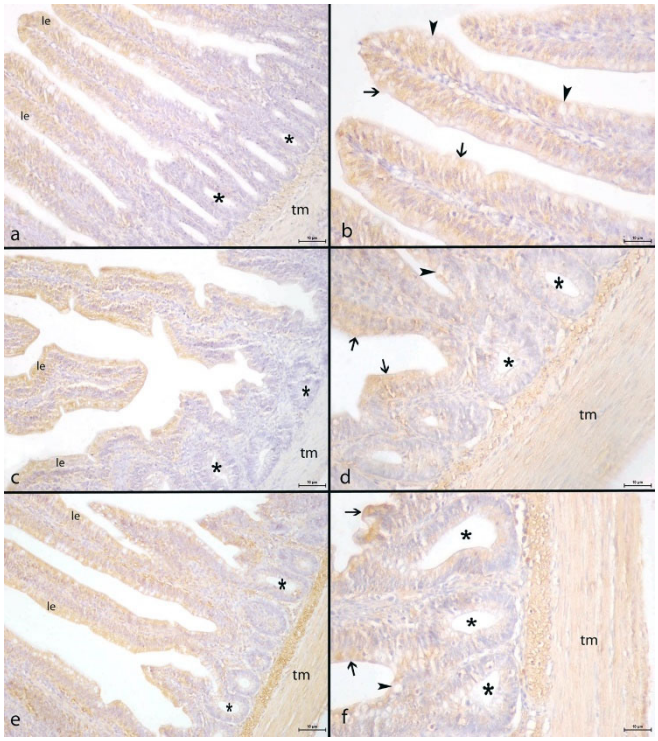

Figure 1. The view of immunohistochemical staining with TLR4 as brown sediment in ileum. (a-b): 7 day old, (c-d): 21 day old, (e-f): 42 day old, le: lamina epithelialis, tm: tunica muscularis, asterix: crypt, arrowhead: goblet cell, arrow: TLR4 positive villus epithelial cell, range bar, 10 µm.

Table 1. The density of TLR4 and VEGF positivity

	TLR4			VEGF		
Groups (n=6)	**Villus epithelial cell**	**Crypt epithelial cell**	**Tunica muscularis**	**Villus epithelial cell**	**Crypt epithelial cell**	**Tunica muscularis**
7	1.2 ± 0.04	0 ± 0	0.75 ± 0.04	1.37 ± 0.07	0.7 ± 0.05	0.65 ± 0.04
21	1.53 ± 0.09*	0.62 ± 0.06**	0.93 ± 0.12	1.82 ± 0.08*	0.72 ± 0.05	0.7 ± 0.06
42	1.73 ± 0.08**	0.88 ± 0.05**,#	1.22 ± 0.08**	1.98 ± 0.09**	0.75 ± 0.08	1.15 ± 0.04**,##

Not: TLR4 (Toll-like receptor 4), VEGF (Vascular endothelial growth factor).
(Data presented as mean ± SEM).
*p<0.01 and **p<0.001 when compared to 7.
#p<0.01 and ##p<0.001 when compared to 21.

VEGF Immunolocalization

Ileum villus epithelial cells, crypt epithelial cells, and smooth muscle cells displayed a brown intracytoplasmic response in sections stained with VEGF. Furthermore, goblet cells did not exhibit any immunoreaction. The reaction in the villus epithelial cells of the ileum was weak on the 7th day, and the epithelial cells that did not stain towards the base of the villi were abundant. Towards the 42nd day, it was determined that the reaction intensity in the villus epithelial cells increased regularly, and more cells reacted in the villus basal. As a remarkable finding, in the upper villus epithelial cells, VEGF protein expression started to rise from the 7th day, and it stained intensely, especially on the 42nd day. Additionally, on the 7th day following post-hatching, weak immunoreaction was observed in crypt epithelial cells; on the 21st and 42nd days, the intensity of the reaction remained unchanged. In addition, it was determined that the density of staining was minimal, the same on the 7th and 21st days in the tunica muscularis layer, and moderate on the 42nd day (Figure 2). Table 1 analyzes and summarizes the immunohistochemistry results.

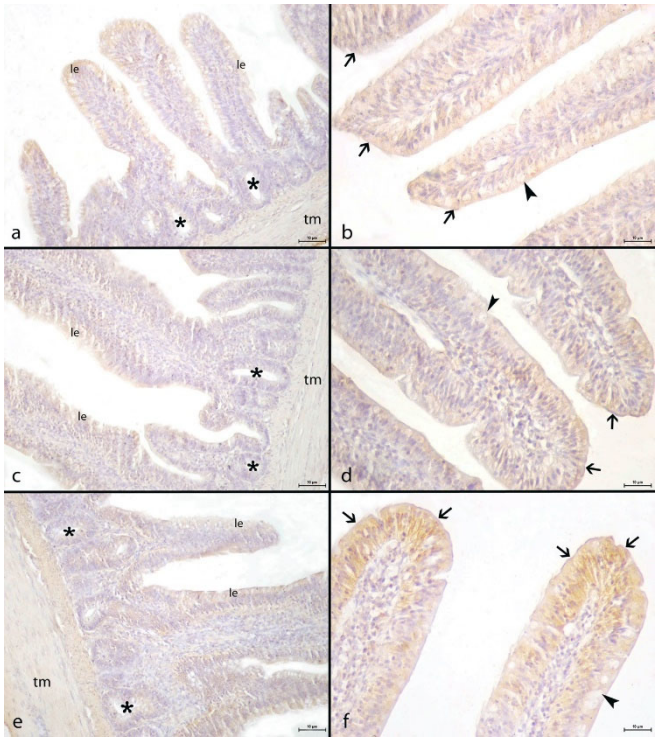

Figure 2. The view of immunohistochemical staining with VEGF as brown sediment in ileum. (a-b): 7 day old, (c-d): 21 day old, (e-f): 42 day old, le: lamina epithelialis, tm: tunica muscularis, asterix: crypt, arrowhead: goblet cell, arrow: VEGF positive villus epithelial cell, range bar, 10 µm.

DISCUSSION AND CONCLUSION

TLR4 and VEGF expression on the brolier ileum during post-hatching development were investigated in this study. During the post-hatching period, TLR4 and VEGF expression increased in the luminal epithelial, crypt epithel, and smooth muscle cells of the ileal tissue. As innate immune sentinels, TLRs are known to be expressed in epithelial cells and play a significant role in tissue-specific inflammation. The principal source of pro-angiogenic factors has been identified as lipopolysaccharide (LPS)-activated macrophages, which are mediated by TLR4 (14). In addition, LPS together with growth factors and cytokines have been observed to cause a significant increase in VEGF levels (15). The TLR4 has been reported to mediate LPS-induced cellular signaling (16). It is known that LPS acts by binding to the intestinal mucosa cell membrane receptor CD14, which acts as a barrier in the body (17). Also, VEGF production has been shown to be synergistically increased by costimulation of macrophages with agonists of TLRs 2, 4, 7, or 9 (18).

TLRs expressed by various cell groups in the intestinal mucosa serves as a link between innate and adaptive immunity and are primarily involved in harmful microorganism product detection and inflammatory signal transmission (19). The increased bacterial count in the small intestine

from birth to adulthood, particularly bacterial colonization by commensal microbes, is thought to act as a trigger for the induction of a hypersensitive state to TLR ligands (20). It is known that TLR4 expression is required for normal growth (including villi height) of the small intestine (21). In addition, in a study, it was reported that significantly reduced villus height was detected in TLR4 deficient mice compared to healthy mice (22). It has also been shown that TLR4 plays an important functional role, particularly in controlling intestinal epithelial homeostasis (23). TLR4 expression has also been found in human and mouse intestine smooth muscle cells and enteric neurons. The presence of TLR4 may play a significant role in the modulation of gastrointestinal motility, according to researchers (24). Furthermore, TLR4 plays an essential role in the development of the gut immune system in the gastrointestinal tract (25). It has been reported that TLR4 expression was diffusely observed in the epithelial layer, propria mucosa, and muscular layer tissues in the one-day-old post-hatch chick cecum (12). Moreover, it has been stated that TLR4 was slightly or weakly expressed on the mucosal surface of the terminal ileum in normal mice, which may be closely related to the immune tolerance of the intestines (26). Inoue et al. (23) have found that the expression of TLR4 in the small intestine increases gradually after birth, and the highest levels of TLR4 gene expression are reached in the ileum on days 3 and 18, respectively. We showed that TLR4 expression was found in the ileum epithelial and smooth muscle layer. In the current study, we demonstrated a relatively increased staining in the epithelium and smooth muscle cells of the ileum during the post-hatching period. Moreover, most of the cells at the ends of the epithelium lining the lumen had a more intense intracytoplasmic staining. Furthermore, we think that this increase in TLR4 expression with the onset of the post-hatching period may be a mechanism by which provides protect against both pathogenic and autoimmune reactions in the ileum.

It has been demonstrated that VEGF expression in the postnatal duodenum promotes villus height and epithelial cell proliferation (27). The expression of VEGF is critical for maintaining gastrointestinal tract homeostasis. VEGF expression is known to be regulated by hypoxia and oxidants. Also, hypoxia-inducible factor-1 alpha (HIF-1α) and reactive oxygen species (ROS) have been shown to activate VEGF. HIF-1α regulates VEGF gene expression and provides the adaptive responses required for gastrointestinal homeostasis maintenance under hypoxia (28). It has been observed that the source of VEGF in the ileocecal region is especially the crypt regions of the surface epithelium. It has been emphasized that this situation may be related to the proliferative activity of crypts (29). VEGF expression in the sheep jejunum, on the other hand, is higher in term animals than in fetal stages, indicating a greater role during postnatal development (10). In a study examining the localization of VEGF during development in the proventriculus of quails, it was reported that VEGF expression started from the 7th day of development and continued throughout development (30). We observed that VEGF expression in epithelial cells, propria mucosa, and muscle tissue sections were weak immunreactions on days 7. Also, we noted that the expression of VEGF increased in ileum tissue steadily from day 7 to day 42. Furthermore, we detected that more cells were stained positively with VEGF in the villus intestinalis epithelium extending into the lumen from the 21. day. We speculated that VEGF, the regulatory cytokine of vasculogenesis and angiogenesis, may be in constant communication with the immune system at all stages of post-hatching period.

In conclusion, this study is the first to describe the expression profile relationship between TLR4 and VEGF in broiler ileum after post-hatch development. In this study, TLR4 and VEGF expression were noted a relatively increased from the 7th to 42nd day after hatching. Thus, it was concluded that the development of innate and adaptive defense systems and angiogenesis mechanisms, including VEGF, continue throughout the post-hatching period. We think that the new data and information reported in this study will help advance both basic and applied developmental biology and the researchers who will research ileum and expression of TLR4 and VEGF.

CONFLICTS OF INTEREST

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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