



AGE-DEPENDENT CHANGES OF GUT-ASSOCIATED LYMPHOID TISSUE IN ONE TO FOUR-MONTH-OLD TURKEYS: A HISTOLOGICAL STUDY

M. H. EFTEKHARI TALAB, S. HAMEDİ & M. R. PARYANI

Department of Basic Sciences, Karaj Branch,
Islamic Azad University, Karaj, Iran

Summary

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Gut-associated lymphoid tissue (GALT) has a pivotal role in the health of birds as the first line of defense against foodborne pathogens. On the other hand, this part of immune system is important for successful development of vaccines. Due to scarcity of knowledge on GALT of turkey as a major industrial species, this study aimed to evaluate histological features of GALT in this species. A total of 40 clinically healthy BUT6 turkeys from both sexes at the age of 1, 2, 3, and 4 months were included in the study. Samples were immediately removed after slaughter from different parts of the gut, including pharyngeal tonsil, cervical oesophagus, oesophageal tonsil, proventriculus, pyloric tonsil, ileum, Meckel's diverticulum, caecal tonsil, the middle and apex of caecum, rectum, and cloaca. After fixation and routine procedures followed by hematoxylin-eosin staining, samples were studied histologically under light microscope. In addition, photomicrographs were taken from sections and were analysed for histomorphometric parameters using Zeiss Axio vision rel.4.8 software. According to the results, in one-month-old turkeys, lymphatic tissues in pharyngeal tonsil, pyloric tonsil, proventriculus, caecal tonsil, middle, and apex of caecum, Meckel's diverticulum, and cloaca were completely developed, while in some other parts such as the cervical oesophagus and the rectum lymphatic structures were absent. In the ileum, the Peyer's patch was observed as an aggregated structure, although the diffuse lymphatic tissue has properly evolved. In two months old turkeys, the dense lymphatic tissue including primary and secondary lymphatic follicles, was present in cervical oesophagus, Peyer's patch, rectum, and cloaca. Many histomorphometric parameters of lymphoid tissue also developed age-dependently. In four months old birds the dense lymphatic tissue was obvious in all examined specimens. Moreover, no signs of tissue atrophy were seen at the age of four months (pre-puberty) in the GALT specimens. In conclusion, the GALT of turkeys was histologically quite similar to those of other birds and these structures showed an age-dependent development from 1 to 4 months of age.

Key words: gut-associated lymphoid tissue, histomorphology, histomorphometry, turkey

INTRODUCTION

The turkey industry is growing very well worldwide so that in 2014 the annual turkey meat production reached about 5,500,000 tonnes globally. At least 70% of the poultry population is reared in dense breeding systems, which increases the yield, but facilitates the spread of infectious diseases (Hünigen *et al.*, 2016). Consumption of turkey meat is recommended for its low cholesterol level to prevent cardiovascular disease and complications associated with high serum lipids (Hashemi & Beigi, 2015; Marangoni *et al.*, 2015).

The control of infectious diseases is one of the major challenges in the turkey industry. It is evident that a healthy immune system is a strategic factor in the prevention of infectious diseases in birds' breeding flocks. In addition, a healthy immune system plays a pivotal role in reducing disease outbreaks and consequently, use of antibiotics in industrial poultry.

While mammals have myriads of lymphoid nodes as an advanced and complex lymphatic organ in the immune system, typical lymphatic nodes are exclusively present in some aquatic birds such as ducks, geese, and swans, and other birds have only small lymph follicles (Hodges, 1974).

The gut plays a critical role in the immune system of birds, especially in combating pathogens from feed origin. Mucosa-associated lymphoid tissue (MALT) is well developed in most birds, consisting of lymphoid cells in the mucosal lamina propria and submucosa of the gut and respiratory tract (Casteleyn *et al.*, 2010).

Considerable knowledge has become available on gut associated lymphoid tissue (GALT) of broilers during past decades. Throughout the gut in birds, the dif-

fuse lymphatic tissue and lymphoid follicles are seen in pharyngeal tonsil, cervical oesophagus, oesophageal tonsil, pyloric tonsil, proventriculus, the Peyer's patch, Meckel's diverticulum, and both caecal tonsils, rectum and proctodeum (Pabst, 2007).

With regard to well-developed GALT in birds, the use of mucosal vaccination strategies in these species is a promising approach (Nochi *et al.*, 2018). In addition, since the ban on growth-promoting antibiotics, research on probiotics and postbiotics selectively stimulating the GALT is increasing. These oral additives affect the GALT regionally and even systemically, as they can stimulate the immune response in other organs through the MALT (Casteleyn *et al.*, 2010). On the other hand, understanding the physiology of the immune system requires a comprehensive knowledge of the basic structures of the lymphoid tissue and its influencing factors such as age and developmental changes. Structural developments of the lymphatic organs of birds might be associated with major changes of their functions. Unlike mammals where extensive knowledge about age-related changes in immune system is available, information in birds is very scarce (Ciriaco *et al.*, 2003).

Taken together, the current study aimed at evaluating the GALT structures of one to four-month-old turkeys by using a histological approach.

MATERIALS AND METHODS

A total of 40 clinically healthy BUT6 turkeys from both sexes at the age of 1, 2, 3 and 4 months (10 birds in each age group), which were reared in similar conditions were purchased and transported to

the Dissection Hall of Karaj branch of Islamic Azad University. Samples were immediately taken after slaughtering from different parts of the gut including pharyngeal tonsil, cervical oesophagus, oesophageal tonsil, proventriculus, pyloric tonsil, ileum, Meckel's diverticulum, caecal tonsil, the middle and apex of caecum, rectum, and cloaca and placed in formalin buffer bottles separately assigned to each bird and then transferred to the histology lab. All methods used in the study were in compliance with the Animal Care and Local Ethics Committee of Islamic Azad University of Veterinary Sciences for use of animals in research.

After fixation the samples were subjected to the routine histological procedures, including dehydration, clearing, and paraffin impregnation, followed by preparation of paraffin molds. Finally, six- μ m-thick transverse sections were made using a rotary microtome. A total number of 10 sections were taken from each sample of each bird and stained with haematoxylin and eosin (H&E) and examined histologically under light microscope (Nikon model Alphaphot/YS). In addition, for histomorphometric study, photomicrographs were taken from glass slides, and were analysed using Axiovision rel.4.8 software. In detail, 15 glass slides were prepared from each specimen, of which five were randomly examined for each parameter. For this purpose, lymphatic follicles were examined, and their thickness was measured in the pharyngeal tonsil, cervical oesophagus, oesophageal tonsil, proventriculus, pyloric tonsil, ileum, Meckel's diverticulum, the middle and apex of caecum, rectum, proctodeum, and cloaca. The nodular unit width and height, fossula height, and follicles number per nodular unit were histomorphometrically examined in caecal tonsil slides. Then the

data were expressed as mean \pm standard deviation. To analyse data, SPSS software version 11.0 by one-way ANOVA followed by Tukey's multiple comparison test were used and the differences were considered statistically significant at $P < 0.05$.

RESULTS

The GALT is typically found in two forms: 1) lymphatic follicles found singular or in small numbers and their adjacent and subepithelial lymphoid tissue; and 2) tonsils.

Pharyngeal tonsil

Diffuse and dense lymphoid cells were found at all ages in the lamina propria and submucosal areas close to the epithelium in the pharyngeal tonsil of turkeys. Accumulation of lymph follicles was also observed among the mucus glands in the lamina propria. Lymphoid tissue was also seen on both the pharyngeal and nasopharyngeal sides of the pharynx (Fig. 1).

Cervical oesophagus

In one month-old turkeys, lymphoid tissue was not found in the cervical oesophagus, but diffuse and dense lymphatic tissue in the oesophageal wall close to mucous glands was seen in two month-old and older turkeys so that the thickest follicle belonged to four month-old turkeys where its difference with those of younger specimens was significant (Fig. 1, Table 1).

Oesophageal tonsil

The oesophageal tonsil was located between the oesophagus and the proventriculus. According to the number of longitudinal folds in the oesophageal wall, the tonsils were divided into several tonsillar units, all located in the mucosa of

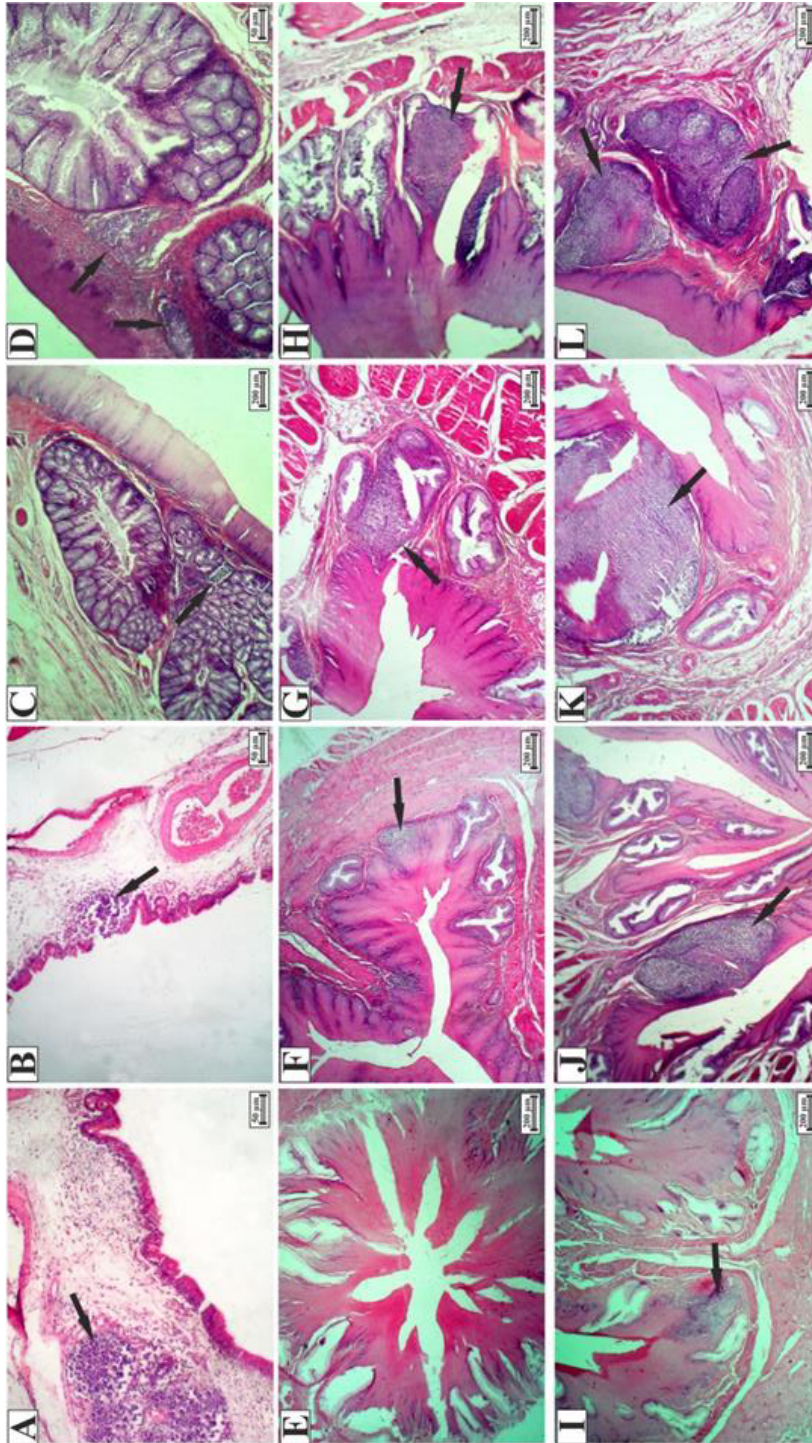


Fig. 1. Histological section of turkeys' pharyngeal tonsil (upper row), cervical oesophagus (middle row), oesophageal tonsil (lower row) at the age of one (A, E, I), two (B, F, J), three (C, G, K) and four (D, H, L) months. Lymphatic tissue (arrows) increased from 1 to 4 months of age, H&E, scale bars: 50 μm (A, B, D) and 200 μm (C, E, F, G, H, I, J, K, L).

Table 1. Follicle width (mean±SD, n=40) of cervical oesophagus, oesophageal tonsil, proventriculus, pyloric tonsil and Peyer’s patch in turkeys at different ages.

	Age, months			
	1	2	3	4
Cervical oesophagus (µm)	–	151.20±28.97 ^a	158.93±27.09 ^a	315.53±63.53 ^b
Oesophageal tonsil (µm)	192.37±26.69 ^a	241.05±26.07 ^a	226.53±28.69 ^a	184.39±24.24 ^a
Proventriculus (µm)	85.95±7.90 ^{a,b}	91.45±6.42 ^{a,b,d}	127.97±15.37 ^{d,c}	124.94±25.27 ^d
Pyloric tonsil (µm)	70.51±5.54 ^a	143.53±7.73 ^b	175.24±16.54 ^c	268.40±6.96 ^d
Peyer’s patches (µm)	97.24±12.74 ^a	159.40±27.67 ^b	176.24±21.33 ^c	209.78±5.45 ^d

Different superscript letters are used to denote significant difference within a row (P<0.05).

the lamina propria, between the two folds so that each unit surrounded a crypt. The crypts were covered by a stratified squamous epithelium that also covered the excretory ducts of the oesophageal mucous glands. Turkeys older than three months had larger tonsils with more lymphatic follicles, but there was no significant difference in the thickness of lymphatic follicles among age groups (Fig. 1, Table 1).

Proventriculus

Larger lymphocyte and follicle accumulations were found in the subepithelium at all ages. Lymphocyte accumulations were also found in the proventriculus adjacent to the glandular ducts (Fig. 2, Table 1).

Pyloric tonsil

It was a longitudinal zone between the gizzard and the duodenum at the pylorus valve level. Lamina propria of the proximal duodenum had a number of primary and secondary lymphatic follicles and interfollicular regions. Lymphocytes and some M-cells were also observed in the epithelium (Fig. 2, Table 1).

Peyer’s patch in the ileum

Lymphoid tissue was found throughout the ileum, but Peyer’s patches were seen in some areas where intestinal villi thickened by numerous lymph cells present in the mucosal and submucosal areas of the lamina propria. The tissue had primary and secondary lymphoid follicles separated by interfollicular areas. The epithelium covering the Peyer’s patches was formed by undifferentiated enterocytes penetrated strongly by lymphoid cells. M-cells were also seen in the epithelium of the Peyer’s patches. This lymphoid tissue was seen at all ages, especially in specimens taken from turkeys older than two months (Fig. 2, Table 1).

Caecal tonsil and the middle and apex of the caecum

The accumulation of lymphatic follicles near the caecal valve is termed caecal tonsil. The lamina propria and submucosal areas contained many lymphatic follicles and tissue. Almost all lymphatic follicles had the same structure composed of nodular units (ND) separated by a thin connective tissue. There was also a distinct fossula within each nodular unit, and almost all M-cells gathered inside the fossula epi-

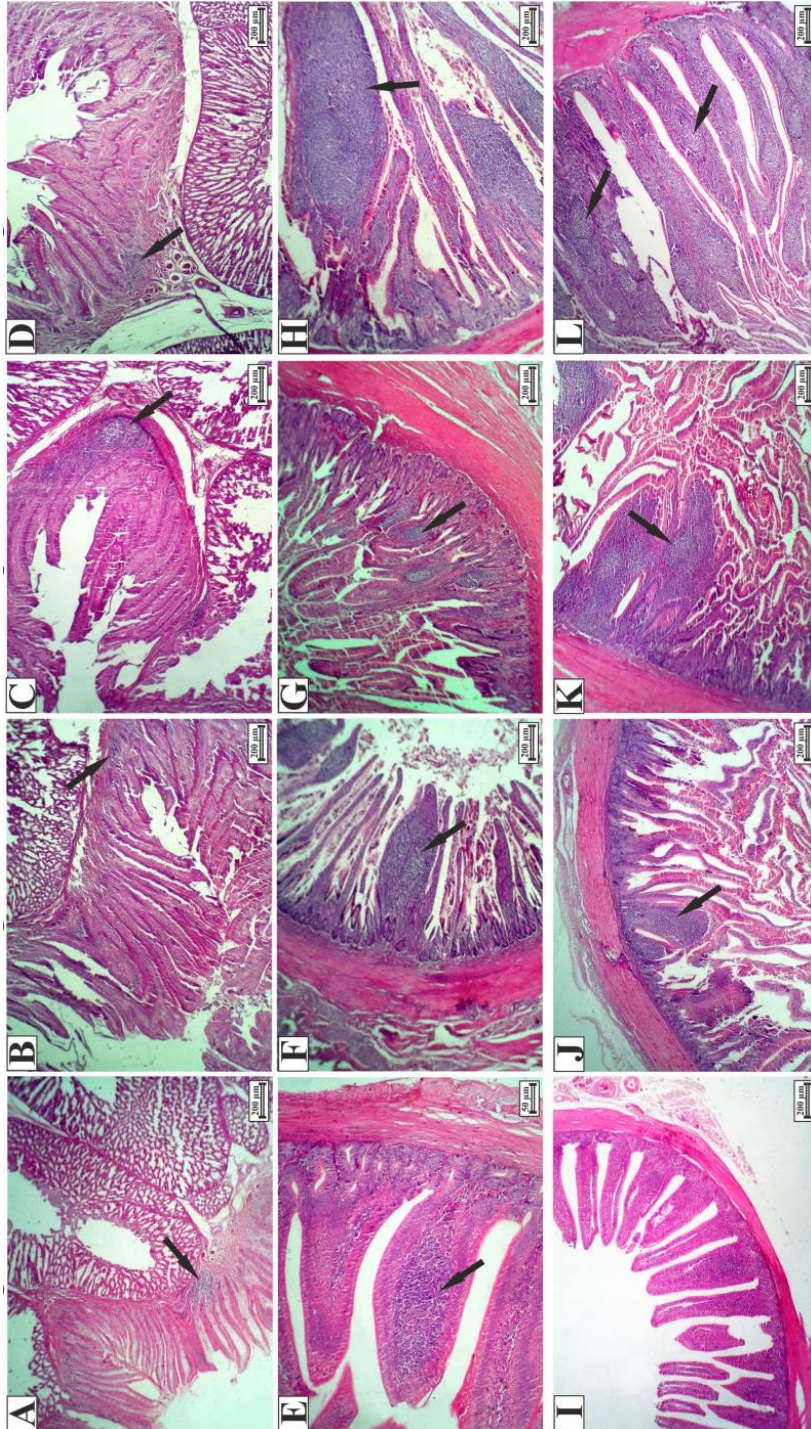


Fig. 2. Histological section of turkeys' proventriculus (upper row), pyloric tonsil (middle row), Peyer's patch (lower row) at the age of one (A, E, I), two (B, F, J), three (C, G, K) and four (D, H, L) months. Lymphatic tissue (arrows) increased from 1 to 4 months of age, H&E, scale bars: 50 μm (E) and 200 μm (A, B, C, D, F, G, H, I, J, K, L).

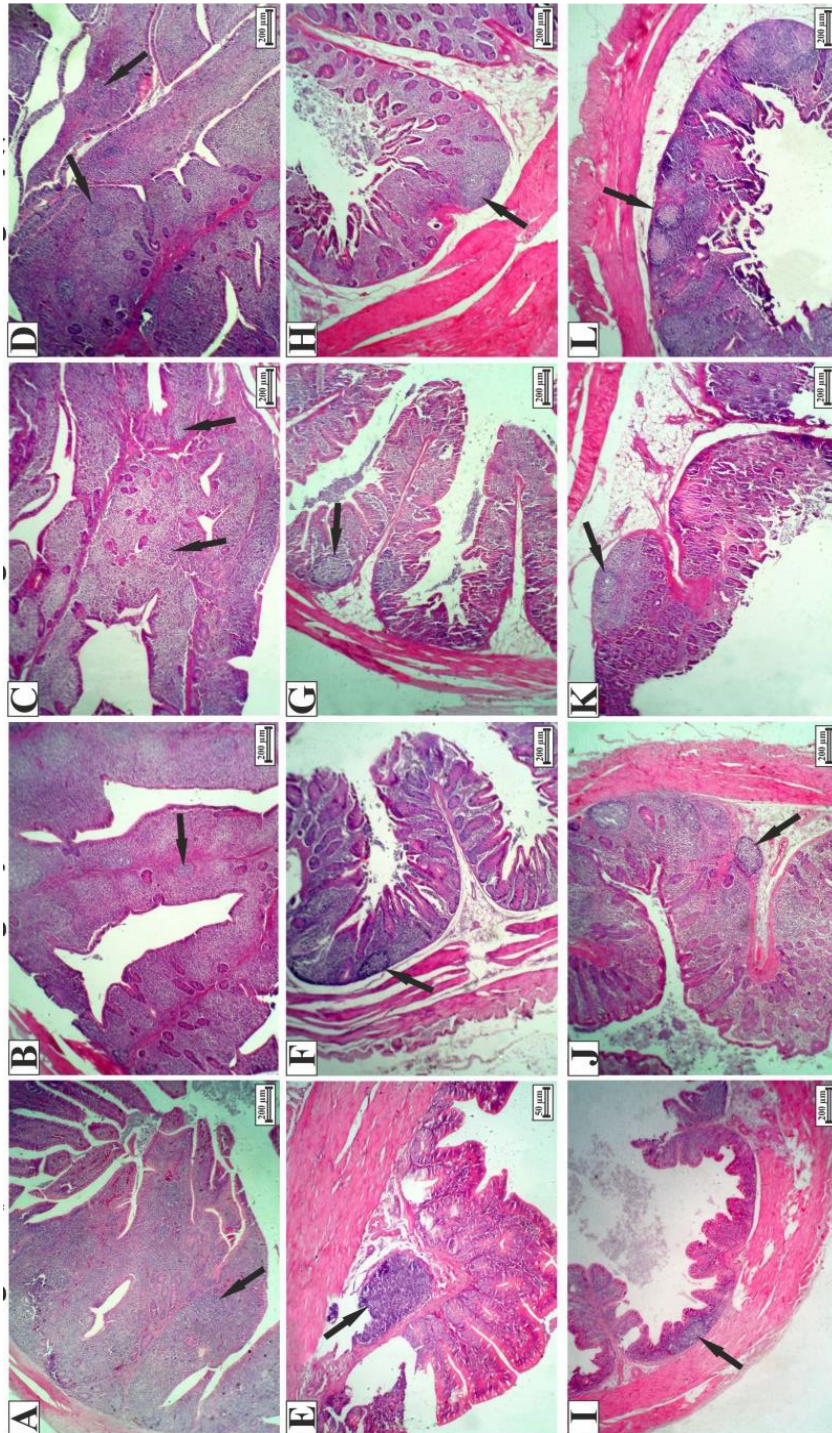


Fig. 3. Histological section of turkeys' caecal tonsil (upper row), middle of caecum (middle row), and apex of caecum (lower row) at the age of one (A, E, I), two (B, F, J), three (C, G, K) and four (D, H, L) months. Lymphatic tissue (arrows) increased from 1 to 4 months of age, H&E, scale bar 200 µm.

Table 2. Histological measurements (mean±SD, n=40) of caecal tonsil, middle and apex of caecum in turkey at different ages.

	Age, months			
	1	2	3	4
Nodular unit height (µm)	1388.69±76.86 ^a	2757.22±422.90 ^b	3308.25±305.55 ^b	3092.65±102.19 ^b
Nodular unit width (µm)	979.76±88.17 ^a	868.53±94.42 ^a	902.47±112.09 ^a	934.27±120.28 ^a
Fossula height (µm)	1135.88±48.51 ^a	2041.27±484.10 ^b	2305.32±225.36 ^b	2286.81±223.57 ^b
Nodular width in caecal tonsil of caecum (µm)	148.87±33.11 ^a	180.11±10.88 ^a	165.61±30.54 ^a	185.94±40.32 ^a
Nodular width in middle of caecum (µm)	105.39±3.97 ^a	131.72±9.84 ^{a,b}	162.75±26.47 ^{b,c}	184.99±8.34 ^c
Nodular width in apex of caecum (µm)	100.00±2.91 ^a	147.75±14.79 ^b	151.28±11.91 ^b	162.01±12.92 ^c

Different superscript letters are used to denote significant difference within a row (P<0.05).

thelium had lighter cytoplasm and larger nuclei. Histomorphometric studies showed that the height of the nodular units and the height of the fossula increased significantly after one month of age, but the width of the follicular units and the thickness of the lymphatic follicles showed no statistically significant difference. Lymphatic follicles were seen throughout the caecum, but their number in the caecal tonsil was much higher than those in other parts of the caecum. In addition, the thickness of the lymphatic follicles increased significantly after one month of age in middle and end parts of caecum (Fig. 3, Table 2).

Meckel's diverticulum

It is a small dome- or worm-shaped bulge on the antimesenteric border of the jejunum (rather close to the distal part of the middle jejunum) which can be detected by naked eyes. The diffuse lymphatic tissue was obvious in the lamina propria in all the studied ages, and lymphatic follicles were also added from two months of age

and their thickness increased significantly with age (Fig. 4, Table 3).

Table 3. Histological measurements of follicles width (mean±SD; n=40) of Meckel's diverticulum and rectum in turkey at different ages.

Age, months	Parameters	
	Meckel's diverticulum (µm)	Rectum (µm)
1	–	–
2	164.19±30.91 ^a	–
3	210.98±37.90 ^{a,b}	127.00±16.74 ^a
4	237.22±10.87 ^b	159.64±25.33 ^a

Different superscript letters are used to denote significant difference in a column (P<0.05).

Rectum

The rectum in turkey, located in the apex of the gut between the ileum and the clo-

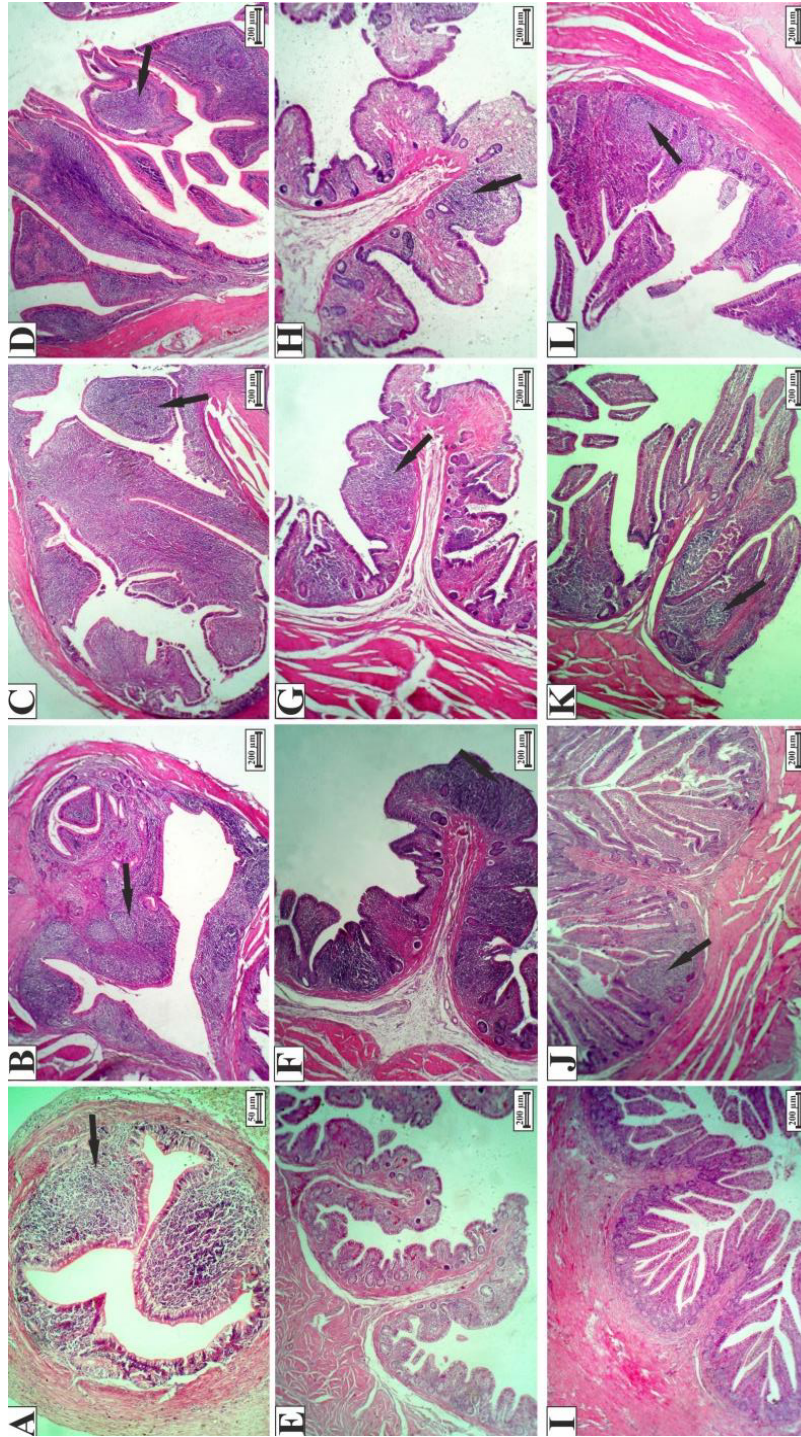


Fig. 4. Histological section of turkeys' Meckel's diverticulum (upper row), cloaca (middle row), and rectum (lower row) at the age of one (A, E, I), two (B, F, J), three (C, G, K) and four (D, H, L) months. Lymphatic tissue (arrows) increased from 1 to 4 months of age, H&E, scale bars: 50 μm (A) and 200 μm (B, C, D, E, F, G, H, I, J, K, L).

aca, had many lymphatic cells in the mucosa of the lamina propria after one month of age. The cells were often organised into small lymphatic follicles at three or four months of age (Fig. 4, Table 3).

Cloaca and proctodeum

In the proctodeum, the lymphoid tissue increased with age. In the mucosa of proctodeum, primary lymphatic follicles were seen in specimens older than two months (Fig. 4).

DISCUSSION

The results of the present study showed that the lymphoid tissue is formed in some parts of the gut of one-month-old turkeys including pharyngeal tonsil, pyloric tonsil, proventriculus, caecal tonsil, middle and apex of the caecum, Meckel's diverticulum, cloaca, and bursa of Fabricius, although it was not present in some regions such as cervical oesophagus and rectum. In the ileum, there were no aggregated Payer's patches in one-month-old turkeys, but diffuse lymphatic tissue was obvious. With advancing age, the GALT developed and the dense lymphatic tissue including primary and secondary lymphatic follicles were seen in the cervical oesophagus, Peyer's patch, rectum, and cloaca. Lymphatic tissue developed until four months of age so that the dense lymphatic tissue was obvious in all examined specimens. Likewise, no signs for tissue atrophy were seen at the age of four months (prepuberty) in the GALT specimens.

Most studies on GALT are related to chickens as prototype industrial bird species. Here we tried to compare the results of our study regarding histological structures of GALT in one to four month-old turkeys to those of other bird species including chickens, quails, etc. based on

previously published materials. The histological features of GALT in turkeys were relatively close to what described in other bird species although some differences were also present in different structures.

In the oropharynx, non-follicular tonsils as an exclusive structure, have an almost wrinkled epithelium that lacks crypt, but lymphatic follicles and interfollicular lymphatic tissue are evident. Some researchers believe that there is no typical tonsil (follicular or non-follicular) in the pharyngeal area of the poultry, and the existing structures should be considered as the pharyngeal lymphatic follicles and that the real tonsil in poultry is only seen in the oesophagus, pylorus, and caecum. In most birds studied, in terms of pharyngeal tonsils, a lymphatic accumulation was seen around Schwann cells, and the infundibulum was mistakenly called pharyngeal tonsil. The results of the current study on turkeys, consistent with studies by Tadjalli *et al.* (2008) on the ostrich and Casteleyn *et al.*, (2010) on broilers, showed that lymphocytes were diffuse and dense in lymphatic follicles, near the epithelium, among the mucous glands in this area.

In agreement with the findings of the study by Arai *et al.* (1988) on broilers, lymphatic cell accumulation in the cervical oesophagus of turkeys was apparent from two months of age. The oesophageal tonsil in turkeys was composed of some tonsillar units with a multitude of lymphatic follicles and a germinal centre and each tonsillar unit surrounded a crypt. The same structures are described in broilers by Oláh *et al.* (2003).

Matsumoto & Hashimoto (2000) studied the proventriculus in broilers for lymphoid tissue and reported that the accumulation of lymph cells in the proventriculus was obvious in three areas of the lamina propria: subepithelium, next to the

duct of deep glands as well as inside the gland. These accumulations were seen during the first week of life in subepithelial regions, while lacking a germinal center, but from the third week of age, when the lymphatic follicles had a germinal center, the accumulations were seen among the glands. Results of the present study, consistent with those of the latter mentioned study, reported the formation of follicular accumulations without germinal centers in the subepithelial tissue of proventriculus from one month of age. On the contrary, no lymphatic follicles were seen among the deep glands of the proventriculus.

Oláh *et al.* (1984) studied the lymphoid tissue of Meckel's diverticulum in broilers and reported that lymphoid accumulations formed in Meckel's diverticulum from the age of two weeks, and longitudinal folds were formed among the lymphoid tissue from two to five months of age. The germinal centers were seen from five to seven months after birth in the lymphatic follicles. Meckel's diverticulum in broilers reaches its maximum development after ten weeks and remained active until the age of 21 months. In the current study, the lymphoid tissue was seen in turkeys' Meckel's diverticula since one month of age and showed germinal centers from the age of two months, where the number of its folds and the thickness of the lymphatic follicles increased by age.

Consistent with the research of Oláh & Nagy (2007) on broilers, subepithelial lymphatic follicles in the current study were seen in the pyloric tonsil of turkeys with interfollicular areas containing small lymphocytes, macrophages, and reticules.

Befus *et al.* (1980) reported that Peyer's patches were located on the antimesenteric side of the ileum in broilers. They were also seen microscopically from

ten days of age. Their sizes increased significantly until 16 weeks of age, and after week 20, they degenerated, and at 52 to 58 weeks of age, only one Peyer's patch, located between the ileum and caecum was seen. In the current study, the Peyer's patch was seen at all ages, especially in specimens taken from turkeys older than two months in line with the study performed by Befus *et al.* (1980).

Rezaian & Hamedí (2007) studied caecal tonsil, bursa, and thymus in broilers before and after puberty, consistently they reported that lymphatic follicles in caecal tonsils were composed of follicular units and a deep epithelial fossula inside the follicular unit form fossula with the M-cells in the epithelium (Rezaian & Hamedí, 2007). Hamedí *et al.* (2013) also studied the caecum of quails from birth to puberty. They stated that lymphatic structures of caecal tonsil in the beginning part of caecum as well as lymphoid tissue in the middle and apex of caecum evolved from birth to puberty as also shown from the findings of the present study. Also, in agreement with the findings of the current study, Amirtaghavi & Hamedí (2019) showed that the caecal tonsil in chukar develops before puberty, the height of the follicular units and the number of its lymphatic follicles increased with age, and no atrophic sign was seen in the studied tissue.

In the turkey, similar to the findings of Hodges' study on broilers, diffuse lymphoid tissue was seen in the rectum until two months old, and then small lymphatic follicles were substituted at 3–4 months of age (Hodges, 1974).

In the proctodeum of turkeys, dense lymphoid tissue is seen from two months old, which expands with age. These findings were consistent with those of Dolfi *et al.* (1988) in chickens.

In conclusion, the structure of GALT in turkeys is histologically quite similar to that of other birds. At the age of one month, GALT is not thoroughly obvious, but from two months of age, it developed. As age increased, all the histomorphometric factors studied on GALT had an increasing trend until puberty, which is significantly similar to other birds such as broilers, quails and chukars.

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Correspondence:

S. Hamed
Department of Basic Sciences,
Faculty of Veterinary Medicine,
Karaj Branch, Islamic Azad University,
Karaj, Iran
e-mail: sahar_hamed@yahoo.com