

Age-related changes in the morphology and the distribution of IgA and IgG in the palatine tonsils of yaks (*Bos grunniens*)

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Summary. This study aimed to describe the age-related morphological changes and the distribution of IgA and IgG antibody-secreting cells (ASCs) in yaks. The palatine tonsils of twenty clinically healthy yaks, viz. newborn juvenile, adult and aged, were studied using histology, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA). The results showed that the palatine tonsils found in two tonsillar sinus were elongated kidney-shaped structures. Some external crypts and internal crypts were present. The palatine tonsils were partially enclosed by a connective tissue capsule and had trabeculae extending into the organ. Within these encapsulated organs, mucous glands were seen very obviously. Each crypt was highly branched and lined with stratified squamous non-keratinized epithelium. Several nonepithelial cells infiltrated between the epithelial cells, forming patches of reticular epithelium or lymphoepithelium. In newborn yaks, the lymphoid follicles were not observed. In other groups, the lymphoid follicles consisted of primary lymphoid follicles and secondary lymphoid follicles. Both IgA and IgG ASCs were distributed in the interfollicular areas, lymphoid follicles, the subepithelial areas of the non-reticular crypt epithelium, and the reticular crypt epithelium, with a few positive cells aggregated around the gland. The density of the two ASCs and the expression of the two proteins gradually increased from

newborn to adult and reached a peak at adult age; they then decreased with age. However, the density of the IgG ASCs and the expression of IgG protein was significantly higher than that of IgA in all groups ($P<0.01$). The results indicated that the palatine tonsils were not only lymphoepithelial structures but also typical secondary lymphoid organs. IgG could be a significant component of mucosal immune responses in the palatine tonsils of yaks.

Key words: Yak, Palatine tonsils, Lymphoid follicles, Interfollicular areas, IgA, IgG

Introduction

Yaks (*Bos grunniens*) are native plateau animals in the central Asian highlands. In China, they are found extensively on the Qinghai-Tibetan plateau, which in turn is subject to strong UV radiation and has atmospheric oxygen content and environmental temperatures that are substantially lower than those at lower elevations. Additionally, yaks provide a critical source of animal products, such as meat, milk, hides, and other by-products for local herdsman (Cui and Yu, 1999).

The palatine tonsils (PT) are located at the junction of the nasopharynx and oropharynx. Functionally, the palatine tonsils provide a significant part of the protective immunological ring at the openings of the gastrointestinal and respiratory tracts (Ogra, 2000) and play a key role in initiating immune responses against

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ingested antigens (Brandtzaeg, 1984). Histologically, the palatine tonsils are lymphoepithelial tissues composed of crypts, lymphoepithelium (LE), lymphoid follicles (LF), interfollicular regions (IF), connective tissue, lymphoid cells (B- and T-lymphocytes), dendritic cells and macrophages. Together, these components provide innate, cellular and humoral immunity at the local and systemic levels (Horter et al., 2003). The palatine tonsils can also form a point of entry and a replication site for some pathogens, of which prions causing bovine spongiform encephalopathy (BSE) are of major importance (Jeffrey et al., 2001; Hunter, 2003; Bellworthy et al., 2005). The structures of the palatine tonsils have been described in various species. Viz. humans (Nave et al., 2001; Jović et al., 2015), bovines (Manesse et al., 1998; Palmer et al., 2009; Zidan and Pabst, 2011), ovine (Casteleyn et al., 2007, 2008, 2010), horses (Kumar and Timoney, 2005), camels (Zidan and Pabst, 2009; Jia et al., 2017), dogs (Belz and Heath, 1995) and porcine (Liu et al., 2012). In addition, it has been reported that in calves, lymphoid follicles are formed in the palatine tonsils at 20 days after birth, and germinal centers are not formed until 2-4 weeks after birth (Schun and Oliphant, 1992; Manesse et al., 1998; Yasuda et al., 2006). In a similar manner, germinal center formation and extrafollicular plasma cells in human tonsils are not recognized until 2 weeks after birth (Brandtzaeg and Halstensen, 1992). The hypofunction of the palatine tonsils with age after adolescence also exists in Bactrian camels (Jia et al., 2017).

Plasma cells are the final B cell differentiation stage, but their involvement in local immunoglobulin A (IgA) and immunoglobulin G (IgG) secretion has not been entirely clarified. The palatine tonsils can produce large numbers of specific IgA and IgG antibody-secreting cells (ASCs) in response to intratonsillar immunization (Quiding-Järbrink et al., 1995). IgA is one of the most important immunoglobulins in the mucosal immune system because it constitutes a first line of defense against foreign antigens, organizes immune tolerance (Hapfelmeier et al., 2010), modulates immune exclusion (Strugnell and Wijburg, 2010), and inhibits inflammation and allergic reactions. In contrast, IgG in the palatine tonsils not only mediates local mucosal immune responses but also affects the humoral immune responses after they are dispersed to other tissues via the circulation (Boyaka et al., 2000). In recent years, several studies have shown that the proportions of ASCs differ among mucosal regions. For example, the ratios of IgA and IgG ASCs in the palatine tonsils of humans and pigs were 1:2 and 1:1, respectively (Korsrud and Brandtzaeg, 1980; Horter et al., 2003). However, the densities of IgA ASCs were significantly higher than those of IgG ASCs in the palatine tonsils of the Bactrian camel, and the densities of the two ASCs populations in the palatine tonsils of the Bactrian camel decreased from puberty to old age without exception (Jia et al., 2017). In the equine palatine tonsils, there are only a few IgA and IgG ASCs

(Kumar and Timoney, 2005).

Previously, the histological structures and the expression of some immune cells in the thymus, spleen, lymph nodes and hemal node have been studied (Zhang et al., 2016, 2017). Despite the important immunological functions and clinical aspects of the palatine tonsils, there are no data available about the structure or function of the palatine tonsils in yaks. The aim of this study is to describe the age-related morphological changes and the distribution of IgA and IgG ASCs in healthy plateau yaks and to compare those changes with those of other animals. This would aid in further studying the immune mechanisms of yak palatine tonsils as a high plateau animal.

Materials and methods

Animals

All experimental animals were handled according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) (No. GSAUAEC-2015-007) of the College of Veterinary Medicine of Gansu Agricultural University.

Twenty yaks (both male and female) were allocated from four age groups (newborn [1-6 days old; n=5], juvenile [5-7 months old; n=5], adult [2-5 years old; n=5], and aged [8-10 years old; n=5]). The animals were obtained from Xining, Qinghai province, China. All yaks were considered clinically healthy on the basis of results of a physical examination and serum biochemical analysis. Each yak was euthanized with sodium pentobarbital (200 mg/kg, IV). The palatine tonsils were harvested immediately after euthanasia.

Anatomical examination

For anatomical analyses, fresh and complete palatine tonsils tissue (newborn [1-6 days old; n=5], juvenile [5-7 months old; n=5], adult [2-5 years old; n=5], and aged [8-10 years old; n=5]) were washed with running tap water, and then the length, width and thickness of each palatine tonsil were measured with a Vernier caliper.

Histological examination

For light microscopy, small specimens of palatine tonsils tissue were fixed in a solution of 4 per cent paraformaldehyde in phosphate buffer (pH, 7.3) for at least 2 weeks. Tissue blocks were processed by routine methods and embedded in conventional paraffin wax. Serial sections (4~5 µm thick) with a 40-µm interval were selected from all tissue blocks. The slides were stained with hematoxylin and eosin (H&E) in conjunction with Beilchowsky's silver impregnation, periodic acid Schiff (PAS) and Masson trichrome methods to examine their structures. Light microscopic observations and image acquisition were performed

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using an Olympus DP73 Light Microscope (including DP control and Image-Pro Express, Japan).

Immunohistochemical examination

The spatial distribution of IgA and IgG ASCs in the fixed palatine tonsils tissue was evaluated by immunohistochemical staining. Fixed tissue specimens were mounted on microscope slides in a routine manner and exposed to primary antibodies against IgA (rabbit anti-cow IgA, Abcam, Cambridge, England, 1:1000 dilution) and IgG (rabbit anti-cow IgG, Abcam, Cambridge, England, 1:2000 dilution) and incubated for 2 hours at 37°C in a moist chamber. A biotinylated anti-rabbit secondary antibody was applied for 10 min. Then, streptavidin-conjugated peroxidase was applied to the slide for 10 min. Reaction products were formed with 3,3-diaminobenzidine tetrahydrochloride. The sections were lightly counterstained with hematoxylin. Negative control had the primary antibody replaced with rabbit serum albumin with all other steps and conditions remaining the same.

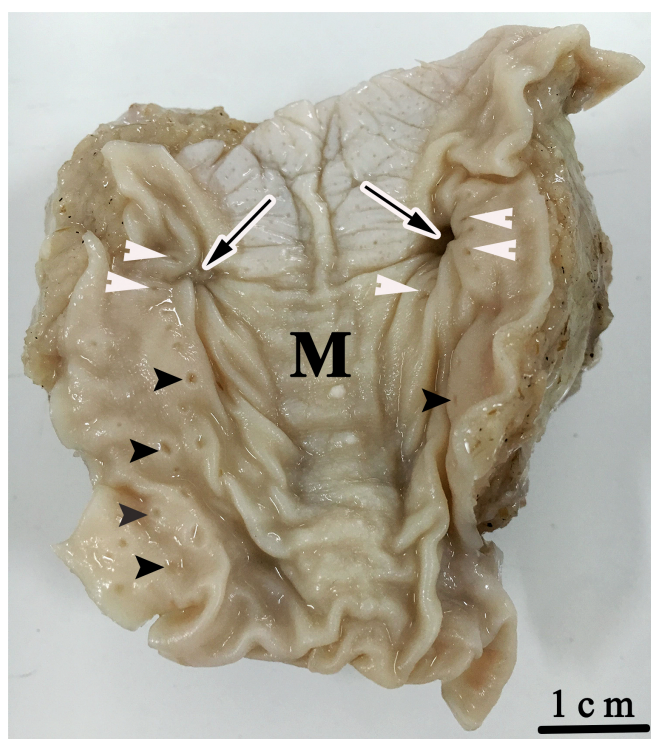


Fig. 1. Palatine tonsil of an adult yak fixed in 4 per cent neutral paraformaldehyde phosphate buffer (pH 7.3). Palatine tonsil had an elongated kidney shape with a central tonsillar fossa leading to a tonsillar sinus and containing mucous secretions (arrows). There were a number of pinhole-sized openings extending into the tonsils forming external crypts (black arrowheads). A number of smaller pinhole-sized internal crypts originated from the tonsillar sinus and were arranged in its surrounding wall (white arrowheads). Oropharyngeal mucosa (M).

Detection of IgA and IgG by ELISA

Expression of IgA and IgG in the palatine tonsils was directly measured by ELISA kits according to the manufacturer's instructions (Bovine IgA ELISA kit, JYM0099Bo, Wuhan ELISA Lab Biotch Co., LTD, Wuhan, China; Bovine IgG ELISA kit, JYM0009Bo, Wuhan ELISA Lab Biotch Co., LTD, Wuhan, China). Tissue samples were frozen in nitrogen and homogenized in ice-cold (4°C) PBS (pH 7.4) (0.1 g/ml). Aliquot the supernatant for ELISA assay (10 µl/well). A well was left empty as a blank control (without sample and HRP-Conjugate reagent). The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The OD value was proportional to the concentration of IgA and IgG. The concentration of IgA and IgG in the samples was determined by comparing the OD of the samples to the IgA and IgG standard curve.

Measurements and statistical analysis

The measurements of the external diameters of the palatine tonsils were performed according to the norm of anatomy and high-altitude medicine. The length of palatine tonsils was measured as the vertical distance from the apex to the bottom, whereas the width was measured as the size of the dorsal maximum diameter. The thickness of the palatine tonsils was measured from the tonsillar sinus to its dorsal vertex.

Ten sections were randomly selected from each palatine tonsil. In each section of the palatine tonsils, five microscopic fields were randomly selected and observed, and the numbers of lymphoid follicles, primary lymphoid follicles and secondary lymphoid follicles were determined. The respective densities of IgA and IgG ASCs were calculated (Image-Pro Plus 6.0). Statistically significant differences among these data were analyzed by one-way ANOVA followed by Duncan's multiple range test. All data were expressed as the mean $\pm$ standard error (SE) and determined using IBM SPSS V.23.0 (SPSS Inc, Chicago, IL, USA). Values of $P < 0.05$ were considered significant, and values of $P < 0.01$ were considered very significant.

Results

Anatomical characteristics

The paired palatine tonsils were embedded in the muscles and connective tissue of the pharyngeal side walls, formed by two central invaginated (tonsillar sinus) elongated kidney-shaped structures (Fig. 1). Each palatine tonsil had a tonsillar sinus opening bilaterally in the pharynx between the palatoglossal and the palate-pharyngeal arch. A white mucoid substance was discharging from the opening of the tonsillar sinus. In addition to the tonsillar sinus, a number of pinhole-sized openings extended into the tonsils and formed external

crypts (Fig. 1). A number of smaller pinhole-sized internal crypts originated from the tonsillar sinus and were arranged in its surrounding wall (Fig. 1). Length, width, thickness, and weight of the palatine tonsils increased with the aging in yak with a peak in adult groups and decrease in old groups (Table. 1).

Histological characteristics

The outer mucosal surface of each palatine tonsil was covered by a keratinized, non-keratinized or parakeratinized stratified squamous epithelium, which invaginated into the lamina propria and formed many

Table 1. The length, width, thickness, and weight of the palatine tonsils in yaks.

Age	Length (cm)	Width (cm)	Thickness (cm)	Weight (g)
Newborn	1.58±0.10c	0.96±0.06c	1.09±0.06b	2.46±0.19c
Juvenile	2.84±0.16b	1.71±0.09b	2.21±0.17a	8.61±0.52b
Adult	3.68±0.03a	2.22±0.07a	2.46±0.11a	13.63±1.04a
Old	3.18±0.23b	1.93±0.06b	2.27±0.04a	10.25±0.42b

Note: Values are mean ± standard error (SE). Ten palatine tonsils of yaks in each age group were analyzed. In the same column, different letters represent a significant difference ($p<0.05$), and the same letter represents a non-significant difference ($p>0.05$).

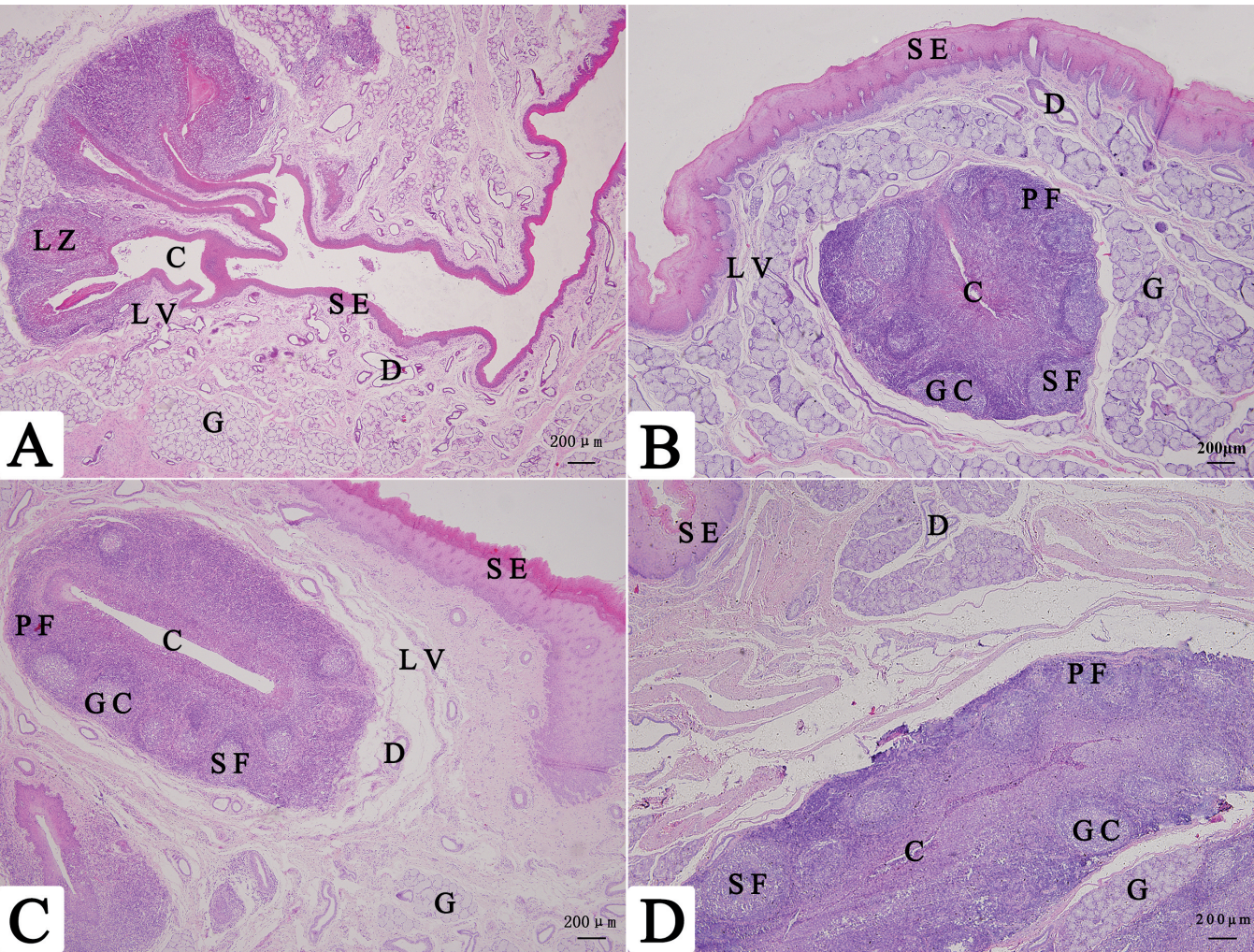


Fig. 2.1. Representative photomicrographs of a section of palatine tonsil [PT] in yak. **A.** Palatine tonsil of newborn yak (1-7 days old); the surface epithelium (SE) of the palatine tonsil (PT) was stratified squamous keratinized epithelium. Parenchyma of the newborn yak PT was formed by diffuse lymphoid tissues (LZ) surrounding the crypt (C), and the lymphoid follicles were not formed yet. Excretory ducts (D) of the mucous glands (G) were seen in the parenchyma near the crypt (C). **B.** Palatine tonsil of juvenile yak (5-7 months old). **C.** Palatine tonsil of adult yak (2-5 years old). **D.** Palatine tonsil of old yak (8-10 years old). B-D: Surface epithelium (SE) of palatine tonsil (PT) was stratified squamous keratinized epithelium. The parenchyma of the palatine tonsil (PT) was formed by primary lymphoid follicles (PF), secondary lymphoid follicles (SF), and interfollicular areas (IF) surrounding the crypt (C). SF had well-developed germinal centers (GC). Excretory ducts (D) of the mucous glands (G) were seen in the parenchyma near the crypt (C).

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highly branched tonsillar crypts. Underlying the epithelium, a dense connective tissue layer extended deep, forming a few trabeculae. There was large number of lymphoid tissues and lymphoid follicles surrounding the crypt of the lamina propria at the epithelial deep surface, which were designated “tonsil follicles” (Fig. 2-1A-D). Interestingly, in newborn yaks, the parenchyma of the palatine tonsils was formed by diffuse lymphoid tissues, and the lymphoid follicles were not formed yet (Fig. 2-1A and Fig. 2-2E-G). In juvenile, adult, and old yaks, the parenchyma of the palatine tonsils was formed by lymphoid follicles and interfollicular areas (Fig. 2-C-D and Fig. 2-2H-J). The tonsil follicles were partially enclosed by a capsule that consisted of fibrous connective tissue, mainly collagen fibers (Fig. 2-2E and Fig. 2-2H). The parenchyma of each palatine tonsil was supported by a reticular fiber network (Fig. 2-2F and Fig. 2-2I). The lymphoid follicles had well-developed germinal centers (GC) and a darkly stained mantle zone (MZ) facing the crypt epithelium in general (Fig. 2-1B-D and Fig. 2-2H-J). Germinal centers had sparse reticular fibers (Fig. 2-2I). Several high endothelial veins (HEVs) lined by plump rounded epithelial cells were seen within the interfollicular areas (Fig. 2-2J). The efferent lymphatic vessels contained a valve extending

into the lamina propria mucosae and under the crypt epithelium. Groups of mucous-secreting glandular tissues were distributed among the connective tissue. The excretory ducts of the mucous glands were obvious in the parenchyma near the crypt (Fig. 2-1A-D).

The epithelium of the tonsillar crypts was of two types, namely, non-reticular epithelium (NR) and the reticular epithelium (R) or the lymphoepithelium (LE) (Fig. 2-1A-D). The non-reticular epithelium of the palatine tonsils was characterized as non-keratinized or parakeratinized stratified epithelium. Within this epithelium, there were rare nonepithelial cells or vascular structures. PAS stained sections demonstrated an intact basement membrane in regions of the non-reticular epithelium (Fig. 2-3K). In contrast, the reticular epithelium or the lymphoepithelium was characterized by epithelial cells altered in shape and cellular content, infiltrative nonepithelial cells, intraepithelial vasculature, and a highly interrupted and discontinuous basement membrane (Fig. 2-3K). Briefly, the reticular epithelium or the lymphoepithelium was infiltrated by many lymphocytes and lesser numbers of macrophages, plasma cells, neutrophils and cells morphologically compatible with dendritic cells (Fig. 2-3L-M). In some regions of the reticular epithelium or the lymphoepithelium,

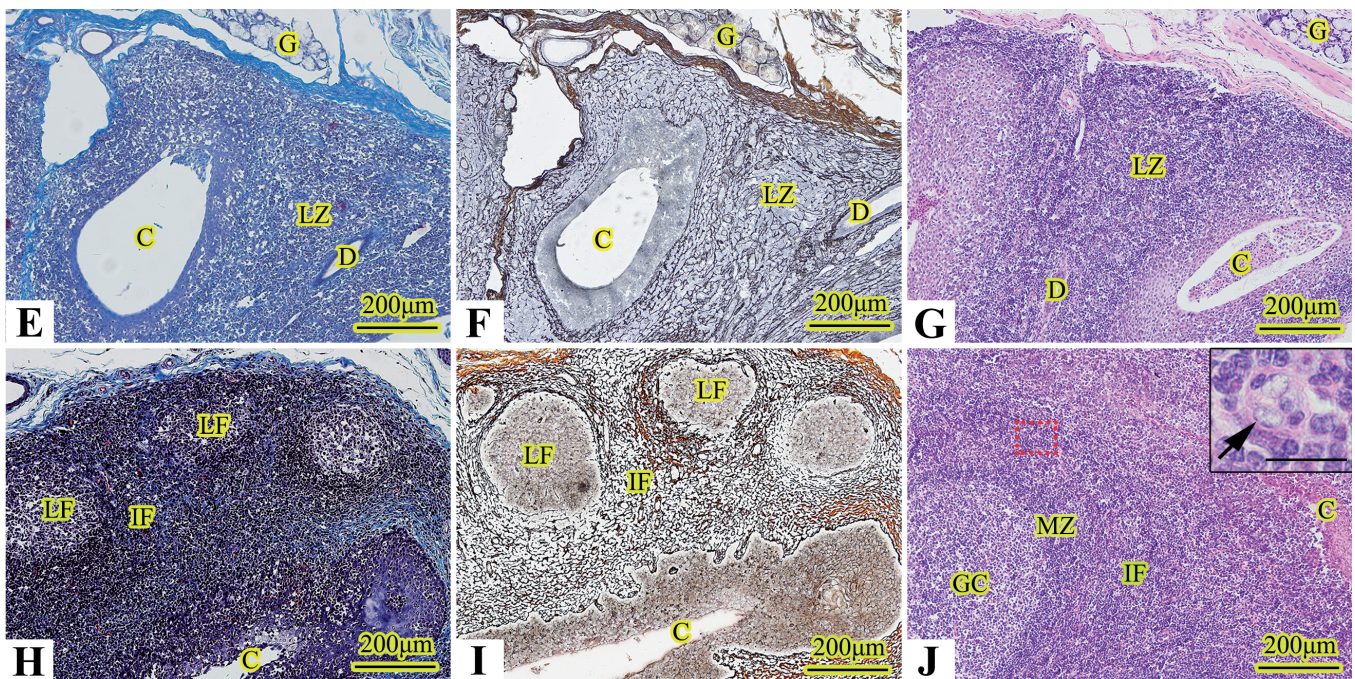


Fig. 2.2. Representative photomicrographs of a section of palatine tonsil (PT) in newborn (E-G) and adult (H-J) yaks. **E, H.** The parenchyma of the palatine tonsil (PT) was partially enclosed by a capsule that consisted of fibrous connective tissue, mainly collagen fibers (blue). Masson trichrome. **F, I.** The parenchyma of each palatine tonsil (PT) was supported by a reticular fiber network. Germinal centers (GC) have sparse reticular fibers (black). Beilschowsky's silver impregnation. **G.** In newborn yaks, the parenchyma of the palatine tonsils was formed by diffuse lymphoid tissues (LZ). **J.** The secondary lymphoid follicles (SF) had well-developed germinal centers (GC) and a darkly stained mantle zone (MZ) facing the crypt epithelium in general. Several high endothelial veins (HEVs) (arrows) were seen within the interfollicular areas (IF). **H.E.** The outlined area is shown at higher magnification by the arrows. Arrows: The high endothelial veins (HEVs) lined by plump rounded epithelial cells was seen within the interfollicular areas (IF). **H.E.**

nonepithelial cell infiltrates extended superficially to the crypt lumen (Fig. 2-1A and Fig. 2-1D).

In newborn yaks, the lymphoid follicles were not formed yet (Fig. 2-1A and Fig. 2-2E-G). In juvenile, adult, and old yaks, there were two kinds of lymphoid follicles, primary lymphoid follicles (PF) that concentrated in the central lymphocyte intensive area, with no germinal center and secondary lymphoid follicles (SF), which had well-developed germinal centers and a darkly stained mantle zone facing the crypt epithelium in general (Fig. 2-1B-D and Fig. H-J). Interestingly, the primary and secondary lymphoid follicles of palatine tonsils increased with the aging of yaks, with its peak in adult groups and decrease in old groups (Fig. 3).

IgA localization and expression

The distributions of IgA ASCs in the four age groups were similar (Fig. 4A-I). IgA ASCs were distributed in the lymphoid tissues (Fig. 4A and Fig. 4E-F). Interestingly, in juvenile, adult, and old yaks, IgA ASCs were occasionally distributed in the interfollicular areas (Fig. 4E). Sometimes, 1 or 2 positive cells were found on the edge of the lymphoid follicles (Fig. 4F), and some of the positive cells aggregated around the gland (Fig. 4B,G). Interestingly, IgA ASCs mainly clustered or scattered in the subepithelial areas of the non-reticular crypt epithelium (Fig. 4C,G), and some of the positive cells were sporadically distributed in the reticular crypt epithelium (Fig. 4D,I). Sometimes, the cells were distributed in the crypt cell debris. In IgA-positive ASCs, immunoreactivity was greatest in the cytoplasmic membrane and along the border of the cytoplasm (Fig. 4A-I). In addition, immunostaining was not observed in lymphoid follicles, interfollicular areas, the reticular

crypt epithelium, or the subepithelial areas of the non-reticular epithelium of the corresponding control sections (Fig. 4J). The distribution densities of IgA ASCs significantly increased with the aging of yaks, with a peak in the adult groups and decrease in the old groups. A significant difference was found between the adult and other groups ($P < 0.01$). There was no significant difference between the juvenile and old groups ($P > 0.05$) (Fig. 5A).

The expression of IgA protein in the four age groups was determined by ELISA, and the statistical analysis of the results demonstrated that the expression of IgA protein also significantly increased with the aging of yaks, with a peak in the adult groups and decrease in the old groups. A significant difference was found between the adult and other groups ($P < 0.01$). There was no significant difference between the juvenile and old groups ($P > 0.05$) (Fig. 6A).

IgG localization and expression

Distributions of IgG ASCs were similar to those of IgA ASCs within the same region in the four age groups (Fig. 7A-I). IgA ASCs were distributed in the lymphoid tissues (Fig. 7A and 4E,F). Interestingly, in juvenile, adult, and old yaks, IgG ASCs were occasionally distributed in the interfollicular areas (Fig. 7E). Positive cells were found on the edge of the lymphoid follicles (Fig. 7F), with a few aggregated around the gland (Fig. 7H). Most of the IgG ASCs clustered or scattered in the subepithelial areas of the non-reticular crypt epithelium (Fig. 7C,H), with a few sporadically distributed in the reticular crypt epithelium (Fig. 7D,I). Sometimes, they were distributed in the crypt cell debris. In IgG-positive ASCs, immunoreactivity was greatest in the cytoplasmic membrane and along the border of the cytoplasm (Fig.

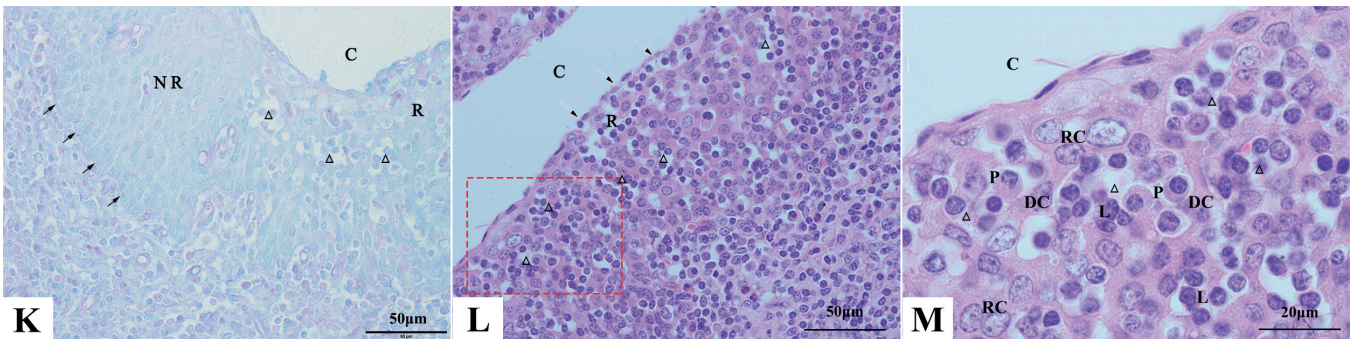


Fig. 2.3. Representative photomicrographs of a section of palatine tonsil in adult yak. **K.** Non-reticular epithelium (NR) of the palatine tonsil demonstrated an intact PAS positive basement membrane (arrows). The reticular epithelium (R) of the palatine tonsil (PT) demonstrated an intact PAS-positive interrupted basement membrane and numerous infiltrative mononuclear non-epithelial cells. Within the reticular epithelium (R) or lymphoepithelium, flattened and angular cells formed pockets (triangles) that contained non-epithelial cells. PAS. **L.** Reticular epithelium (R) or lymphoepithelium of the palatine tonsil characterized by infiltrates of lymphocytes and other nonepithelial cells. In some areas, non-epithelial cells were separated from the crypt lumen only by a thin cytoplasmic process (arrow). Flattened and angular cells formed pockets (triangles) that contained non-epithelial cells. H.E. **M.** Shown at higher magnification in the red box of Fig L. The pockets (triangles) contained non-epithelial cells, such as reticular cells (RC), lymphocytes (L), plasma cells (P), and dendritic cells (DC). H.E.

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7A-I). In addition, immunostaining was not observed in lymphoid follicles, interfollicular areas, the reticular crypt epithelium, or the subepithelial areas of the non-reticular epithelium of the corresponding control sections (Fig. 7J). The distribution densities of IgG ASCs significantly increased with the aging of yaks, with a peak in the adult groups and decrease in the old groups. A significant difference was found between the adult and other groups ($P<0.01$). There was no significant difference between the juvenile and old groups ($P>0.05$). The distribution densities of IgG ASCs in juveniles was significantly higher than that in the old and newborn groups. However, there was no significant difference between the newborn and old groups ($P>0.05$) (Fig. 5B). The distribution densities of IgG ASCs was significantly higher ($P<0.01$) than the distribution densities of IgG ASCs in all groups. The proportion of IgA and IgG ASCs was about 1:3.35 (Fig. 5A,B).

The expression of IgG protein in the four age groups

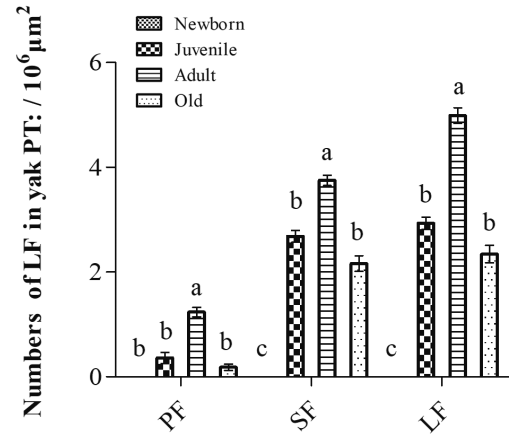


Fig. 3. Lymphoid follicles (LF) consisted of primary follicles (PF) and secondary follicles (SF). Different letters represent a significant difference ($P<0.05$), and the same letter represent a non-significant difference ($P>0.05$). PT: the palatine tonsil.

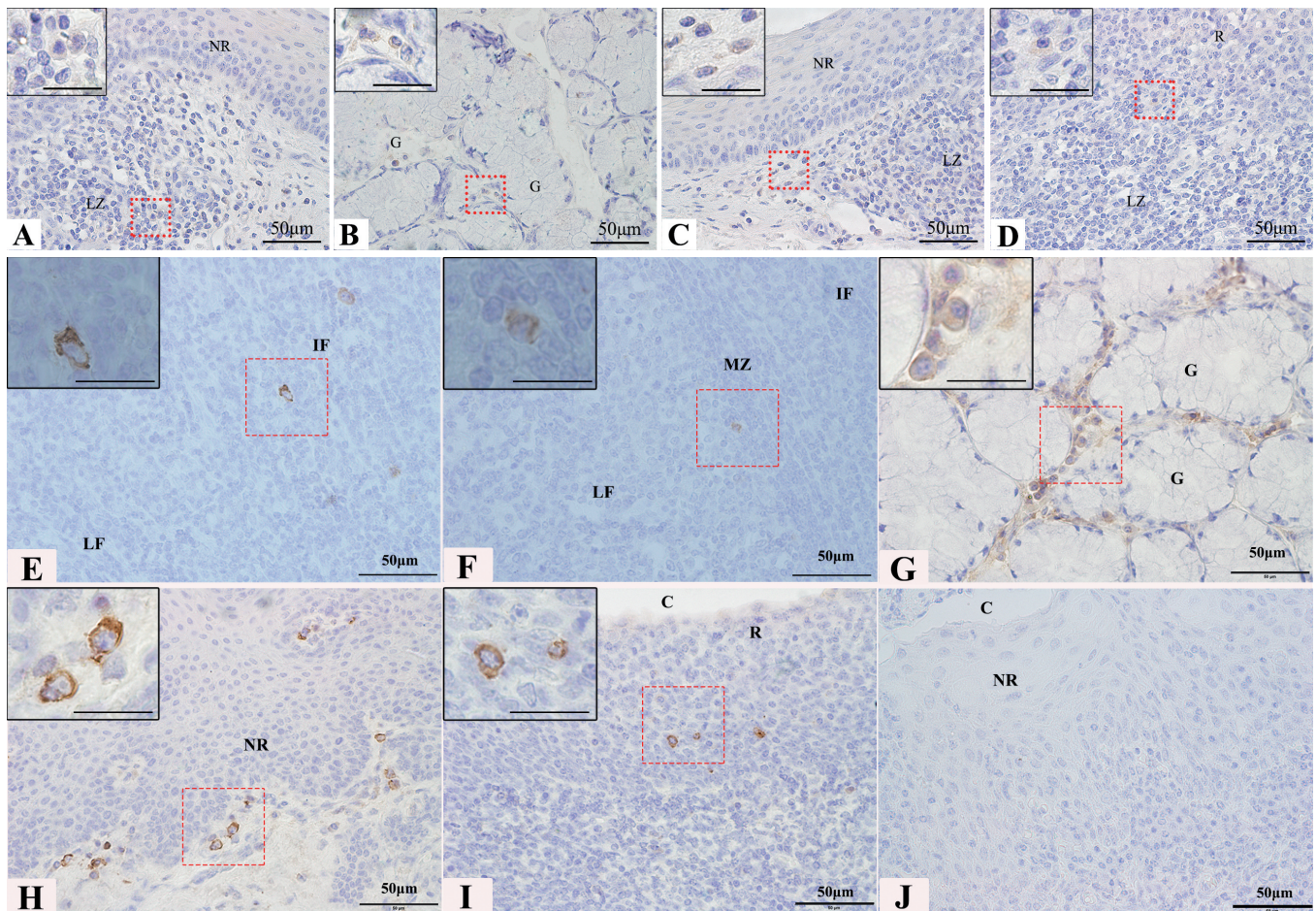


Fig. 4. Representative photomicrographs of palatine tonsil [PT] sections from healthy newborn (A-D) and adult (E-I) yaks that were stained for IgA. Notice that the IgA positive cells were located in lymphoid tissues [LZ] (A), interfollicular areas [IF] (E), lymphoid follicles [LF] (F), between glands [G] (B and G), the subepithelial areas of the non-reticular crypt epithelium [NR] (C and H), and the reticular crypt epithelium [R] (D and I). The outlined area is shown at higher magnification in the red box. Red box: The immunoreactivity was greatest in the cytoplasmic membrane of plasma cells [P]. Control for IgA expression in the subepithelial areas of the non-reticular crypt epithelium [NR] (J) in yaks.

was determined by ELISA, and the statistical analysis of the results demonstrated that the expression of IgG also significantly increased with aging in yaks with a peak in the adult groups and decrease in the old groups. A very significant difference was found between the adult and other groups ($P < 0.01$). The expression of IgG protein in juveniles was significantly higher than that in the old and newborn groups. However, there was no significant difference between the newborn and old groups ($P > 0.05$) (Fig. 6B). The expression of IgG protein was significantly higher ($P < 0.01$) than the expression of IgA protein in all groups (Fig. 6A-B).

Discussion

Anatomical structures of the palatine tonsils in yak were partially similar to those of cows and buffalo

(Palmer et al., 2009; Zidan and Pabst, 2011). However, they were different from the palatine tonsils of camels (Zidan and Pabst, 2009; Jia et al., 2017). The paired palatine tonsils of yaks were formed by two central invaginations resulting in elongated kidney-shaped structures in the tonsillar sinus. A number of external crypts extended into the tonsils, while a number of internal crypts originated from the tonsillar sinus and were arranged in its surrounding wall. The unique arrangement of the external and internal crypts was similar to that of the buffalo (Zidan and Pabst, 2011) but had not been recorded in other species (Lieblertorio and Pabst, 2006; Casteleyn et al., 2011). Tonsil sinus and crypts were the necessary structures leading to superficial lymphoid tissue. These structures greatly increased the efficiency of the palatine tonsils by expanding the epithelial surface area exposed to the

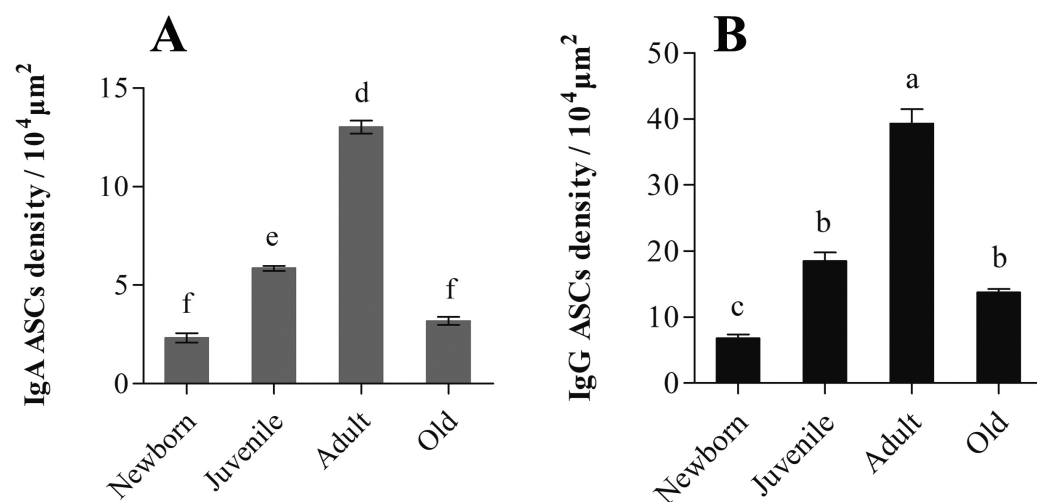


Fig. 5. The distribution densities of IgA (A) and IgG (B) ASCs in the yak PT. Different letters represent a very significant difference ($P < 0.01$), and the same letter represents a non-significant difference ($P > 0.05$).

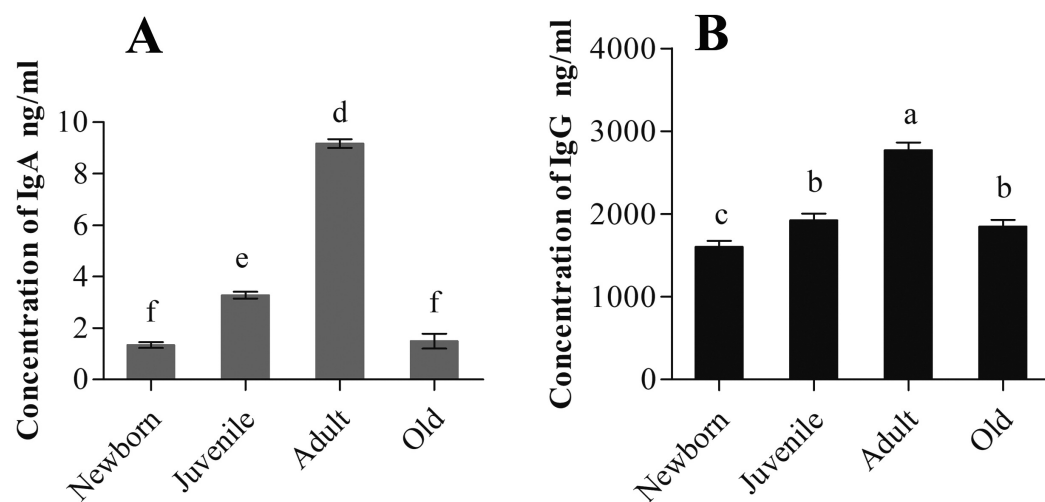


Fig. 6. IgA (A) and IgG (B) protein expression in the yak PT were measured by ELISA. Different letters represent a very significant difference ($P < 0.01$), and the same letter represents a non-significant difference ($P > 0.05$).

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antigen (Casteleyn et al., 2008; Palmer et al., 2009). Tonsillar surface area, which was important for the exposure of the immune system to foreign material, was directly related to the number of crypts (Casteleyn et al., 2007). The crypts played an important role in the immune response by trapping antigens (Nave et al., 2001). Compared to the internal crypt, the exposure of the external crypts to antigen would be direct and would last for a shorter period of time. Palatine tonsils of the yak can be classified as tonsils with crypts, as seen in other ruminants, viz. cattle and ovine (Manesse et al., 1998; Casteleyn et al., 2007, 2008, 2010; Palmer et al., 2009; Zidan and Pabst, 2011), human (Nave et al., 2001; Jović et al., 2015), horses (Kumar and Timoney, 2005) and swine (Liu et al., 2012) but not in carnivores (Belz and Heath, 1995), which lack the crypts.

Palatine tonsils were not only lymphoepithelial structures but also typical secondary lymphoid organs

(Pabst, 2007), which had no afferent lymphatics; antigens entered by their surface structures (Nave et al., 2001; Brandtzaeg, 2003). Crypt epithelium of yaks was formed by stratified squamous epithelium (NR) and reticular epithelium (R) or the lymphoepithelium (LE) with patches. This arrangement might be associated with antigenic stimulation, where the less strongly stimulated area might still retain as stratified squamous non-keratinized epithelium. Reticular epithelium was a modified epithelium in which lymphocytes and other mononuclear cells transformed the squamous epithelium into a reticular network of intercellular passageways (Perry, 1994). This lymphoepithelial barrier sampled and translocated antigens to the underlying lymphoid tissue (Perry and Whyte, 1998). Several previous studies recorded that the lymphoepithelial facilitated the uptake of prions responsible for BSE (Bernstein et al., 2005; Bellworthy et al., 2005). This might have important

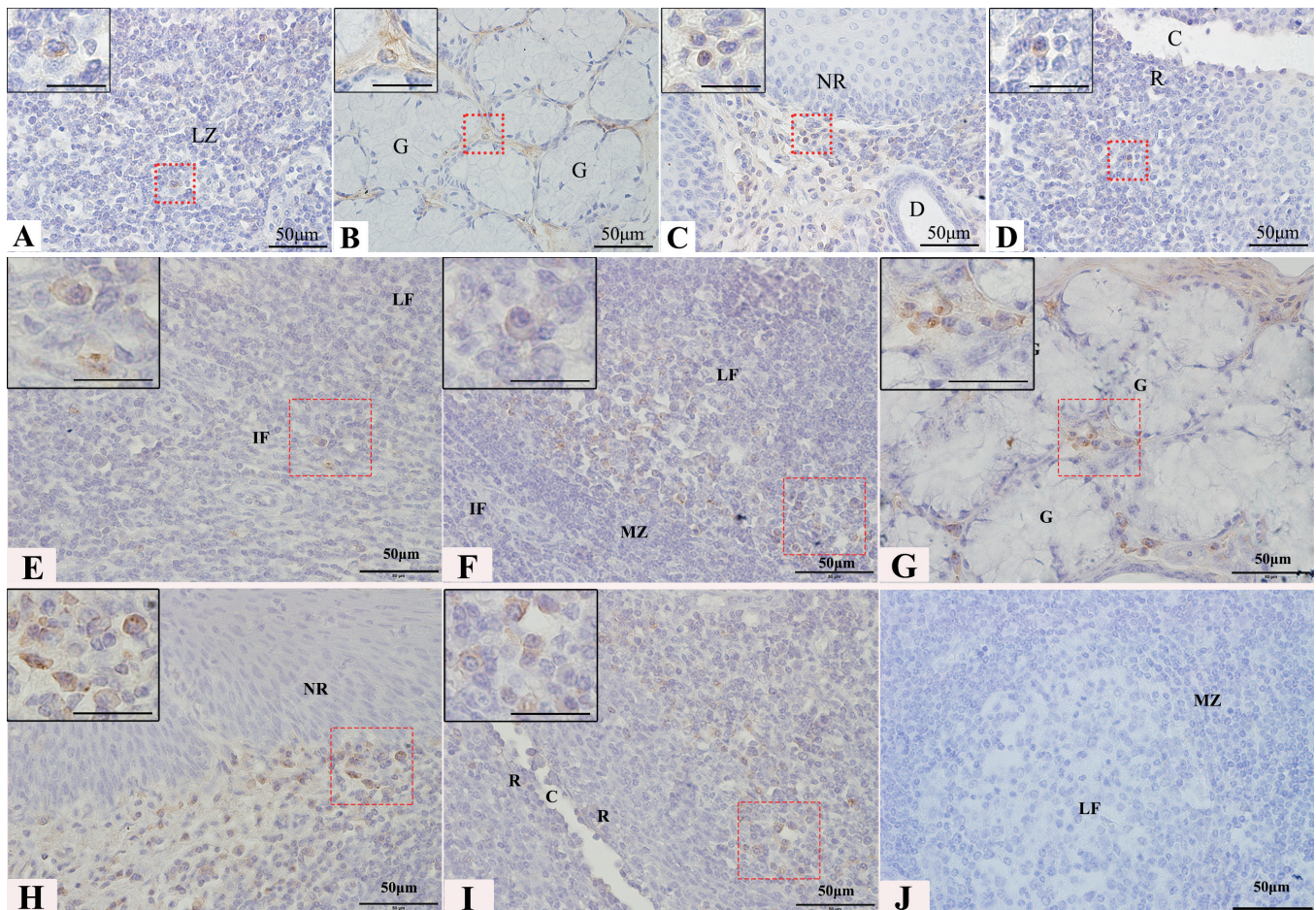


Fig. 7. Representative photomicrographs of palatine tonsil sections from healthy newborn (A-D) and adult (E-I) yaks that were stained for IgG. Notice that the IgA positive cells were located in lymphoid tissues [LZ] (A), interfollicular areas [IF] (E), lymphoid follicles [LF] (F), between glands [G] (B and G), the subepithelial areas of the non-reticular crypt epithelium [NR] (C and H), and the reticular crypt epithelium [R] (D and I). The outlined area is shown at higher magnification in the red box. Red box: The immunoreactivity was greatest in the cytoplasmic membrane of plasma cells [P]. Control for IgA expression in the subepithelial areas of the lymphoid follicles [LF] (J) in yaks.

clinical significance, as the prions can be detected in tonsils one year before the expected onset of clinical prion diseases (Roels et al., 1999; Thuring et al., 2005).

Parenchyma of the palatine tonsils in yak was mainly composed of tonsillar follicles that were formed by the lymphoid follicles and interfollicular areas surrounding the crypt. These were similar to the palatine tonsils of humans, bovines, ovine, camels and other domestic ruminants (Manesse et al., 1998; Nave et al., 2001; Kumar and Timoney, 2005; Casteleyn et al., 2007, 2008, 2010, 2011; Zidan and Pabst, 2011; Jović et al., 2015; Jia et al., 2017). In agreement with Kumar and Timoney (2005), the results of the present study indicated that the parenchyma of palatine tonsils in newborn yak was formed by diffuse lymphoid tissues as the lymphoid follicles were yet to form. The other three age groups of palatine tonsils had the primary and secondary lymphoid follicles. The secondary lymphoid follicles had well-developed germinal centers and a darkly stained mantle zone facing the crypt epithelium. The clear germinal centers indicated that the palatine tonsils in yak played an important role in immune responses. Interestingly, the primary and secondary lymphoid follicles of the palatine tonsils increased with aging in the yaks with a peak in the adult groups and decrease in the old groups. Additionally, consistent with previous studies, there were a number of HEVs distributed in both the interfollicular regions and underlying the reticular epithelium. Few migrating lymphocytes were also observed in the lining endothelium. HEVs were specialized vessels that supported active lymphocyte transmigration from the peripheral blood to the secondary lymphoid organs (Indrasingh et al., 2002), communicating between local systemic and mucosal adaptive immunity.

Palatine tonsils were located at the entrance to the digestive and respiratory tracts, forming a first line of defense against foreign antigens (Nave et al., 2001; Wilson et al., 2005). The results of the present study assumed it as a source of crucial immunologic effector and barrier cells, with the distribution characteristics of IgA and IgG ASCs being the same in the palatine tonsils of yaks at different ages. The two types of ASCs were mainly clustered or scattered in the subepithelial areas of the crypt epithelium, occasionally distributed in the interfollicular areas. Positive cells were also found in the edge of the lymphoid follicles, with a few aggregated around the gland. However, there was no distribution underlying the outer mucosal epithelium. Because the epithelium was thick, it was not easy for the pathogens and antigens to be transferred through the outer mucosal epithelium for antigen capture and response. Distribution characteristics of IgA and IgG ASCs suggested that the palatine tonsils had characteristics of effector sites and were important for maintaining the stability of the palatine tonsils immunological functions. In addition, ELISA showed that the expression of IgG ASCs was significantly higher than that of IgA ASCs in the same site. These results were consistent with the distribution

of two ASCs in the palatine tonsils of pigs (Horter et al., 2003; Li et al., 2010), horses (Kumar and Timoney, 2005) and human (Hoefakker et al., 1993; Boyaka et al., 2000; Nave et al., 2001) but were significantly different from that in Bactrian camels (Jia et al., 2017). Furthermore, as a secondary lymphoid organ, the palatine tonsils produced a large number of IgG ASCs and a few IgA ASCs to promote mucosal immunity. IgG could be a significant component of the mucosal immune response in the palatine tonsils of yaks. In the present study, the populations of two ASCs gradually increased from newborn to adult, reached a peak at adult age, thereafter decreasing with age.

Conclusion

In conclusion, the palatine tonsils in yaks are an important part of Waldeyer's ring, which are not only typical secondary lymphoid organs, but also an important part of the mucosa-associated lymphoid tissue (MALT). In newborn yaks, the lymphoid follicles were not observed. In other groups, the lymphoid follicles consisted of primary lymphoid follicles and secondary lymphoid follicles. Both IgA and IgG ASCs were distributed in the interfollicular areas, lymphoid follicles, the subepithelial areas of the non-reticular crypt epithelium, and the reticular crypt epithelium, with a few positive cells aggregated around the gland. The density of the two ASCs and the expression of the two proteins gradually increased from newborn to adult and reached a peak at adult age; they then decreased with age. However, the density of IgG ASCs and the expression of IgG protein was significantly higher than that of IgA in all groups. IgG could be a significant component of mucosal immune responses in the palatine tonsils of yaks. This result was consistent with the number of lymphoid follicles in different ages of palatine tonsils in yak.

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Conflict of interest. The authors declare that there is no conflict of interest.

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