

Original Research

New Insights into the Splenic Stroma of the Barki Sheep: A Histological, Immunohistochemical, Ultrastructural and Morphometrical Study

Omnya Elhussieny^{1*}, Mohamed Abdelaziz², Eman Khalifa³

¹ Department of Histology and Cytology, Faculty of Veterinary Medicine, Matrouh University, Fuka, 51744, Matrouh, Egypt

¹ Department of Histology and Cytology, Faculty of Veterinary Medicine, Alexandria University, 21944, Alexandria, Egypt.

¹ Department of Microbiology, Faculty of Veterinary Medicine, Matrouh University, Fuka, 51744, Matrouh, Egypt.

Correspondence*

Omnya Elhussieny, Department of Histology and Cytology, Faculty of Veterinary Medicine, Matrouh University, Fuka, 51744, Matrouh, Egypt.

Email omnya.elhussieny@mau.edu.eg

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ABSTRACT

Introduction: Spleen is the largest secondary lymphatic organ in the body designed to respond immunologically to antigens circulating in the blood. **Aim:** describe the histoarchitectural features and morphometrical characters of the stroma of the Barki sheep's spleen. **Materials and Methods:** the splenic stroma of 30 clinically healthy Barki sheep was studied using different histological, immunohistochemical, ultrastructural, and morphometrical techniques. **Results:** A prominent layer of smooth muscles with few sporadic collagen fibers form the capsule which was 676.29 μm and has a subcapsular sinus. Meanwhile, the trabeculae which were composed mainly of smooth muscle cells originate from the capsule into the splenic parenchyma branch into primary (517.095 μm thick), secondary (470.609 μm thick), and tertiary trabeculae (382.27 μm thick). The subcapsular blood sinuses and the peritrabecular blood sinuses all are connected and are engorged with blood cells mainly CD3+ T lymphocytes. **Conclusion:** the high amount of smooth muscles and very thick stroma is unique to Barki sheep's spleen denoting an efficient storage function beside the defensive function. CD3 T lymphocytes in subcapsular and peritrabecular sinuses are to form a link between primed T lymphocytes and the underlying stromal elements, creating microenvironments suitable for effector responses.

Keywords: : Barki sheep, intermediate spleen, capsule, trabeculae, smooth muscles

INTRODUCTION

The spleen is the secondary lymphoid organ where antibodies and active lymphocytes are produced and conveyed directly to the blood (Mescher, 2018). Unlike lymph nodes, the spleen lacks afferent lymphatic vessels so all cells and antigens enter the spleen via the blood (Lewis et al., 2019). The spleen is surrounded by a thick capsule of connective tissue invested by the peritoneum. The capsule is composed of two layers: a layer of dense irregular connective tissue and a layer of smooth muscle. The total thickness and relative amount of smooth muscle vary with the species. The Trabeculae extend from the capsule and the hilus into the parenchyma (Eurell and Frappier, 2013). The spleen of both sheep and goats consists of two surfaces: the parietal and visceral surface. The parietal surface is convex while the visceral surface is concave with hilus at its dorsal end of the cranial border. The spleen of sheep is triangular while it is roughly quadrangular in outlines with blunt edges

in the case of goats (Gnanadevi et al., 2019). The stroma of the spleen was described in dromedary camel (Zidan et al., 2000) horses, cows, carnivores, pigs (Bacha Jr and Bacha, 2012), other types of sheep and goats (Gnanadevi et al., 2019), and the water buffalo (Rashad et al., 2020) but not in the Barki sheep. So, the present study aimed to describe the histoarchitectural features and age-related morphometrical characters of the stroma of, intermediate type of spleen, the Barki sheep spleen.

MATERIALS AND METHODS

Samples: The present study was done on 30 clinically healthy Barki sheep slaughtered for human consumption according to the rules of the Egyptian Veterinary Authorities in the abattoir of Marsa Matrouh, Matrouh, Egypt with an age range from 6 months to 5 years. The animals were divided into 3 groups, each group contain 10 animals: Group A: 6-9

months' Barki sheep, Group B: 1.5-2 years Barki sheep, Group C: 3-5 years Barki sheep. Fresh tissue samples were obtained from peripheries, centers, and hilus of the spleen of each animal. The samples were prepared for histological, immunohistochemical, ultrastructural, and morphometrical examination as follow: **Histological study:** The tissue samples were fixed in 10% phosphate-buffered formaldehyde (Rhodes 2013). The fixed specimens were processed for paraffin sectioning. Serial sections (5µm) were prepared as outlined by Spencer & Bancroft (2013) and stained using the following stains: 1. Mayer's hematoxylin and eosin (H&E) stain for general studies (Mayer 1903). 2. Gomori's trichrome staining protocol for connective tissue (Gomori 1950). **Immunohistochemical study:** Deparaffinized sections were obtained on positive slides for immunohistochemical staining then hydrated in descending grades series of alcohol solutions. Sections were incubated in antigen retrieval (boiling the sections at 98°C for 20 minutes in 10 mmol/L sodium citrate buffer), treated with 3% H₂O₂ to block endogenous peroxidase. Monoclonal antibody for mouse anti-human CD3 was applied on the slides and incubated in the humid chamber overnight in the refrigerator at 4°C (Dabbs, 2017). The human CD3 antibodies are cross-reacted with T cells of sheep, cattle, goats, rats, and mice (Ramos-Vara et al., 1994). **Transmission electron microscopy (TEM):** Fresh tissue samples, about 1mm³ in size, were obtained from different parts of spleens and were immediately fixed in 3% phosphate-buffered glutaraldehyde at pH 7.2 and stored at 4°C. The specimens were processed in the electron microscope unit, Faculty of Science, Alexandria University. After fixation, the tissues were washed in several changes of cold (4°C) 0.1 M phosphate buffer every 15 minutes for 2 hours. Then the tissues were post-fixed in a 1% solution of phosphate-buffered osmium tetroxide (2% osmic acid 5 ml and phosphate buffer 5 ml) for 2 hours at room temperature. Then they were rapidly dehydrated through ascending grades of ethyl alcohol series (30, 50, 70, 90, and 100% for 2 changes) for 30 minutes in each. Then transferred to propylene oxide and placed overnight in a 1:1 mixture of propylene and epoxy araldite. Then they were embedded in epoxy araldite (Hayat 1986). Polymerization of embedding mixture and the tissue blocks were done in an oven for 5 days as follows: at 35°C for 24 hours, at 45°C for 24 hours, and lastly at 60°C for 3 days. Semithin sections (1µm) were cut firstly and stained with toluidine blue and examined with a light microscope to select the suitable areas for the electron microscopic examination. Then the ultrathin sections (60 - 100 nm) were cut with a glass knife on LBK ultramicrotome, then they were stained with uranyl acetate followed by lead citrate (Hayat 1986). The sections were examined with transmission electron microscope JEM-1400 PLUS present in electron microscope unit, Faculty of science, Alexandria University. **Morphometrical Study:** Morphometric measurements of the thickness of the capsule and trabeculae (primary, secondary and tertiary) were done on H and E sections of five animals from each age group. 3

microscopic fields were chosen randomly from each section of each animal and analyzed using Image J software (NIH) (Alsanosy et al., 2021). The thickness of the capsule and trabeculae is compared at different ages.

RESULTS

The spleen of the Barki sheep is located against the left abdominal wall and is usually covered by the ribs. It is triangular in shape and reddish-brown in color in the fresh state. It has two surfaces; parietal and visceral surface. The parietal surface is convex while the visceral surface is concave containing the hilus which is present close to the dorsal end of the cranial border of the visceral surface (Fig: 1). It is covered with the parietal and visceral layers of the peritoneum (Fig: 2). The spleen of Barki sheep has a reddish-brown color on the cut surface also (Fig: 3). The stroma of the spleen of the Barki sheep is composed of capsule and trabeculae. The capsule of the spleen of the Barki sheep is composed of a prominent layer of smooth muscles and a few sporadic collagen fibers and has a subcapsular sinus which is engorged with blood cells mainly lymphocytes and erythrocytes. These lymphocytes are CD3+ T cells (Figs. 4-8). The Barki sheep have a thick capsule and its thickness ranges from 608.428 to 776.004 µm (Table 1 and diagram 1). The trabeculae originate from the capsule (Fig. 4) into the splenic parenchyma branch into primary, secondary, and tertiary trabeculae and all are composed mainly of smooth muscle cells with very few and sporadic collagen fibers. The trabeculae (primary, secondary, and tertiary) are surrounded by peritrabecular Most trabeculae studied in the spleen of the Barki sheep were avascular trabeculae, we do not find vascular trabeculae in the spleen of the Barki sheep. The subcapsular blood sinuses and the peritrabecular blood sinuses all are connected and continuous with each other (Figs. 9-13). Regarding the fine structure of longitudinal and cross-sections of the smooth muscles present in the trabeculae, we found myofilaments and dense bodies which are unique to smooth muscles (Figs. 14-15). The peritrabecular sinuses are engorged with blood cells mainly CD3+ T lymphocytes (Fig. 16). The thickness of the splenic primary, secondary and tertiary trabeculae are described in tables and diagrams (2-4).

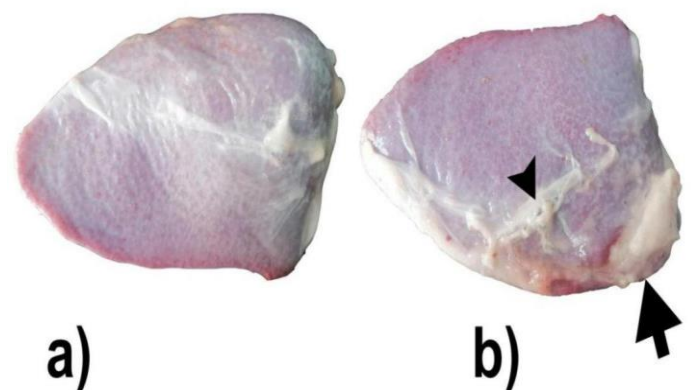


Fig. (1): A photograph of the spleen of Barki sheep in the fresh state showing that the spleen is triangular in shape,

reddish-brown in color and has two surfaces; parietal surface (a) and visceral surface (b) containing the hilus (arrow). Splenic artery (arrowhead) (arrowhead).

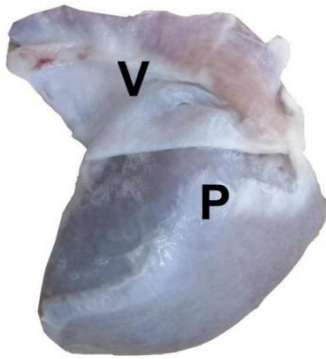


Fig. (2): A photograph of the spleen of Barki sheep showing that the spleen is covered with the parietal (P) and visceral (V) layers of the peritoneum.



Fig. (3): A cut surface of the spleen of Barki sheep showing the splenic artery (arrow) and its branches. The splenic corpuscles (arrowhead) can be seen on the cut surface but the red pulp and white pulp do not have the red and white colors. The spleen of Barki sheep has a reddish-brown color on the cut surface also.

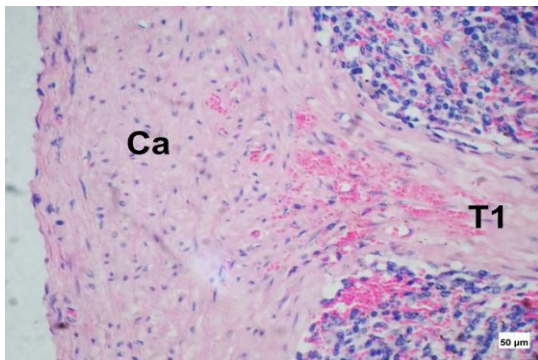


Fig. (4): A photomicrograph of the spleen of the Barki sheep showing the capsule (Ca) and primary trabeculae (T1) extended from it. H&E stain. Bar = 50 μm.

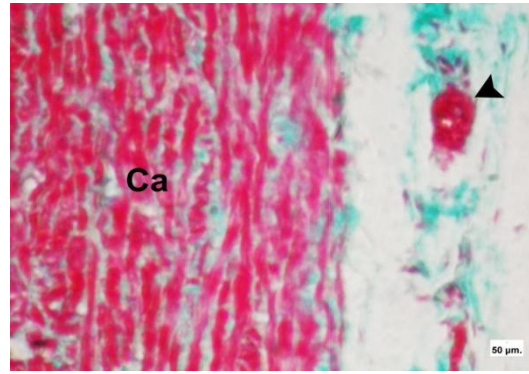


Fig. (5): A photomicrograph of the spleen of the Barki sheep showing that its capsule (Ca) consists mainly of smooth muscle cells (red color) and a few sporadic collagen fibers (green color). Arrowheads= capsular blood vessels. Gomori trichrome stain. Bar = 50 μm.

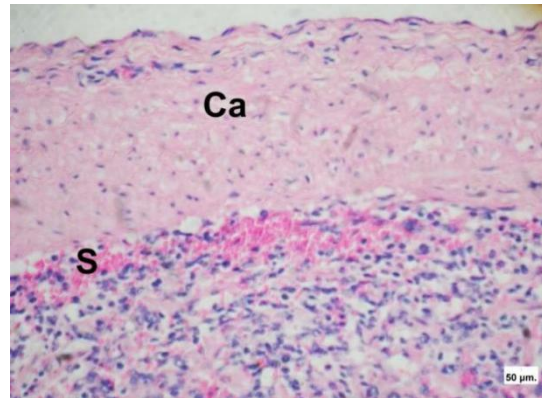


Fig. (6): A photomicrograph of the splenic capsule (Ca) of the Barki sheep denoting that the subcapsular sinus (S) is engorged with blood cells mainly lymphocytes and erythrocytes. H&E stain. Bar = 50 μm.

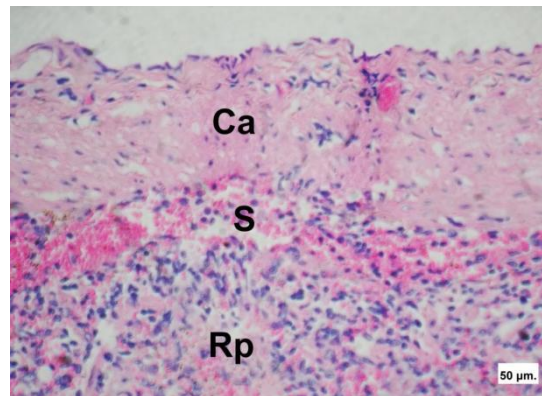
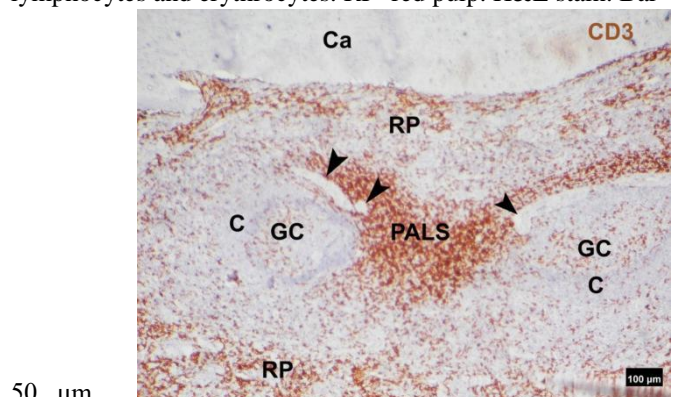


Fig. (7): A continuation of the previous photomicrograph showing that the splenic capsule (Ca) of the Barki sheep containing subcapsular sinus (S) engorged with blood cells mainly lymphocytes and erythrocytes. RP=red pulp. H&E stain. Bar= 50 μm.



50 μm.

Fig. (8): Tissue section of the spleen of the Barki sheep showing that CD3+ T lymphocytes are distributed under the capsule (Ca) in the subcapsular sinus, sparsely present in the germinal center (GC) and corona (C) of the secondary lymphoid nodule while heavily distributed around the central artery (Arrowheads) forming the periarterial lymphatic sheath (PALS). RP =Red pulp. Bar= 100µm.

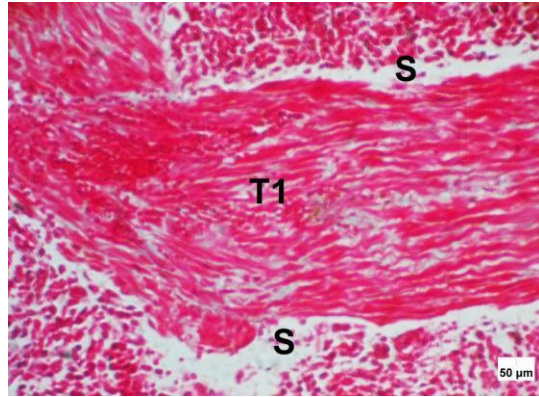


Fig. (9): A photomicrograph of the splenic primary trabeculae (T1) of the Barki sheep showing that they consist mainly of smooth muscle cells (red color) with very few sporadic collagen fibers (green color). S= peritrabecular sinus. Gomori trichrome stain. Bar = 50µm.

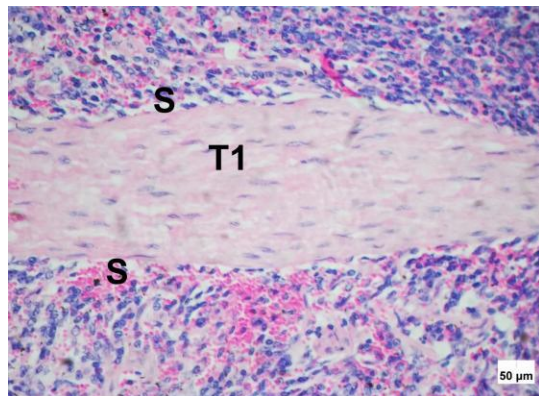


Fig. (10): A photomicrograph of the spleen of the Barki sheep showing that the primary trabeculae (T1) have a peritrabecular sinus (S) engorged with blood cells. H&E stain. Bar = 50 µm.

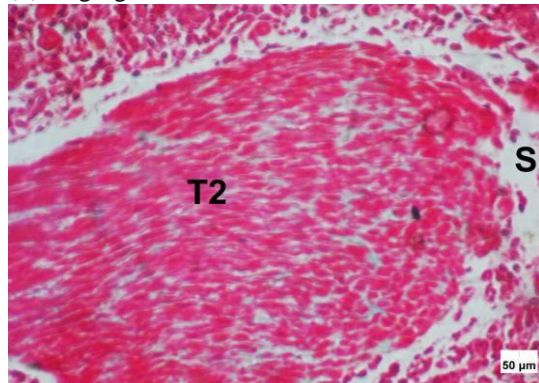


Fig. (11): A photomicrograph of the secondary trabeculae (T2) of the spleen of the Barki sheep denoting that they consist mainly of smooth muscle cells (red color) with few sporadic collagen fibers (green color). S= peritrabecular sinus. Gomori trichrome stain. Bar= 50 µm.

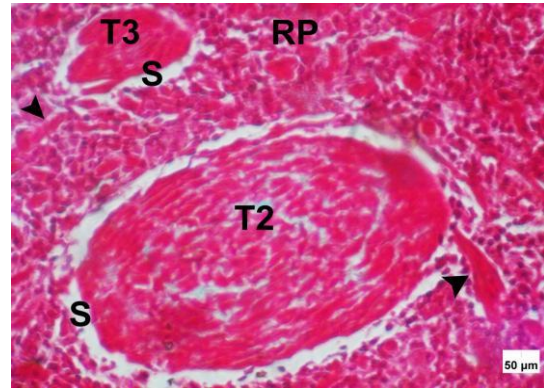


Fig. (12): A photomicrograph of the spleen of the Barki sheep showing that the secondary (T2) and tertiary trabeculae (T3) consist mainly of smooth muscle cells (red color) and few sporadic collagen fibers (green color). The trabeculae have a peritrabecular sinus (S). The red pulp contains smooth muscle cells (arrowheads). Gomori trichrome stain. Bar = 50 µm.

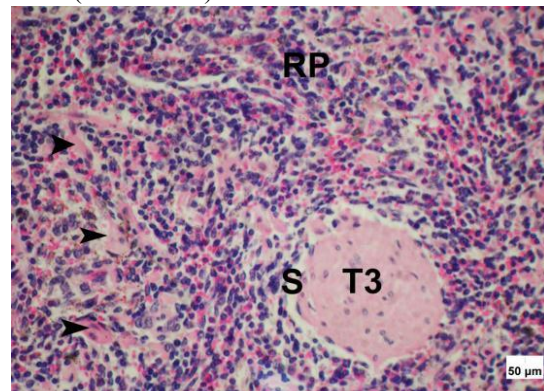


Fig. (13): A photomicrograph of the Barki sheep splenic tertiary trabeculae (T3) surrounded by peritrabecular sinus (S) and the red pulp (RP) containing many smooth muscle cells (arrowheads). H&E stain. Bar= 50 µm.

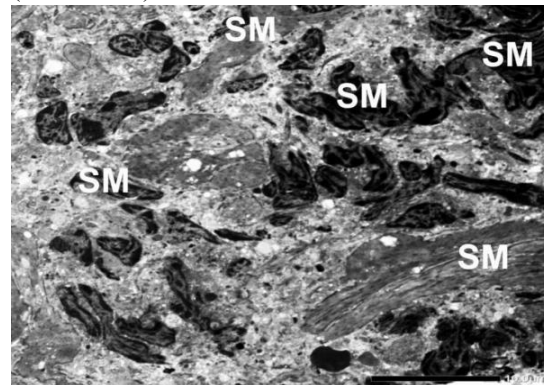


Fig. (14): Transmission Electron microscopic micrograph of the trabecula of the spleen of Barki sheep denoting mainly smooth muscles (SM) rich in myofilaments occupying the center and periphery of the trabecula. Bar= 10 µm.

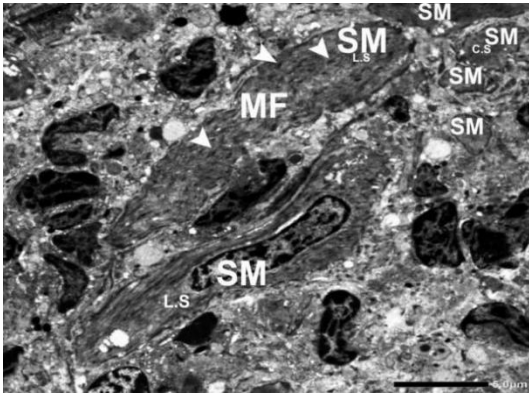


Fig. (15):

A higher magnification of the previous electron micrograph denoting longitudinal (L.S) and cross (C.S) sections of the smooth muscles (SM) which contain dense bodies (arrowheads) and myofilaments (MF) which are unique to smooth muscles. Bar = 0.5µm.

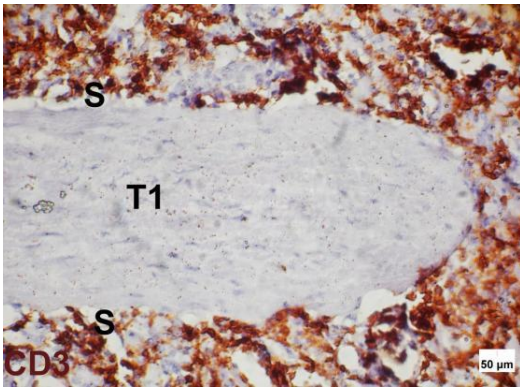


Fig. (16): A

photomicrograph of the Barki sheep spleen depicting CD3+ T lymphocytes in the peritrabecular sinus (S) surrounding the primary trabecula (T1). Bar = 50 µm. **Table 1: Thickness of the splenic Capsule of the Barki Sheep (µm)**

Mean 776.004 644.428 608.428 SD (standard deviation) 87.649 173.174 157.079 Min 660 330.055 408 Max 948 996 840 Mean of all ages 676.29 **Table 2: Thickness of the splenic primary trabeculae of the Barki sheep (µm)**

Mean 430.405 614.403 506.476 SD (standard deviation) 72.693 166.215 236.539 Min 282 330 265.767 Max 516 828 924 Mean of all ages 517.095 **Table 3: Thickness of the splenic secondary trabeculae of the Barki sheep (µm)**

Mean 517.2 432.803 461.823 SD (standard deviation) 109.635 118.595 109.455 Min 306 264 255.265 Max 720 666 720 Mean of all ages 470.609 **Table 4: Thickness of the splenic tertiary trabeculae of the Barki sheep (µm)**

Mean 370.003 317.2 459.605 SD (standard deviation) 109.246 100.345 232.77 Min 180 150 156 Max 552 468 906 Mean of all ages 382.27

Diagram (1): illustrating thickness of the splenic capsule of the Barki sheep (µm)

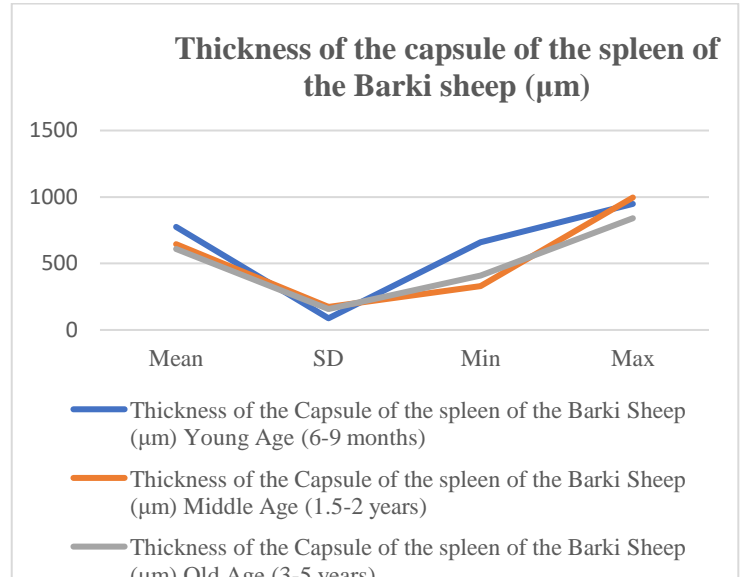


Diagram (2): illustrating the thickness of the splenic primary trabeculae of the Barki sheep (µm)

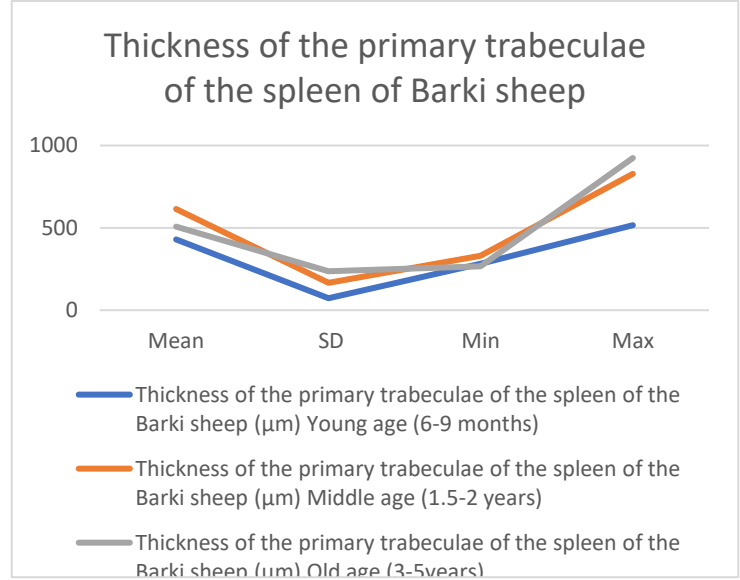


Diagram (3): illustrating the thickness of the splenic secondary trabeculae of the Barki sheep (µm)

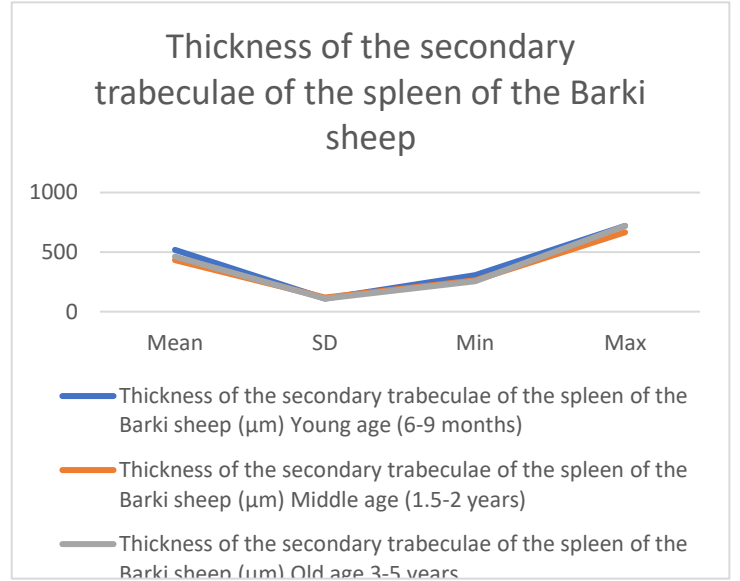
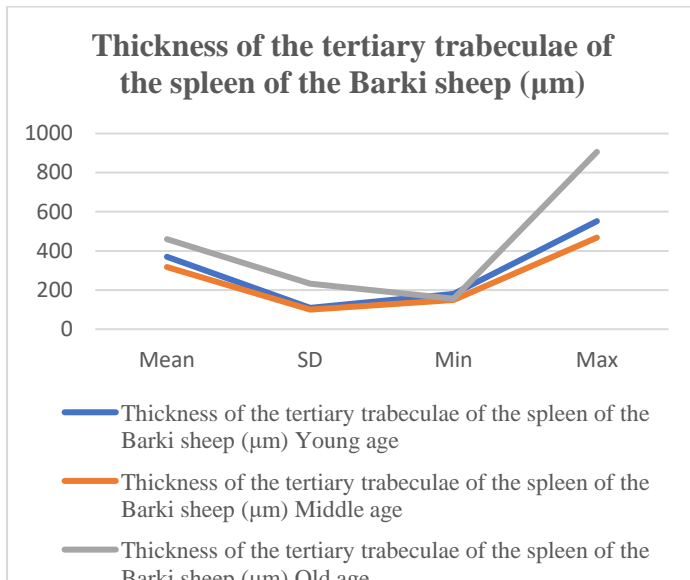


Diagram (4): illustrating the thickness of the splenic tertiary trabeculae of the Barki sheep (µm)



DISCUSSION

The present study described the normal histological structure of the splenic stroma of the Barki sheep, using different histological, immunohistochemical, ultrastructural, and morphometrical studies. We found that the spleen of the Barki sheep was located against the left abdominal wall and usually covered by the ribs. Thus, the Barki sheep spleen is similar to other domestic animals in located against the left abdominal wall and usually covered by the ribs except for the pig and carnivores and to a lesser degree the horse where the position of the spleen depending on the fullness of the stomach, for example, the spleen of the dog may be located caudally to the flank when the stomach is greatly distended (Nickel et al., 1979). The present work denotes the triangular shape of the Barki sheep spleen which is similar to other species of sheep, on the contrary, it is different from that of the ox which is a long oval, and the goat which is more rectangular in shape (Nickel et al., 1979). In the present study, the reddish-brown color of the fresh Barki sheep spleen, is similar to other species of sheep, although the goat spleen is reddish-gray in color. The color of the spleen of Barki sheep differs from that of the bovine spleen where the color of the bovine spleen varies according to the age and sex of the animal. In bovine calves, the spleen is reddish-brown to bluish-red while in bulls and steers the spleen is dark red or reddish-brown and is bluish-gray or gray in cows (Nickel et al., 1979). In the present work, the capsule of the spleen of the Barki sheep is composed mainly of a prominent layer of smooth muscles and a few sporadic collagen fibers and has a subcapsular sinus engorged with blood cells mainly CD3+ T lymphocytes. Thus, differ from other domestic animals where the capsule is composed of two layers: a layer of dense irregular connective tissue and a layer of smooth muscle. The total thickness and relative amount of smooth muscle vary with the species (Eurell and

Frapplier, 2013). In cows, two or three layers of muscle are oriented perpendicular to each other, while in carnivores and pigs the smooth muscle fibers are interwoven (Bacha Jr and Bacha, 2012). On the contrary, the spleens of other species of sheep and goats are covered by thick capsules mostly made up of collagen and smooth muscle fibers (Gnanadevi et al., 2019), meanwhile, the capsule of the spleen of water buffalo (*Bubalus bubalis*) is thick fibromuscular connective tissue (Rashad et al., 2020). As well as the dromedary camel has a thick capsule divided into an outer layer composed mainly of connective tissue with very few smooth muscles and an inner layer composed mainly of smooth muscles supported by connective tissue (Zidan et al., 2000). The splenic capsule of the Barki sheep (676.29 μm) was thicker than that of the dromedary camel (292 μm) (Zidan et al., 2000), *Capra hircus* goat (251.44 μm), *Bos indicus* cow (196.88 μm) (Alim et al., 2012) Iraqi sheep (140.5 \pm 13.712 μm) (Khalel, 2010), the goat (146.25 μm), the buffalo (145.50 μm), the human (108.00 μm), and the rabbit (33.33 μm) per (Rahman et al., 2016). The present work revealed that the trabeculae of the spleen of the Barki sheep were composed mainly of smooth muscle cells with very few sporadic collagen fibers. Regarding the fine structure of longitudinal and cross-sections of the smooth muscles. So, the trabeculae of the spleen of the Barki sheep was similar to the avascular trabeculae of the dromedary camel (Zidan et al., 2000) and the water buffalo (Rashad et al., 2020) which were composed mainly of smooth muscle cells and were different from the vascular trabeculae of the dromedary camel which composed mainly of connective tissue (Zidan et al., 2000). Most trabeculae studied in the spleen of the Barki sheep were avascular trabeculae, we do not find vascular trabeculae in the spleen of the Barki sheep. The trabeculae of the Barki were similar to trabeculae of other species of sheep (Gnanadevi et al., 2019). We studied the thickness of the trabeculae of the spleen of the Barki sheep (primary trabeculae: 517.095 μm ; secondary trabeculae: 470.095 μm ; tertiary trabeculae: 382.27 μm) which were different from that of the sheep 104.35 μm and goat 224.67 μm per Suri et al. (2017) also differ from that of sheep 134 μm and goat 105 μm per Gnanadevi et al. (2019). In species where the capsule and trabeculae rich in smooth muscles like dromedary camel (Zidan et al., 2000), equine and feline (Tablin et al., 2002), sheep and goats, (Gnanadevi et al., 2019), cows (Bacha Jr and Bacha, 2012), and water buffalo (Gnanadevi et al., 2019) these spleens are termed "storage spleens" as the contraction of the capsule and trabeculae leads to delivery of large reserves of blood into the circulation according to the needs of the body, e.g. on extreme physical activity or in haemorrhagic conditions. On the contrary, the "defense spleen" that are present in humans, rabbits, dogs, and mice because of their less contractile ability and quite small erythrocyte reserves, however, holding a large number of platelets in ready reserve. The defense spleens have a greater immunological and antimicrobial capacity (Tablin et al., 2002). The present work denoted that, the trabeculae of the spleen of Barki

sheep have peritrabecular sinuses engorged with blood cells mainly CD3 lymphocytes. These subcapsular blood sinuses and the peritrabecular blood sinuses surrounding the primary, secondary, and tertiary trabeculae all are connected and continue with each other. In agreement with the results of **Gnanadevi et al. (2019)** who reported the subcapsular and peritrabecular blood sinuses presence in sheep and goats and the dromedary camel (**Zidan et al., 2000**), and the water buffalo (**Rashad et al., 2020**). We suggest that the peritrabecular and subcapsular sinuses play a great role in gathering the venous blood from the spleen to the splenic vein also migrating of primed T cells to the circulation. These came in accordance with the results of **Kim et al. (2007)** who propose that the function of CD3 T lymphocytes in peritrabecular sinuses is to form a link between primed T lymphocytes and the underlying stromal elements, creating microenvironments suitable for effector responses.

CONCLUSION

We concluded that the presence of a lot number of smooth muscles and the very thick capsule and trabeculae is unique to Barki sheep splenic stroma denoting an efficient storage function in addition to defensive function. The function of CD3 T lymphocytes in subcapsular and peritrabecular sinuses is to form a link between primed T lymphocytes and the underlying stromal elements, creating microenvironments suitable for effector responses.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this report.

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