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Research article

Early-life development of spleen in white rabbit (*Oryctolagus cuniculus*): A morphometric and histochemical analysis

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Abstract

The spleen's significant immunological and protective role has long inspired researchers to explore its developmental characteristics. This study intended to understand the splenic morphological developmental changes during postnatal life. Samples obtained form 25 (*Oryctolagus cuniculus*) white rabbits, divided into five age groups in :1, 10, 15, 30, and 90 postnatal days. The samples were measured to determine the length, breadth, thickness, and weight. afterward, the samples sectioned and stained using hematoxylin and eosin (H&E) and Masson's trichrome stains (MTs). And for histochemical analysis, additional slides were stained with Periodic acid schiff (PAS) and Alcian blue (AB) 2.5 pH stains. The histological measurements included the thickness of the capsule and trabeculae, diameter of white pulp follicle, and central artery, and the ratio of white and red pulps. The macroscopic findings showed a significant increase in dimensional measurements. Histologically, the capsule and trabeculae exhibited thickening, and the diameter of the white pulp follicles and central arteries expanded. And the splenic sinuses enlarged with age progression. The histochemical evaluation revealed intense staining with PAS in the capsule, trabeculae, and walls of splenic arteries in first postnatal day group, with moderate staining affinity in the other groups. Furthermore, there was positive staining with AB in the white pulp follicles of the first postnatal day group compared to weaker staining affinity in the older age groups. This study demonstrated that the critical development period begins after birth and continues until 30 postnatal days. During this period, the development of the immune response triggers significant alterations in the spleen's structural, morphometric, and histochemical parameters.

Keywords: Histochemical, Morphometric analysis, Postnatal development, Rabbit, Spleen

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INTRODUCTION

Decades ago, studies widely acknowledged the importance of the spleen. Its physiological value derived from the hematopoietic and immunological roles the animal body, including the elimination of bloodborne pathogens, storage of blood cells and iron, and regulation of B and T cell activity (Lewis et al., 2019; Promket and Ruangwittayanusorn., 2021). Moreover, recent reports highlight its importance in lipid metabolism (Tarantino et al., 2011).

The prenatal development of the spleen begins when the mesenchymal cells accumulate around the dorsal mesogastrium to form the primordial spleen, the splenic stroma is formed by the differentiation of mesenchymal cells, while the hematopoietic components come from other vascular centers such as the aorta. As the stomach undergoes leftward rotation, the mesogastrium fold transforms into the gastrosplenic ligament, during this developmental stage, the lymphocytes migrate to the spleen from the bone marrow and thymus (Schoenwolf et al., 2020).

The development of the spleen in laboratory animals has been described by many authors. (Cesta., 2006; Seymour et al., 2006), found that the hematopoietic components of the spleen are first recognized in rats on day 17 after birth and in mice on day 15. The lymphocytes accumulate in the splenic parenchyma between the 2nd and 5th postnatal day, while B lymphocyte progenitors appear by day 5 in both rats and mice. The reports mentioned that immunological activity initiates around postnatal day 14 when animals come into contact with pathogens.

(Parker et al., 2015), found that the development of the spleen in rats continues up to the age of 35 postnatal days and they noted the absence of lymphoid follicles and periarterial lymphoid sheath in the spleen by 7 postnatal day. (Khalil et al., 2009), provided a description of morphological events in the spleens of domestic chickens following birth, (Quyen et al., 2024) also described splenic microstructure in adult pigs and mentioned that splenic parenchyma and lymphatic components effected by aging and diseases. The spleen of rabbits was studied during late postnatal life by (Rahmoun et al., 2019). However, critical early-life developmental stages remain unidentified. And their study lacked comprehensive coverage of microscopic details and histochemical changes associated with splenic growth.

Nevertheless, there is a consensus among most authors that understanding the morphological growth of laboratory animals holds potential experimental advantages for understanding the lymphatic organs and spleen ontogeny in humans and other animal species (Cesta., 2006; Seymour et al., 2006; Khalil et al., 2009; Parker et al., 2015; Rahmoun et al., 2019; Farghali et al.,2020).

While the majority of the studies concentrating on the fetal development of the spleen in laboratory animals, the macroscopic, microscopic, and histochemical developmental events of the spleen in rabbits' kittens during early postnatal life remain unknown. Accordingly, the current study aims to explain the anatomical, microscopic, and histochemical developmental attributes in the spleens of rabbits.

MATERIALS AND METHODS

Sample preparation and ethical approvement

Twenty-five disease-free (*Oryctolagus cuniculus*) white rabbits were utilized in this study. The animals were obtained from the animal farm of the Veterinary Medicine College at Mosul University – Iraq, during April 2023, regardless of gender. They were divided according to age into 5 different groups: one, ten, fifteen, thirty, and ninety postnatal days (PNDs), The animals were provided with optimal feeding and living conditions. The euthanasia process was performed using 150 mg/kg of pentobarbital (Nembutal®) administered through intraperitoneal injections in compliance with AVMA guidelines (Underwood and Anthony, 2020). The approval for the current study was granted by the Institutional Animal Care and

Use Committee (IACUC) of the college. The coelomic cavity was exposed, and the spleens were collected. The samples rinsed with normal saline to remove blood and tissue debris, then measured separately with a digital caliper (LOUISWARE, China) to determine the thickness, length, and breadth of the spleen. The samples were weighed using a compact balance (EK-EW, Australia), and all data were meticulously recorded. The samples were then kept for 72 hours in containers with 10% buffered formalin.

Histological processing

The spleen samples were washed, cut, and placed into serial graded containers contain 70%, 80%, 90%, and 100% concentrations of alcohol for dehydration. After dehydration, the samples were cleared for 30 minutes using xylene solvent. Afterward, the impregnation process was achieved using molten paraffin at a temperature of 58°C for a duration of 3 hours, later samples were cast into blocks. The blocks were then trimmed with a rotary microtome (BIOBASE BK-2218, China) to prepare 5-micrometer sections. The sections were stained with hematoxylin and eosin as well as Masson's trichome stains following Bancroft's staining method (Suvarna et al., 2019). Additional sections were stained with periodic acid Schiff (PAS) and Alcian blue (AB) 2.5pH stains, for acidic and neutral glycoprotein detection. lastly, the histological evaluation was conducted using a light microscope (Olympus-CX21, Japan).

Histological morphometry

The measurements were obtained from randomly selected sets of 5 slides in each age group. Furthermore, the examination was conducted in 10 microscopic fields, magnified under 100X power. The measurements were performed using image j software (developed by NIH, USA). The measured parameters included: splenic capsular thickness, trabecular thickness, diameter of central arteries, the number and diameter of white pulp follicles.

To analyze the proportions of red pulp to white pulps, a 17x23 pointed grid was implemented over the captured pictures from 5 different slides in each group. Using an enumeration method, the total of 391 points covering the area of white and red pulp was calculated, and the mean value was assessed in each group. The stromal connective tissue was excluded from the analysis (Figure 1) (Ungor et al., 2006).

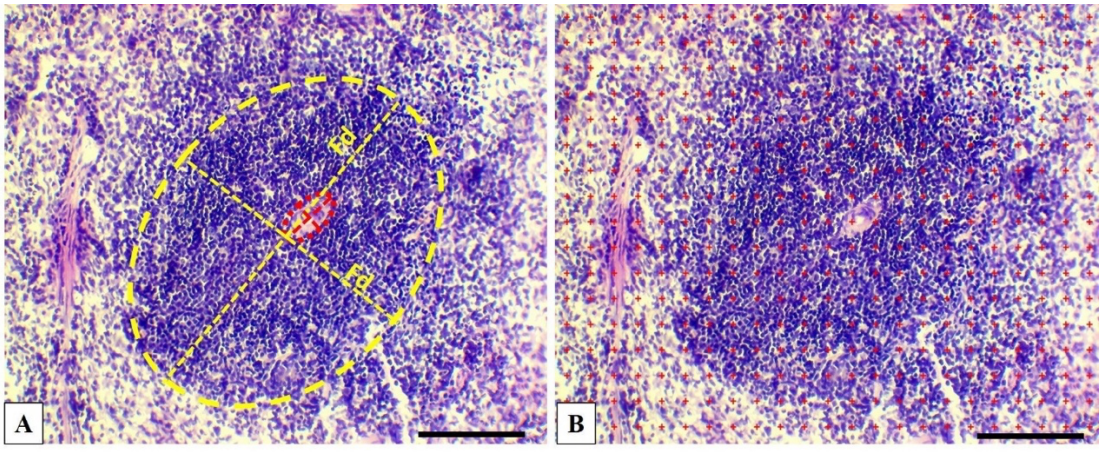

Figure 1 Photomicrograph illustrating the histological measurements executed on the sections of the spleen. (A) highlight the white pulp follicle's vertical and horizontal diameters (Fd) , and the central arteries diameter (red dashed circle). (B) highlight the application of a 17x23 pointed grid using ImageJ to determine the ratio of white and red pulps, (H&E, Scale bar =100 µm).

Statistical analysis

The percentages, means, and standard error, were computed from the macroscopic and microscopic data using (IBM SPSS V.25, UK) software. The data confirmed to be normally distributed according the Shapiro-Wilk test. The significant variations in the splenic parameters within groups were identified using the parametric one way analysis of variance (ANOVA) and the Duncan post hoc test, at a significance level of P≤0.05 (Petrie and Watson, 2013).

RESULTS

The anatomical observations

The visual inspection of the spleen in most animal groups revealed that the spleen appeared dark reddish and had an elongated sickle-like shape. The spleen consisted of two ends (ventral and dorsal), two surfaces (partial and visceral), and two borderlines (medial and lateral). The spleen positioned at the left side of the abdominal cavity, attached laterodorsally to the gastric greater curvature, the visceral aspect of the spleen connected with the stomach by the gastrosplenic ligament and surrounded by the adjacent viscera

The length of the spleen varied significantly among different age groups. In rabbits, it measured 11.21±0.39 mm on the first postnatal day and 33.81 ± 0.37 mm in the postnatal day 90 age group. Additionally, the breadth of the spleen showed significant differences among age groups, ranging from 3.57 ± 0.19 mm on the first postnatal day to 8.20 ± 0.15 mm in the postnatal day 90 age group. The spleen appeared compressed and thin, and the mean thickness increased progressively with advancing age. The majority of the gross measurements of the spleen showed a statistical difference at a significance level of p ≤ 0.05 (Table 1).

Table 1 The macroscopic spleen measurements in various postantal age groups

Animal groups	Spleen dimensional measurements				
	Length M ± SEM (mm.)	Breadth M ± SEM (mm.)	Thickness M ± SEM (mm.)	Weight M ± SEM (g.)	Relative weight M ± SEM (%)
Postnatal day 1	11.21 ± 0.39 a	3.57 ± 0.19 a	0.54 ± 0.04 a	0.087 ± 0.03 a	3.46±0.13 a
Postnatal day 10	18.39 ± 0.21 b	4.13 ± 0.14 b	1.63 ± 0.18 b	0.127 ± 0.19b	2.33±0.12 a
Postnatal day 15	23.3 ± 0.15 c	4.79 ± 0.17 c	1.85 ± 0.11 b	0.175 ± 0.15c	1.03±0.19 b
Postnatal day 30	26.41 ± 0.26 d	6.08 ± 0.11 d	2.83 ± 0.06 c	0.248 ± 0.11d	0.74±0.15 c
Postnatal day 90	33.81 ± 0.37 e	8.20 ± 0.15 e	3.09 ± 0.08 d	0.414 ± 0.22 e	0.06±0.20 d

Statistical differences are denoted by unique letters within each column among the animal groups at a significance level of $P \leq 0.05$

The histological observations

The microscopic examination of the spleen in most rabbit groups revealed that the spleen was enclosed in a bilayer capsule. The internal layer mainly consisted of a thin layer of smooth muscle fibers, while the external layer was primarily composed of collagen and elastic fibers arranged in a wavy pattern.

The composition and arrangement of the fibrous content varied among different animal age groups. In the first postnatal day group, the layer of collagen fibers was thin, with reticular fibers and fibroblasts being the predominant components (Fig. 2A). It was observed that the thickness of the fibrous layer increased steadily with advancing age. Furthermore, the muscular layer was absent in the first postnatal day group. However, the thickness increased with age progression and forming a multiple intersected layer, especially in the postnatal day 30 and 90 age groups (Figure 2B). In the older age groups, the muscular layer was intermingled with bundles of fibrous tissue and lymphocytes, providing the spleen

with flexibility for contraction and expansion. The histomorphometric data revealed a significant and rapid increase in the capsular thickness among the different age groups (Table 2).

The histological examination of the trabeculae revealed that they share similar microscopic components with the capsule, including the collagen, elastic and smooth muscle fibers. The trabeculae extend through the splenic parenchyma to provide pathways for blood vascularization within the splenic tissue. Notably, the length and number of trabeculae exhibited variations among age groups, both parameters increased with age advancement.

Moreover, the trabecular thickness varied among animals. In the first postnatal day age group, the trabeculae were thinner, and composed of minimal collagen and muscle fibers. while, the older age groups showed thicker fibrous content. On the other hand, the trabecular vessels were larger in the postnatal day 30 and 90 animal groups, compared to the younger age groups (Figure 2C,D). Furthermore, the trabecular vessels were surrounded by numerous fibroblasts, particularly in the 90 postnatal day rabbits. The trabecular thickness ranged from 3.27 ± 0.21 µm in the first postnatal day kittens to 35.93 ± 1.03 µm in the postnatal day 90 rabbits. The progressive increase in trabecular thickness was found to be significant among the different age groups (Table 2).

The observations of the spleen showed that the splenic parenchyma consists of two distinct areas, namely the white and red pulp, which were observed in most of the animal groups. The development of the white pulp initiated as small, disorganized aggregations of lymphocyte patches distributed throughout the splenic parenchyma in the first postnatal day animals, These patches appeared darkly stained with hematoxylin and eosin surrounding minute central arteries (Figure 3A)(Figure 5A).The number , size and diameter of the white pulp follicles increased progressively in older age groups The typical white pulp follicles were distinctly observed in postnatal day 15 kittens (Figure 3C). The size of the follicles further increased and became more circular in the postnatal day 30 and 90 age groups. Additionally, their density increased compared to the younger age groups. The diameter of the central arteries increased with age progression, and their wall thickened (Figure 3D,E) (Figure 4B) (Figure 5B).

The developed white pulp follicles in postnatal day 30 and 90 groups were composed from two distinct zones ;the mental zone ,located at the center of the follicle and mainly comprised from small lymphocytes and the marginal zone, located at the periphery between the white and red pulp and composed from large lymphocytes ,as well as macrophages and the reticular cells. The average diameter of the white pulp follicles showed significant variations among age groups ($p<0.5$) (Figure 4B) (Figure 5B) (Table 2).

On the other hand, the microscopic examination of the red pulp revealed that this area comprises two distinct elements: the splenic cords and the splenic sinuses. The splenic cords are organized from various types of cells, including reticular cells, macrophages, plasma cells and mast cells and the cellular density increased with age progression. In contrast, the splenic sinuses exhibited irregular narrow spaces in the first and 10 postnatal day groups. Furthermore, the size of the sinuses tended to grow and expand progressively with age advancement. the sinuses in the postnatal day 30 and 90 groups enlarged, dilated and interconnected with each other (Figure 6A, B).

Table 2 The microscopic spleen measurements in various postnatal age groups

Animal groups	Diameter of white pulp follicles M ± SEM (µm.)	Thickness of capsule M ± SEM (µm.)	Thickness of trabeculae M ± SEM (µm.)	Diameter of central artery M ± SEM (µm.)
Postnatal day 1	91.06 ± 1.87 a	6.13 ± 0.32 a	3.27 ± 0.21 a	10.63 ± 0.27 a
Postnatal day 10	241.3 ± 3.15 b	10.65 ± 0.27 b	7.82 ± 0.13 b	17.63 ± 0.31 b
Postnatal day 15	287.9 ± 2.11 c	19.07 ± 0.29 c	15.06 ± 0.43 c	22.21 ± 0.46 c
Postnatal day 30	355.2 ± 2.26 d	26.6 ± 0.43 d	32.95 ± 0.37 d	27.78 ± 0.34 d
Postnatal day 90	379.3 ± 2.25 e	33.17 ± 0.65 e	35.93 ± 1.03 e	53.24 ± 0.64 e

Statistical differences are denoted by unique letters within each column among the animal groups at a significance level of $P \leq 0.05$

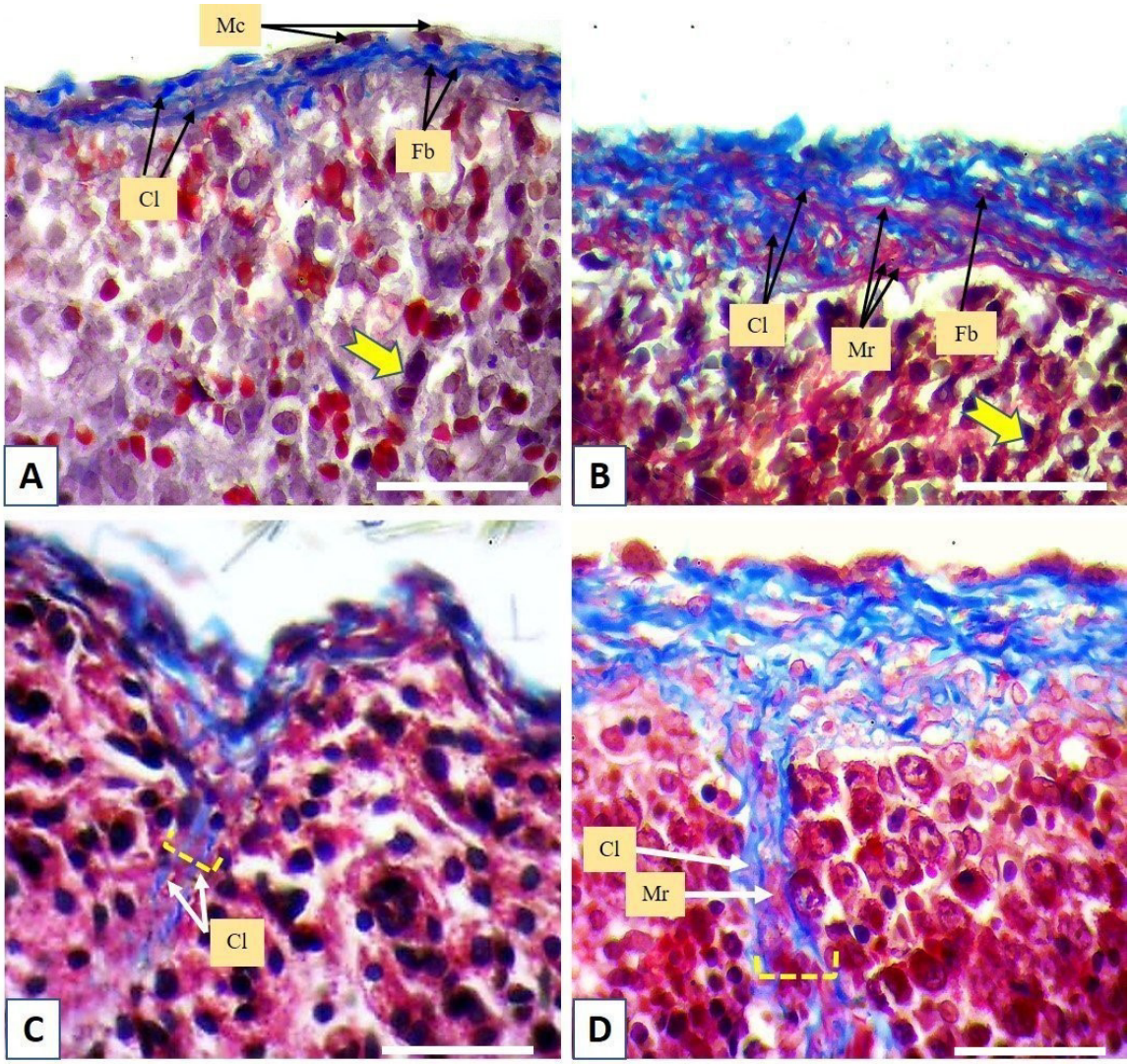

Figure 2 Photomicrograph illustrating the components of the splenic capsule in first postnatal day (A), in 90 postnatal day age (B), images highlight the mesothelial cells (Mc), fibroblast cells (Fb), the smooth muscle fibers (Mr), the collagen fibers (Cl), and the splenic cord (arrow). The figures in the second row depict the splenic trabeculae. in first postnatal day (C), in 90 postnatal day age (D), trabeculae (dashed lines), (MTs, Scale bar =30µm).

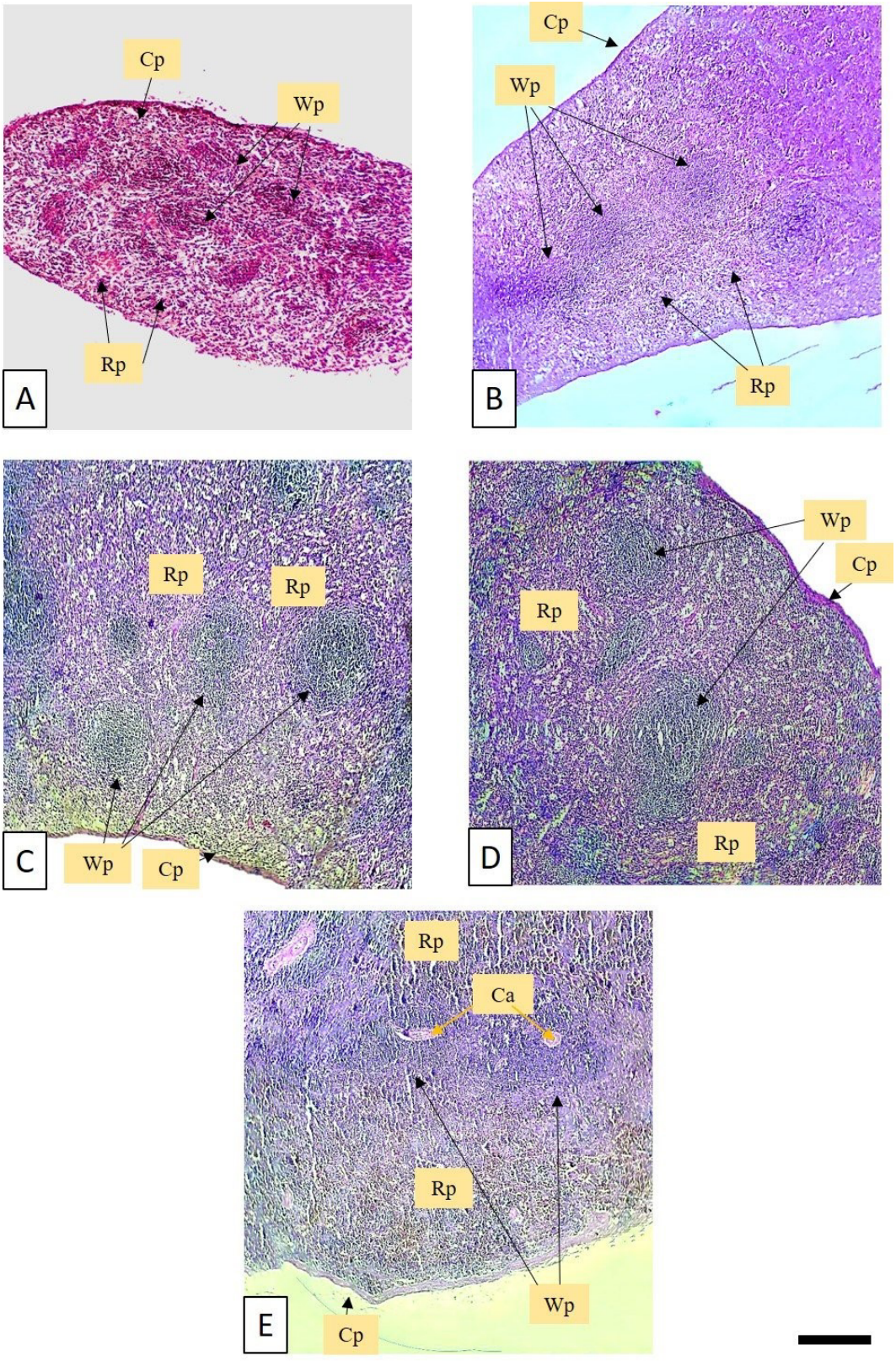

Figure 3 Photomicrograph show the spleen in various age groups, in first postnatal day (A), 10 postnatal days (B) , 15 postnatal days (C) 30 postnatal days (D), and 90 postnatal days(E) , the images highlight the splenic capsule (Cp) , central artery (Ca) , white pulp follicles (Wp) , and red pulp (Rp) , (H&E, Scale bar =150 µm)

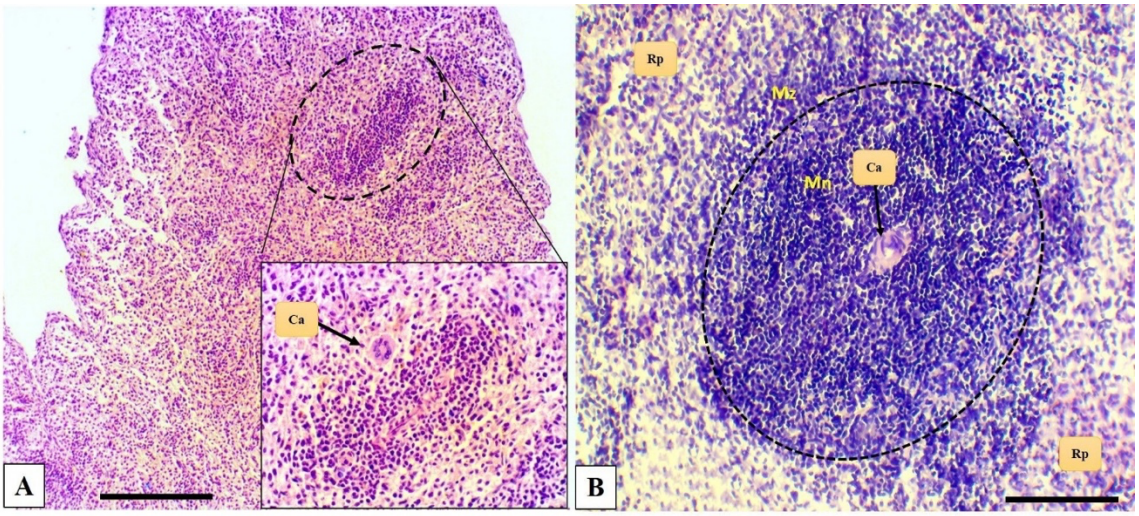

Figure 4 Photomicrograph illustrating the splenic parenchyma including the white and red pulp in first postnatal day (A), and 90 postnatal days (B) , the images highlight the mantle zone (Mn) , marginal zone (Mz) , red pulp (Rp) , and central artery (Ca), (H&E , Scale bar =100 µm)

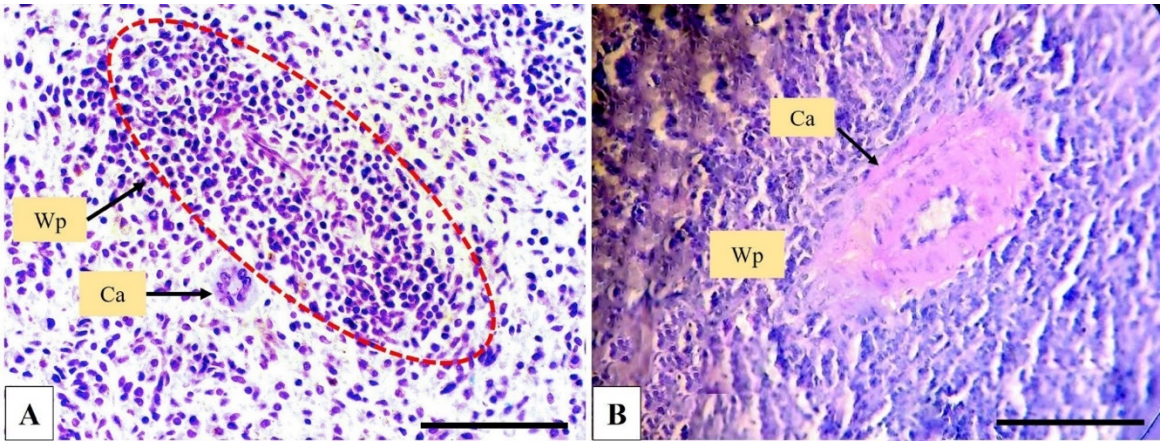

Figure 5 Photomicrograph illustrating the white pulp arteries: in first postnatal day (A), in 90 postnatal days (B), the white pulp follicles (Wp), and central artery (Ca), (H&E, Scale bar =30 µm)

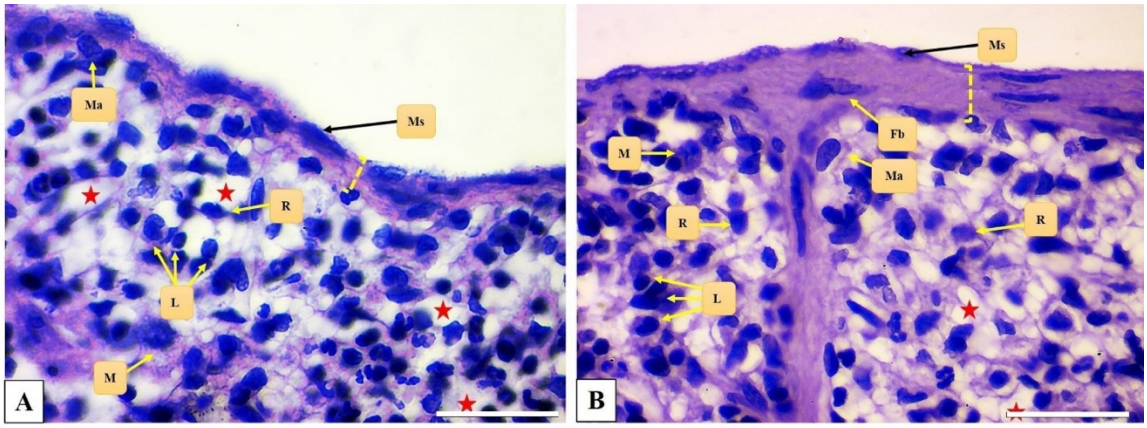

Figure 6 Photomicrograph illustrating the splenic red pulp components : in first postnatal day (A) and in 90 postnatal days (B); the image point out the splenic capsule (dashed lines), mesothelial cells (Ms), macrophages (M), reticular cells (R), lymphocytes (L), fibroblasts (Fb), and the splenic sinuses (stars), (H&E, Scale bar =30 µm)

The histochemical analysis

The histochemical analysis of the splenic carbohydrate was conducted using the periodic acid Schiff (PAS) and Alcian blue 2.5 pH stains. The findings indicated the presence of acidic and neutral glycoproteins. The splenic capsule exhibited moderate staining affinity to the PAS stain in the first postnatal day age group and intensity increased in older age groups (Figure 7A, B). Furthermore, the walls of the central arteries showed positive staining with PAS in all animal groups. The red pulp also showed moderate reactivity with PAS stain in the first and 10 postnatal day groups, and weak reactivity in older age groups (Figure 8A, B). The white pulp follicles were stained with Alcian blue (AB) stain, and the affinity was intense in the first and 10 postnatal day groups, but decreased gradually with age advancements. The AB stain was negative in other splenic components (Figure 9A, B).

The volumetric evaluation of the splenic parenchyma was performed to determine the proportion of white pulp to red pulp. The ratio was 0.12 ± 0.32 % in the first postnatal day group and 0.49 ± 0.36 % in 90 postnatal day group (Table 3).

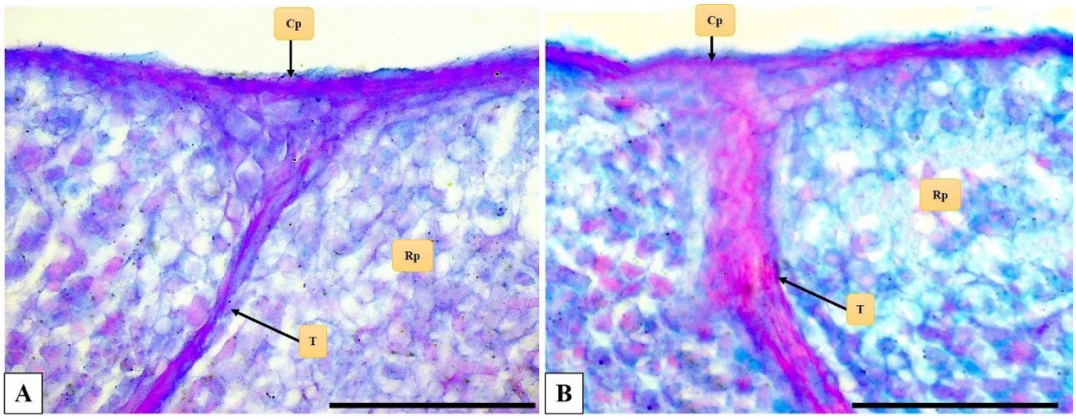

Figure 7 Photomicrograph illustrating the capsule and trabeculae: in first postnatal day (A), in 90 postnatal days (B), the capsule (Cp), trabecula (T), and the red pulp (RP), (AB/PAS, Scale bar =100 µm)

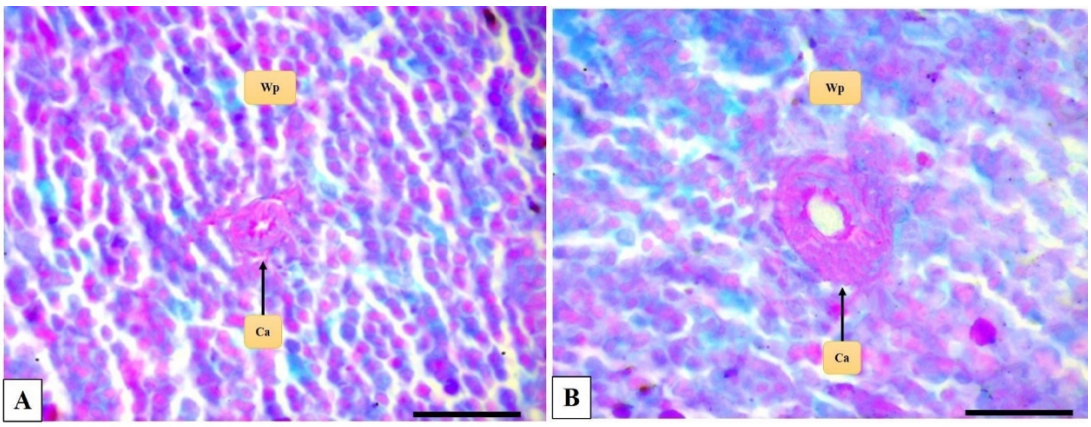

Figure 8 Photomicrograph illustrating the white pulp arteries: in first postnatal day (A), in 90 postnatal days (B), the splenic white pulp (WP), the central artery (Ca), (AB/PAS, Scale bar =30 µm)

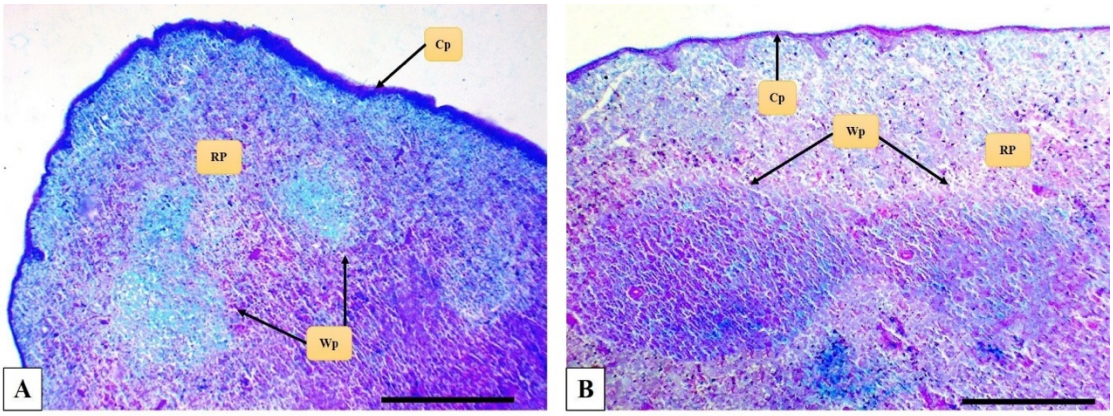

Figure 9 Photomicrograph illustrating the splenic parenchyma: in first postnatal day (A), in 90 postnatal days (B), the red pulp (Rp), white pulp follicles (Wp), and the capsule (Cp), (AB/PAS, Scale bar =150 µm)

Table 3 The ratio of white and red pulp in various postnatal age groups

Animal groups	Numbers of white pulps follicles in x40 field M ± SEM	Proportion of white pulp area M ± SEM (%)	Proportion of red pulp area M ± SEM (%)	white pulp / red pulp ratio M ± SEM (%)
Postnatal day 1	16 ± 0.31 a	10.93 ± 0.46 a	89.07 ± 0.45 a	0.12 ± 0.32
Postnatal day 10	7 ± 0.47 b	15.91 ± 0.27 b	84.09 ± 0.24 a,b	0.18 ± 0.27
Postnatal day 15	7 ± 0.39 b	18.96 ± 0.44 b	81.04 ± 0.49 a,b	0.23 ± 0.41
Postnatal day 30	4 ±0.30 b	25.47 ± 0.26 b,c	74.53 ± 0.27 b	0.34 ± 0.25
Postnatal day 90	3 ± 0.37 c	33.13 ± 0.38 c	66.87 ± 0.38 c	0.49 ± 0.36

Statistical differences are denoted by unique letters within each column among the animal groups at a significance level of $P \leq 0.05$

DISCUSSION

The macroscopic features and position of the spleen, as described in this study, align with the findings of (Rahmoun et al., 2019; Farghali et al., 2020) in rabbits, (Hasan et al., 2023) in hamsters and (Ikpegbu et al., 2019) in rats. The development of the spleen during early postnatal life revealed a progressive increase in splenic length, breadth, and weight. Consistent observations have been documented by (Geetha et al., 2001) in rats, mice, and guinea pigs, (Rahmoun et al., 2019) and in rabbits.

The microscopic findings showed that the splenic capsule was bilayered in most age groups, composed of fibrous and muscular components, and that capsular thickness increased gradually with age advancement. Similar findings were noted in rats, mice, and guinea pigs by (Geetha et al., 2001), in chickens by (Ayman et al., 2021), in hamsters by (Hasan et al., 2023) and in pigs by (Quyen et al., 2024). Furthermore, (Geetha et al., 2001; Waghaye, 2007; Ayman et al., 2021), reported that the collagenous content and fibrous mass thickened within the splenic capsule and trabeculae in relation to age progression in goats and laboratory animals and chicken. Except that splenic trabeculae were absent in chickens until 56 post - hatching age.

The progressive increase in the splenic capsule and trabecular thickness might be attributed to the continuous increase in splenic size, which reduces the spaces around it and causing it to be impacted among the abdominal viscera.

Additionally, the continuous gastric movements post-weaning of the animal might widen the frictional surface of the splenic faces.

The present observations showed that the parenchyma of the spleen comprised white and red pulps in most animal groups. The white pulp follicles were small, irregular patches of lymphocyte aggregations on the first postnatal day and developed gradually to reach complete morphological configuration by the 30th postnatal day.

The findings mentioned by (Parker et al., 2015). indicated that on the 5th postnatal day, the splenic white pulp follicles in rats were composed of small, disorganized accumulations of lymphocytes, with unidentified germinal, mantle, and marginal zones.

(Parker et al., 2015), also report that complete follicular growth continues until the 28th postnatal day, and (Yaglova and Obernikhin, 2014), mentioned that white pulp follicles reach complete developmental configuration by the 45th postnatal day in mice.

(Ayman et al., 2021), also mentioned that white pulp in chick's spleen until 42 post-hatching day age was scattered with no identified marginal zone, distributed within splenic parenchyma, except that sheathed splenic artery were developed early and clearly recognized at 14 post-hatching age

The structural development of the white pulp follicles and the lymphocytic population in 30 postnatal day rabbits suggests that it occurs as a result of the elevation of splenic immunological activity and the possibility of exposure to infection as kittens depart the burrow for the first time. It is noteworthy that the red pulp sinuses undergo current developmental alterations. They were enlarged, anastomosed and interconnected with each other, similar observations recorded by (Malik et al., 2001; Waghaye, 2007), who noticed that sinuses expanded and anastomosed in the spleen of goats in late postnatal aged animals. Parker et al. reported that the development of the splenic sinuses initiated on the day 6 after birth and continues until the day 30. During this period, the sinuses expand to facilitate increased blood flow to the splenic tissue.

Moreover, the proportion of the white pulp to the red pulp increased gradually with age development. Similar findings by (Yaglova and Obernikhin, 2014; Hasan et al., 2023), mentioned that the follicular width and volume ratio of white pulp follicles in mice increased from the 17th to the 40th postnatal day age.

The histochemical analysis revealed strong staining affinity of the splenic capsule and arteries walls to PAS stain on the first postnatal day with a moderate staining in other age groups. The white pulp follicles stained intensely with Alcian blue during the (1-10) postnatal age, and the intensity decreased gradually with age advancement.

(Waghaye, 2007), found that the intensity of staining with PAS was high from day 1 to 10 postnatal age animals, which decreased gradually with age progression. This suggests that the presence of a large quantity of glycogen in the splenic capsule, trabeculae, and wall of blood arteries during this period, essential for the physiological and functional development of the spleen in early postnatal life. (Waghaye, 2007; Malik et al., 2001), also reported moderate Alcian blue staining of the white pulp follicles in the first postnatal day age and negative staining activity in other age groups, and suggested that the presence of acidic glycoprotein during this period is necessary to provide optimal physiological growth conditions and protection for the spleen

CONCLUSIONS

The paramount protective function of the spleen reflects the scientific interest in studying the morphological, developmental events during early postnatal life in white rabbits. The current study showed that the critical developmental period initiates immediately after birth and continues until 30 postnatal days. During this

period, the weight and size of the spleen increased, and the splenic stroma thickened. The follicular configuration of the white pulp was completed, and the lymphatic tissue expanded. Using the spleen in white rabbits as a model has great potential benefits for understanding the morphological and histochemical development of the spleen in other animal species and humans. This broader perspective fosters a comprehensive investigation of other lymphatic organs' ontogeny and its implications for future anatomy and medical research.

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AUTHOR CONTRIBUTIONS

Omar y. altaey: design the study and write the initial draft and the data analysis. Ali A.hasan: perform the anatomical and macroscopic measurements. Ammar G.Alhaaik: perform the histological and the microscopic analysis. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The author declares that there are no conflicts of interest

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