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STEREOLOGICAL AND MORPHOMETRIC ANALYSIS OF SPLEEN ON NEW ZEALAND WHITE RABBITS (*ORYCTOLAGUS CUNICULUS*)

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ABSTRACT

The aim of this research was to examine the total volume and diameter of the spleen, white pulp and red pulp volume fractions, capsule thickness, germinal core surface area and diameter and investigate morphological differences between female and male White New Zealand rabbits. 9 male and 9 female rabbit spleens were used. Spleens were individually weighed and the length, thickness and width of spleen were measured using a digital caliper. The fixed samples after the routine histological procedure, serial sections of 10 µm thickness were taken and stained with hematoxylin eosin. All specimens were examined under the light microscope and photographed. Cavalieri's method was used in area and volume calculations. According to results, there was no statistical difference between spleen volumes, spleen density, red pulp and white pulp volumes of female and male rabbits. It was determined that length and width of spleen in female rabbit spleens were greater than male but there was no difference between the thickness and weights of spleen. Germinal center diameter was found to be greater in female rabbits, while no difference was found in capsule thickness and germinal center areas. These morphometric data would significantly help the physician in the diagnosis and treatment of spleen diseases.

Keywords: Spleen, Cavalieri's method, Stereology, White New Zealand rabbits

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INTRODUCTION

The spleen is the largest lymphatic organ in the mammalian body that stores blood and gives it to the circulation when necessary. It is located on the left side of the abdominal cavity, under the last rib (Benter *et al*., 2011; Dursun, 2008). A thick layer of dense connective tissue (capsule) is covered and there is no spleen cortex and medulla. On histological sections, it is seen to be arranged in two regions (white pulp and red pulp). The central arteries pass through the white pulp and lead to sinuses in the marginal area. Around this central artery is PALS, where T cells concentrate and form a sheath. The red pulp consists of reticular fibers with macrophages and all other blood specific cells. It filters antigens and particulate materials, engulfs aged red blood cells, and acts as a reservoir for erythrocytes and platelets (Barone, 1997; Cesta, 2006; Linden *et al*., 2012).

Rabbits have been preferred in laboratories for years to investigate immunological problems and develop immunological techniques. (Pinheiro *et al*., 2016; Weber *et al*., 2017). Rabbit antibodies are more preferred in immunological studies compared to other rodents because they can recognize epitopes on human antigens and increase the total number of targetable epitopes. Although rodents other than rabbits show immunity to the therapeutic agents used in humans and most mouse strains are interbred, the scarcity of inbred rabbit species is another factor that increases the use of rabbits. Additionally, the larger blood volume of rabbits compared to other rodents is an advantage for studies. (Ayyar *et al*., 2015; Weber *et al*., 2017; Yu *et al*., 2015). The shape and volume of the spleen are used in the diagnosis of many diseases such as splenomegaly, traumatic lesions of spleen rupture, spleen pseudoaneurysms, hematoma in the splenic parenchyma, infarctions of the spleen, splenic lymphomas (Benter *et al*., 2011). Therefore, it needs a more detailed description of the volume and micro-anatomical structure of the spleen and its components in rabbits.

Morphometric studies of the spleen have been performed in humans (Almenar *et al*., 2019; Linden *et al*., 2012) on a wide variety of species, including domestic animals such as sheep and goat (Gnanadevi *et al*., 2019), camel (Jaji *et al*., 2019), pig (Shringi *et al*., 2017), as well as cats (Maher *et al*., 2020) and rodents such as rabbits (Dimitrov, 2012; Qasem *et al*., 2015; Rahmoun *et al*., 2019; Takeda *et al*., 2007), guinea pig (Qasem *et al*., 2015) and mice (Linden *et al*., 2012). Studies on the spleen were generally carried out as gross anatomical measurements and determination of the location of the spleen, but no research on the micro-anatomical and stereological based was found.

Stereological methods have many unbiased approaches to avoid subjective measurements. This method produces the most accurate and reliable data by using the resources at the optimum level by giving the tissues equal sampling chance (Bolat, 2018; Gundersen *et al*., 1999).

This study aims to examine the total volume and diameter of the spleen, white pulp and red pulp volume fractions, capsule thickness, germinal core surface area and diameter in the rabbit. Therefore, the findings from this study about the general morphometric properties of the spleen using stereological methods may contribute to the expansion of comparative anatomy knowledge and possibly help the development of experimental studies in this field and the progress of the diagnosis and treatment of diseases in the spleen.

MATERIALS AND METHODS

Materials: Eighteen healthy White New Zealand rabbits, (9 males, 9 females, and 14 months old) were used. This study was approved by Karamanoglu Mehmetbey University Faculty of Health Sciences Ethics Committee (No:2019-10/52). All the rabbits were given standard rabbit diet and ad libitum water and housed under the same conditions. Animals were anesthetized by administering xylazine hydrochloride (10 mg/kg, IM) and ketamine hydrochloride (30 mg/kg, IM) (Flecknell, 2015). An incision was made over the linea alba in the supine position and was exposed by displacing the intestines. The blood was drained by attaching a cannula to the vena cava caudalis, and euthanasia was performed. With this vena 10%, neutral formalin solution was perfused and the spleen was removed after euthanasia (Bolat *et al*., 2011).

Morphometric measurements: After the adipose tissue was removed, spleens were individually weighed and the length, thickness and width of spleen were measured using a digital caliper. The density of each spleen was calculated by dividing the weight to volume. Relative organ weight was calculated by dividing the spleen weight by the whole body weight.

Histological process and analysis: To make the spleen suitable for histological procedures, spleens were sliced into three equal parts on millimeter paper and were fixed in 10% neutral buffered formaldehyde for 24 hours at room temperature. After the fixed samples were washed overnight, the samples were dehydrated, cleared, and embedded in paraffin. Following the routine histological procedure, serial sections of 10 µm thickness were taken from the entire spleen after routine histological procedures. Sections were sampled at a ratio of 1/300 and stained with hematoxylin-eosin (H&E) (Table 1). All specimens were examined under the light microscope and were photographed. In the histological examination of spleen sections, six different regions were randomly selected with ImageJ program and mean capsule thickness was obtained from the measurements. When measuring the diameter of the germinal center, large and small diameters were measured and the average diameter was obtained by taking the square root of multiplying the measured values.

Area and volume calculations of spleen: Cavalieri's method was used in area and volume calculations. For this, a point counting grid that had a distance of 0.1 mm between two points was applied on the spleen images with the ImageJ program and the points on spleen, red and white pulp, and germinal centers were counted separately (Figure 1).

The volumes were estimated as $V = a(p) \times \Sigma p \times t$ formula. In this formula, V is the volume of the structure concerned, a(p) is the area of the one point on the grid (this value is 0,01 mm^2 in the study), Σp is the sum of the points on the structure of interest and t is the section thickness (Gundersen *et al*., 1999; Selçuk and Tıpırdamaz, 2020). Coefficient of error (CE) was calculated according to the study of Gundersen *et al*. 1999. Red and white pulp volume ratios were obtained by dividing related spleen section to the volume of total spleen.

Statistical analysis: IBM SPSS 21 program was used for statistical evaluation. First, a pre-test was applied to the groups to determine whether they show normal distribution using the Shapiro-Wilk Normality Test. Since our data showed normal distribution according to the pre-test data, it was evaluated with the independent T test. The results were accepted within the 95% confidence interval and those with a P value less than 0.05 were considered significant. Results were expressed as mean and standard error (mean ± SE).

Table1: Hematoxylin-eosin staining procedure (Selcuk and Colakoglu, 2020).

Staining Stages					
1	Xylene, 5 min.	**9**	Tap water for 5 min.	**21**	80% alcohol dip once
2	Xylene, 5 min.	**10**	Hematoxylin solution, 2 min.	**22**	96% alcohol dip once
3	100% alcohol, 3 min.	**11**	Tap water for 1 min.	**23**	96% alcohol dip once
4	100% alcohol, 3 min.	**12**	3% HCl with 70% alcohol dip once	**24**	100% alcohol, 2 min.
5	96% alcohol, 3 min.	**13**	Tap water for 1 min.	**25**	100% alcohol, 2 min.
6	80% alcohol, 3 min.	**14**	Eosin solution, 2 min.	**26**	Xylene, 2 min.
7	70% alcohol, 3 min.	**15**	Tap water for 2 min.	**27**	Xylene, 2 min.

8	Rinse in distilled water	**16**	70% alcohol dip once

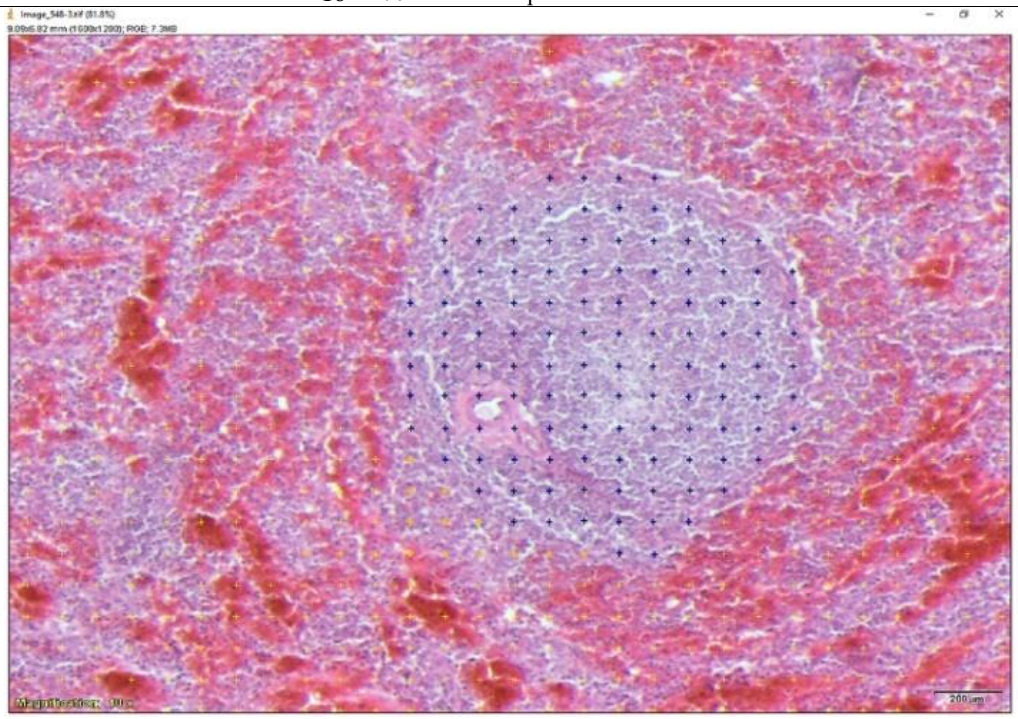

Figure 1: Application of point counting grid with ImageJ on germinal center of the spleen.

RESULTS

The spleen was found to be dark red, an elongated organ with two faces and two sharp edges, its parietal face was found to be attached to the great curvature of the stomach, adjacent to the diaphragm and the lateral wall of the abdominal cavity. It was observed that the visceral face was divided by the lineal hilus and was adjacent to the intestines.

The weights of the female and male White New Zealand rabbits used in the study are 3275.56 ± 169.81 gr and 2714.22 ± 77.61 gr, respectively. The average spleen weight measured in female White New Zealand rabbits is 1.54 ± 0.18 g, while it is 1.10 ± 0.11 g in male White New Zealand rabbits.

The volumes of the spleen and its constituent structures calculated by the Cavalieri's method in female and male White New Zealand rabbits were given in Table 2 and Table 3. It was determined that the spleen in females was composed of 16.65% white pulp and 83.35% red pulp. These rates were found to be 13.14% and 86.86%, respectively in male White New Zealand rabbits. There was no statistical difference between spleen volumes, spleen density, red pulp and white pulp volumes of female and male White New Zealand rabbits (p> 0.05).

Table 2: Female White New Zealand Rabbits Data.

Animal Numbers	Body Weight (g)	Spleen Weight (g)	Relative Organ Weight (%)	Spleen Volume (mm^3)	Red Pulp Volume (mm^3)	Red Pulp ratio (%)	White Pulp Volume (mm^3)	White Pulp Ratio (%)	Density of Spleen (g/mm^3)
F1	3100	0.8989	0.029	394.50	333.06	84.43	61.44	15.57	7.86
F2	2940	1.0989	0.037	522	402.99	77.21	119.01	22.79	5.63
F3	3350	1.7359	0.052	746.25	641.34	85.94	104.91	14.06	4.49
F4	4410	1.1718	0.027	680.25	566.73	83.31	113.52	16.69	6.48
F5	3370	1.4173	0.042	644.25	531.75	82.54	112.50	17.46	5.23
F6	3550	1.3059	0.037	605.25	455.13	75.20	150.12	24.80	5.87
F7	3130	2.0821	0.067	942.75	843.60	89.48	99.15	10.52	3.32
F8	3050	1.5032	0.049	761.25	620.55	81.52	140.70	18.48	4.01
F9	2580	2.6414	0.100	667.50	604.44	90.55	63.06	9.45	3.87
Mean± SE	3275.56± 169.81	1,54± 0.18	0.049± 0.008	662.67± 51.48	555.51± 50.07	83.35± 1.69	107.16± 10.06	16.65± 1.69	5.19± 0.48

Table 3: Male White New Zealand Rabbits Data.

Animal Numbers	Body Weight (g)	Spleen Weight (g)	Relative Organ Weight (%)	Spleen Volume(mm^3)	Red Pulp Volume (mm^3)	Red Pulp ratio (%)	White Pulp Volume (mm^3)	White Pulp ratio (%)	Density of Spleen (g/mm^3)
M1	2750	0.5454	0.020	332.58	273.80	82.33	58.78	17.67	8.27
M2	2790	0.9775	0.035	441	349.20	79.18	91.80	20.82	6.33
M3	2510	1.3624	0.054	656.25	575.31	87.67	80.94	12.33	3.83
M4	2440	1.4588	0.059	674.25	579.00	85.87	95.25	14.13	3.62
M5	2740	1.2908	0.047	591.75	523.71	88.51	68.04	11.49	4.63
M6	2838	1.428	0.050	576.75	527.34	91.43	49.41	8.57	4.92
M7	2360	1.0845	0.046	512.25	457.83	89.38	54.42	10.62	4.61
M8	2950	0.7158	0.024	346.50	302.58	87.32	43.92	12.68	8.51
M9	3050	0.9964	0.033	444.75	400.38	90.02	44.37	9.98	6.86
Mean±	2714.22±	1.10±	0.041±	508.45±	443.24±	86.86±	65.21±	13.14±	5.73±
SE	77.61	0.11	0.005	41.95	38.87	1.29	6.63	1.29	0.61

Table 4: Histomorphometric Features and Diameter Measurements of The Spleen (Mean±SE).

Animal Gender	Capsule Thickness (µm)	Germinal center diameter (µm)	Germinal Center Areas (mm^2)	Length of Spleen (mm)	Thickness of Spleen (mm)	Width of Spleen (mm)
Female	77.34±3.18	437.57±10.85^a	0.13±0.01	49.20±3.04^a	4.94±0.37	10.21±0.42^a
Male	68.89±2.55	355.86±6.94^b	0.11±0.01	44.60±1.48^b	4.49±0.36	8.08±0.18^b

Different letters in the same row (a, b) indicate statically significant differences ($p<0.05$).

The histomorphometric properties, diameter and length measurements of the spleen in female and male White New Zealand rabbits are given in Table 4. It was determined that length and width of spleen in female White New Zealand rabbit spleens were greater than male White New Zealand rabbits and were statistically significant ($p <0.05$). There was no statistical difference between the thickness of spleen and spleen weights of female and male White New Zealand rabbits ($p> 0.05$). Germinal center diameter was found to be greater in female White New Zealand rabbits than male White New Zealand rabbits ($p <0.05$), while no difference was found in capsule thickness and germinal center areas ($p> 0.05$).

The quality and accuracy of measurements made in stereological studies and the conformity of the sampling strategy could be observed by establishing the CE. A value of 5% or less of the CE was required for the results of the study to be considered reliable (Gundersen *et al* 1999). The CE in the study were less than 5% (Figure 2).

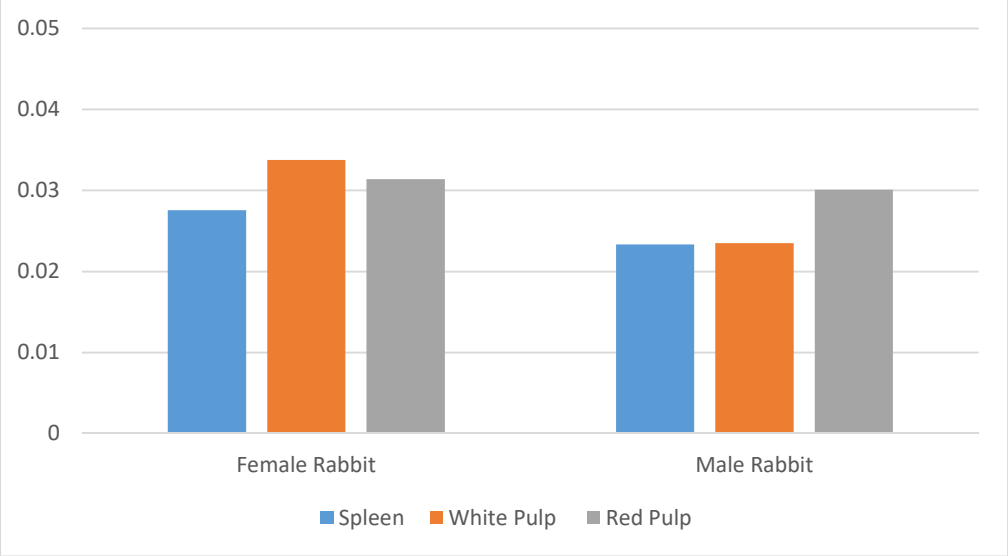

Figure 2: Coefficient of error values spleen and its structures

DISCUSSION

In addition to being the largest lymphoid organ in our body, the spleen reacts to blood-borne antigens by producing antibodies and is the main source of circulating antibodies. It removes defective red blood cells and platelets from circulation (Linden *et al*., 2012). The rate at which lymphocytes leave the blood in the spleen is faster than in other organs. Thus, the spleen, with its enormous phagocytic capacity, receives T and B cells from the recirculating pool and separates them into different compartments of the white pulp, allowing them to interact with antigen presenting dendritic cells or macrophages. Therefore, the spleen is a very important organ for the immune system (Pabst, 2020; Weber *et al*., 2017). Splenomegaly can be seen in many pathological conditions of the spleen or liver diseases, bacterial and viral infections, and metabolic diseases. In acute infection, spleen cells become activated and proliferate, causing hyperplastic enlargement. The reason for enlargement in liver diseases is the increase in venous pressure by trapping blood in the sinusoids of the spleen. About a third of the circulating platelets are stored in the spleen. In the case of splenomegaly, it can destroy more platelets than normal and this can have life-threatening consequences such as thrombocytopenia. Also, enlargements in the spleen are more prone to rupture of the spleen formation compared to healthy individuals (Rotbain *et al*., 2017; Lv *et al*., 2017; Palmiere *et al*., 2018).

In previous studies, it had been reported that the spleen was long, dark red in the left cranial part of the stomach (Maher *et al*., 2020; Qasem *et al*., 2015), its convex side connected the diaphragm (Benter *et al*., 2011), its concave side was in contact with the stomach, left kidney, and colon flexure, (Benter *et al*., 2011; Cesta, 2006; Hristov *et al*., 2006), and the side facing the organs was divided into two unequal parts by the hilum (Qasem *et al*., 2015). In our study, it was observed that the topographic location of the spleen was similar to other studies.

In a study using fifty healthy males in a local rabbit breed of the Souk-Ahras region in Algeria, 15 months old, it was found that the rabbit weight was 3900 ± 100 g, the spleen weight was 1.86 ± 0.05 g, the length was 71.2 ± 1.19 mm, the width was 9.98 ± 0.18 mm (Rahmoun *et al*., 2019). In a study in which had different age groups by Hristov *et al*. (2006) on twenty New Zealand rabbits, the rabbit weight was between 1500-3680 g, the length of the spleen was 40-70 mm and the width was 10-30 mm, without specifying the gender. Takeda *et al*. (2007) reported in their study using ultrasonography on 5 female 13- to 21-week-old white Japanese rabbits that the spleen length varied between 2.77-8.46 mm. Dimitrov (2012) found the rabbit weight between 2800-3200 g, the length of the spleen as 56.2 ± 0.73mm, the thickness of the spleen as 5.6 ± 0.09 mm, and the width of the spleen as 9.8 ± 0.12 mm, in their study on 8-month-old twelve New Zealand rabbits. Qasem *et al*. (2015) reported that the rabbit weight was between 800-1000 g, the spleen weight was 0.547±0.035 g, the length of the spleen was 33.14 ± 1.6 mm, the width was 5.44 ± 0.40, and the thickness was 1.28 ± 0.7 mm in their study on 15 male New Zealand rabbits. In present study, the mean weight of female and male White New Zealand rabbits used were 3275.56±169.81 and 2714.22±77.61 g, spleen weights were 1.54 ± 0.18 and 1.10 ± 0.11 g, the lengths of the spleen were 49.20±3.04 and 44.60±1.48 mm, the widths were 10.21 ± 0.42 and 8.08 ± 0.18 mm, the thicknesses was 4.94 ± 0.37 and 4.49 ± 0.36 mm respectively. Our study and Dimitrov *et al*. (2012) study showed compliance. The current difference between other studies and our study is thought to be due to the methodological difference, the lack of age and racial discrimination of the rabbits used, and the fact that the data were not given according to sexual dimorphism.

Qasem *et al*. (2015) reported the spleen volume as 0.488 ± 0.03 mm^3 and the relative organ weight as 0.06782 ± 0.002. In present study, the spleen volumes were 662.67 ± 51.48 mm^3 in female, 508.45 ± 41.95 mm^3 in male. Relative weights of the spleen were in female and male 0.049 ± 0.008 and 0.041 ± 0.005 respectively. The difference between the two studies was thought to be due to the lack of sexual dimorphism in the study conducted by Qasem *et al*. (2015).

Rahman *et al*. (2016) stated in their study without specifying the rabbit breed, that the spleen capsule thickness was 33.33 µm. In our study, these values were determined as 77.34 ± 3.18 µm in the female White New Zealand rabbits and 68.89 ± 2.55 µm in the male White New Zealand rabbits.

As a result of this study, morphometric studies of the spleen were very limited in literature reviews, and morphometric data could not be reached in rabbits except for a few parameters. However, these morphometric data would significantly help the physician in the diagnosis and treatment of spleen diseases. It is thought that the data obtained from the present study and the stereological method used would contribute to the elimination of the deficiencies in these issues. In the study, morphometric measurements were made on the spleens of animals before (macro-anatomical) and after (micro-anatomical) histological procedures, and these values were presented separately in tables and figures and by comparing some parts. It is thought that these data would significantly contribute to future research on the subject and new research is needed on this subject.

Statement of novelty about the manuscript: As a result of this study, morphometric studies of the spleen were

very limited in literature reviews, and morphometric data could not be reached in rabbits except for a few parameters. However, these morphometric data will significantly help the physician in the diagnosis and treatment of spleen diseases. It was thought that the data obtained as a result of this study would constitute the basis for the studies on the spleen and would guide physicians in the treatment of splenic diseases.

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