A stereological and histological analysis of spleen on obese female rats, fed with high fat diet

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ABSTRACT

Objective: To determine if there is an association between fatty diet induced obesity and spleen enlargement by means of Cavalieri principle, unbiased stereological method and light microscopic examination.

Methods: In this study, we used 16 adult female Sprague Dawley rats, weighing between 150-200 g. All animals were obtained from the Ataturk University Experimental Research and Applying Center, Turkey in 2005. We performed rat models, fed with normal or high-fat diet for duration of 3 months. After this controlled nutritional process, spleens are removed from all anesthetized rats and performed by routine histological process. Stereologically, we estimated the spleen volumes in consecutive serial sections using Cavalieri method in control and treatment groups. Then, we examined histologically all those sections by a light microscope with camera attachment.

Results: Mean spleen volumes were 1.40 ml in the control and 2.03 ml in the treatment group, suggesting splenomegaly. Volumes of spleens in 2 groups revealed statistical significant difference ($p<0.05$, independent samples t-test). In studying spleen slices, many macrophages and necrotic figures were defined. Also, sinusoidal dilatation and hemosiderin deposits were observed and we found macrophages, filled with hemosiderin droplets. In some sections, especially around small vessels, eosinophilic aggregations and lipid accumulations in dilated sinusoids were detected.

Conclusions: Spleen enlargement at significant levels (38%) in obese patients was determined by Cavalieri stereologic volume calculation method; an unbiased stereological method. Finally, our results clearly indicated that high fat diet caused to splenomegaly via sinusoidal dilatation and intra-cellular or intercellular deposits.

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Obesity is a severe metabolic disorder, characterized with increases in energy intake and a decrease in energy output concerning body weight and glucose metabolism.[1] Obesity is associated with many important complications such as diabetes and coronary heart disease, diseases of the gallbladder, liver and spleen.[2,3] The spleen is an organ involved in the production and maintenance of red blood cells, the production of certain circulating white blood cells, and is a part of the lymph system and the immune system. Because of its wide variety of functions, the spleen may be enlarged by many conditions involving the blood or lymph system, and by infection, malignancies, liver disease, and parasites.[4-6] Also previous studies were indicated that splenomegaly was occurred in obesity cases.[7] This splenomegaly was a few times confirmed with different techniques.[8,9] But there is not any quantitative or histological data clarifying this subject. The Cavalieri principle, a well-established stereological technique, uses interpolation between samples to estimate volume of three-dimensional (3D) objects. To estimate volumes with this method, a few simple conditions are required: (i) 2-dimensional slices or images that are obtained through the object have to be parallel to each other; (ii) these slices or images must be separated by a known distance; (iii) the samples must be chosen using a systematic random rule; and finally (iv) a pilot study should be conducted before the beginning of the study in order to set both coefficient of error and variation and this method is reliable for volume estimation.[10-12] We previously reported that there was a significant liver injury, including micro vesicular steatosis, fibrosis, necrosis and mononuclear cell infiltration and so forth in obese rats, fed with high fat diet.[13] Our current purpose is to determine if there is an association between fatty diet induced obesity and spleen enlargement by means of Cavalieri principle, unbiased stereological method and light microscopic examination.

Methods. Sixteen adult female Sprague Dawley rats, weighing between 150-200 g, were used. All animals were obtained from the Ataturk University Experimental Research and Applying Center in 2005. The rats were maintained in laboratory under controlled environmental conditions (12 hours in the light/dark cycle and room temperature 22-24^{0}C). Rats were housed in plastic cages (2 animals per cage) and given food and water ad libitium. Diet contents used in this study were reported previously.[13] Standard diet was used in supervision of the Animal Care Committee of the Ataturk University. Control rats (n=8 animals) were fed with the standard commercial chow. Then, high fat diet was prepared and necessary vitamins-minerals were added. In high fat diet, the standard chow in powder form was mixed by adding animal abdominal fat (milted) up to 30% per total kilocalorie. until become homogenous in a dough-like consistency. This dough was shaped and the obtained chow blocks were dried and used for feeding. All animals were randomly divided into control and treatment groups. While control rats (n=8 animals) were maintained on the standard chow and the treatment group (n=8 animals) were fed with the specially prepared chow. Animals' weight was measured every 10 day in order to determine the possible putting on weight for 3 months. At the end of this period, animals were anesthetized by Sevorane® (Abbott, Ultane; Canada). Anesthetized animals' nasoanal length was calculated for detecting body mass index (BMI). Thus, the status of the animals as to being obese or not, were confirmed. Spleens were removed from all rats, and prepared to estimate spleen volumes and examine spleen structure by light microscope with camera attachment (Olympus BH2 microscope with Sanyo WC-6975 P camera attachment). Removed spleens were placed in fixative solution, formaldehyde consisting of 10 ml formaldehyde, 90 ml distilled water for a duration of 48 or 60 hours. On the basis of a pilot study, it was decided to select every 5th spleen section through a set of consecutive sections from each spleen. Choosing the first section was carried out randomly. Fifteen to twenty sections were sampled from each spleen in a systematic random manner. Sampled sections were photographed with a modified light microscope, a camera attachment and dial indicators (**Figures 1a & 1b**).[14] The Cavalieri principle was used for the estimation of the volume of spleens.[15,16] A point counting test grid was used for the estimation of sectioned area of spleens (**Figure 1b**). The point density of the point counting grid was designed to obtain an appropriate coefficient of error (CE) for images of the serial sections.[17] Coefficient of error and coefficient of variation (CV) were estimated according to Gundersen and Jensen' formula.[18] The test grid with systematic array of points is randomly placed on screen of PC. The volumes of the spleens were estimated with the following formula:

$$\text{Volume} = t \times a/p \times \sum P.$$

Where, "t" is the section thickness; "a/p" is the representing area of each point on the point counting grid and "$\sum$P" is the total number of the points hitting the sections surface areas.

For histological examination at the light microscopical level, removed spleens were fixed by formaldehyde solutions (10 ml formaldehyde, 90 ml distilled water) during 48-60 hours. Following these tissue samples were processed through ethyl alcohol and xylene series and embedded in paraffin blocks. Hematoxyline and eosin was used for staining of serial spleen slices. Then, it were covered with Canada balsam, and viewed by light microscope with camera attachment (Olympus U-PMTVC-BH2, Japan).

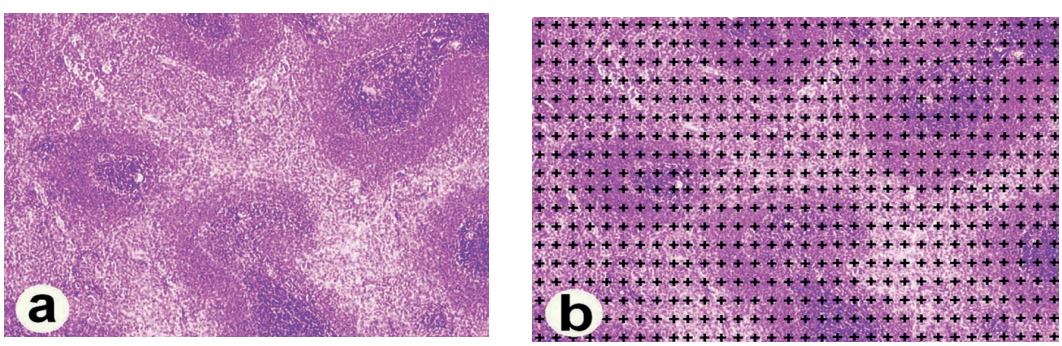

Figure 1 - Photomicrograph of **a)** selected spleen section and **b)** selected spleen section with superimposed point-counting grid.

Microsoft® SPSS Version 13.0 for Windows was used for statistical analyses and Mean Whitney U Test was applied. All statistical values under 0.05 was considered as to be significant.

Results. There were no sign of death during the experimental period. Exposure of animals to the high fat diet led to a significant increase (p<0.05) in amount of diet consumed by the treatment group. Mean BMI values were 3.2 ± 0.075 kg/m^2 in the control group and 5.6 ± 0.125 kg/m^2 in the treatment group. The difference between BMI values of the 2 groups was statistically significant (p<0,05). All volumetric values were summarized in **Table 1**. From series of hematoxyline and eosin stained sections, spleens were outlined in control and treated rats, fed with high fat die (**Figure 1**). Volumes were estimated using Cavalieri principle (**Table 1**). In our experiment, the mean CE was lesser than CV. We analyzed more large spleens in the treatment group compared with the control group. Considering volume of spleens, control and treated groups were significantly different (p<0,05). In the control group, white pulp consisting lymphatic tissue mostly lymphocytes was appeared as circular. Within the white pulp, the branch of the splenic artery is called the central artery. Essentially, red pulp including splenic sinuses was separated by splenic cords. In the treatment group, we found spleen slices around the blood vessel and between splenic cords hemosiderin deposits (**Figures 2a-2b, 3b, & 4b**). Sinusoids in both red pulp and white

Table 1 - All volumetric values of spleens in control and treatment groups.

Animal number	Control Group	Coefficient of error (%)	Treatment Group	Coefficient of error (%)
1	1.23	3.44	2.06	4.34
2	1,61	2.82	1.9	6.03
3	1,53	4.41	1.97	3.27
4	1,32	3,58	1.75	4.52
5	1.07	5.11	2.19	3.55
6	1.352	4.27	2.20	4.41
7	1.62	4.88	2.34	3.89
8	1.48	3.96	1.83	5.02
Mean	1.40	4.05875	2.03	4.37875

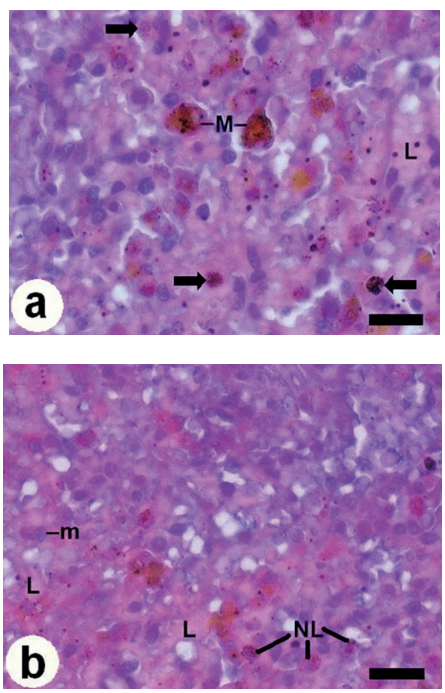

Figure 2 - Sections of treated spleens. **a)** M - hemosiderin filled macrophage; L - lipid accumulations; arrows - necrotic lymphoid cells. **b)** m - monocyte; L - lipid accumulations; NL - necrotic lymphoid cell. Magnification bars: 25 µm.

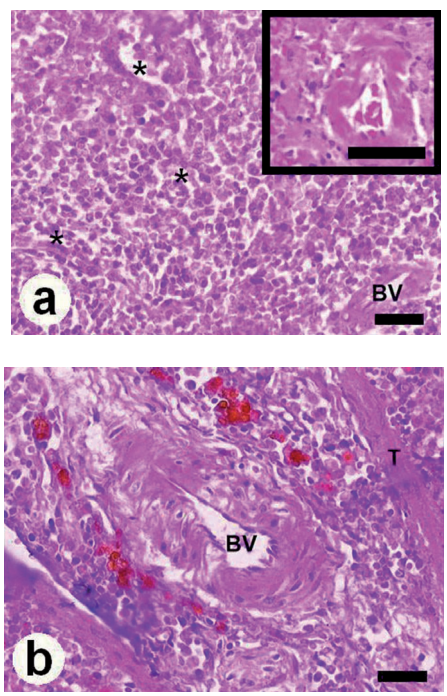

Figure 3 - Sections of treated spleens. **a)** BV - blood vessel; asterisks - dilated sinusoids. Inset reveals to eosinophil dyed amyloid droplets Magnification Bar: 80 µm. **b)** BV - blood vessel with vacuolated wall; T - trabecula. Perivascular, degenerated erythrocytes were seen.

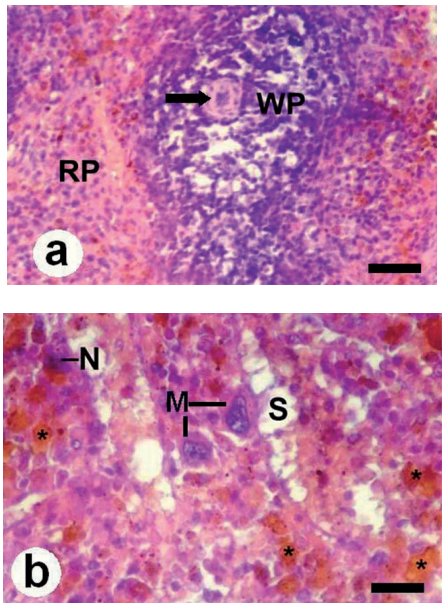

Figure 4 - Sections of treated spleens. **a)** RP - red pulp; WP - white pulp (dilated); arrow - central artery. Magnification Bar: 150 µm. b) S - dilated sinusoids; M - macrophages; N - neutrophil; asterisks - hemosiderin deposits. Magnification Bar: 30 µm.

pulp were dilated (**Figures 3a, & 4a-4b**). In this dilated sinusoids, lipid accumulations were observed (**Figures 2a-2b**). Eosinophilic droplets around blood vessels were detected (**Figure 3a inset**). Many macrophages, filled with hemosiderin were defined (**Figures 2a, 4b**). In spleen slices, among healthy cells, there were many necrotic lymphoid cells (**Figures 2a-2b**). Also, neutrophils and monocytes were seen (**Figures 2b, 4b**). We noticed that vacuolizations occurred in the wall of the blood vessel belongs to the treatment group (**Figure 3b**).

Discussion. Stereology is a number of mathematical and statistical methods that permit the evaluation of 3-dimensional structural information from 2-dimensional sections (or histological slices). Thus, researchers obtain important quantitative structural information, such as the volume, surface area or numbers of cells within described regional lines. The need for such quantitative information biological studies is of importance when evaluating the effect of various experimental treatments on any specific organs, tissues and cells in the body. Knowledge of such changes has given important data into the high fat diet that may be responsible for the functional and structural in consequences of this experiment. Stereological technique, used in this study, based on the Cavalieri principle has traditionally been used in histological and pathological studies to obtain accurate and unbiased estimates of volumes of anatomically or pathologically defined structures. Spleen volumes were significantly increased, from animals, fed with high fat diet. Obesity has been recognized as an epidemic in the USA for more than 2 decades; still the proportion of overweight and obese adults in the population continues to grow.[19] The prevalence of obesity, detected as a BMI of >30 kg/m^2, also reached dramatically from 23-31% during the same period. It is estimated that the prevalence of obesity in adults will rise to 39% by the year 2008.[20] This trend has an alarming health and economic implications, because obesity is associated with major causes of morbidity and mortality such as diabetes, coronary heart disease, diseases of the gallbladder, liver and spleen.[21-25] Recent findings have established an association between obesity and immune dysfunction. According to Morrison et al,[26] the first phase was the result of the immunological response and the second and hypersplenigism are very much pronounced as the disease progresses. Hemorrhagic lesions, congestion, absence phase represented the disorganized and depleted lymphoid system. A more or less similar phase of reaction is observed of germinal centers, hemosiderosis, increase in follicular cells, and focal necrosis. Formation of giant cells due to aggregation of histiocytes starts developing around the sinusoids also reported by Uche and Jones.[27] We asked whether histological defects on spleen, an important immunological organ, also occur in diet-induced obesity. Specifically, we focused on the effect of fatty diet induced obesity on spleen structure and we tried to understand what histological change may cause of splenomegaly present in obese. Body mass index and the amount of diet consumed of rats in the treament group were 2 times higher compared with the control group (p<0.05). Thus, we evaluated that the treated rats was become obese due to our diet model. In studying spleen slices, macrophages and necrotic figures needs to be defined and sinusoidal dilatation and hemosiderin deposits were observed. There were many macrophages filled with hemosiderin droplets in these sections. We obsereved an extra cellular lipid aggregations in dilated sinusoids. Moreover, perivascular eosinophilic areas were accessed as amyloid. According to our volumetric results, the mean spleen volume in the treatment group was higher than those of the control group indicating splenomegaly. If this stereological data combined with light microscopical examination; we suggest that fatty diet induced obesity may be cause of splenomegaly via sinusoidal dilatation and extra cellular deposits such as lipid and hemosyderin.

Finally, our results clearly indicated that high fat diet caused to splenomegaly via sinusoidal dilatation and intra-cellular or intercellular deposits.

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