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# HISTOCHEMICAL EFFECTS OF BRODIFACOUM ON RAT SPLEEN

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**ABSTRACT.** In this study, the histochemical effects of Brodifacoum, an anticoagulant used against rodents, on the spleen are examined under a light microscope using CD4 and CD8 histochemical staining methods. A single dose of 0.2 mg Brodifacoum was dissolved in Dimethyl Sulfoxide (DMSO) and was given orally to mature male rats. Spleen samples were collected under ether anesthesia after 24 h, 72 h, 14 days, and 30 days from the rats in the experimental groups and after 14 days from the rats in the control group. In this light microscope study, it was observed that the capsule, white pulp, and red pulp zones in the rat spleen were constructed normally and as their natural structures primary and secondary follicles (germinal center) they were few, and CD4 and CD8 lymphocytes were spherically structured. In the 24 h spleens of the rats, the diameters of germinal centers were expanded and deterioration of the structure of CD4 and CD8 cells was observed. Related to the increase in time (72 h and 14 days) it was determined that primary follicles increased in number and the diameters of germinal centers expanded. In addition to this, after30 days, the rate of CD4:CD8 of the brodifacoum applied rat spleens were approximately the rate of the control group, and the improvement of the structures of the cells was reported as an effect of regeneration. As a result of this study, it was found that Brodifacoum caused immunohistochemical abnormalities in the rat spleen, affected the morphological structure of CD4 and CD8 T lymphocytes and created an immune response in rats. It is thought that the obtained results will be a source for the studies on Brodifacoum.

## 1. INTRODUCTION

Colloquially known as rat poison, Rodenticides are a chemicals used for pest and rodent population control [1]. There are two groups of rodenticides

*Keywords*. Brodifacoum, Rattus norvegicus, spleen, histochemistry, anticoagulant, CD4 T lymphocyte, CD8 T lymphocyte, light microscope

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commercially available. These are acute and anticoagulant rodenticides. Anticoagulant rodenticides are among the most important pesticides used for the control of harmful rodent populations [2]. The hydroxycoumarine derivatives Difenacoum, Brodifacoum, and Bromadiolone belong to the group of second-generation anticoagulant rodenticides. Second-generation anticoagulants (4-anticoagulants) inhibit one or more enzymes related to blood IX, II, VII, and K synthesis. The lipophilic structure of long-acting anticoagulant rodenticides facilitates the tight binding of the enzymes to the cell membranes they target [3]. Anticoagulant rodenticides are stored in the liver until the microsomal binding sites are saturated [4]. It has been reported many times that rodenticides have toxic effects on non-target organisms. Therefore, it is imperative to investigate the possible effects of these dangerous chemicals on organisms. Second-generation anticoagulant rodenticides have been reported multiple times to contaminate non-target wildlife, particularly poisoning primary predator birds and mammals, and their routes of exposure are not yet understood [5]. However, it has been reported that the poison accumulates in the body because of its consumption [6]. In a study with rabbits, one of the non-target animals, the clearance times of Warfarin, Brodifacoum and Difenacoum from plasma were compared and it was shown that Brodifacoum was cleared more slowly than warfarin, but Difenacoum spread to larger volumes [7]. Predators are at a risk of secondary poisoning due to the persistent accumulation of anticoagulants in the liver [8]. In mammalian predator population studies, for example, anticoagulant rodenticide residues were found in 84% of the red fox (*Vulpes vulpes*) population [9]. The presence of anticoagulant rodenticides was investigated in liver samples taken from red-tailed hawk (*Buteo jamaicensis*) and great-horned owls (*Bubo virginianus*), and they were found in 81% of red-tailed hawks and 82% of great-horned owls [10].

The spleen plays an important role in host defense. It is the organ where antigens from the blood are cleared. The spleen is an essential organ for blood homeostasis [11]. These functions are performed by two structurally and cellularly different components of the spleen, white pulp, and red pulp. The spleen is a vascular organ as it acts as a blood filter. The arterial branches were surrounded by lymphoid tissues. The main arteries feed the red pulp, and the smaller arteries feed the white pulp [12]. Lymphocytes involved in the immune system consist of two main groups, B and T. T lymphocytes get their name from the Thymus from which they are formed about 80% of all lymphocytes in the peripheral blood. T lymphocytes have several subgroups. One of these groups, helper T lymphocytes, is recognized by the CD4 marker protein on their surface, while another group of cytotoxic

T lymphocytes is recognized by the CD8 marker protein. These cells play important tasks in the immunity of the organism. Helper T lymphocytes secrete the cytokine hormone, which initiates immune system reactions. Cytotoxic T lymphocytes, on the other hand, neutralize pathogenic organisms or molecules with some enzymes they secrete [13]. The development of the primary immune response involving T lymphocytes may take up to 14 days, depending on the antigen and the region where it enters the organism [14]. There are 30%-40% T lymphocytes in the spleen. According to Langeveld et al.'s (2006) study, it has been suggested that CD8 T lymphocytes are more abundant than CD4 T lymphocytes in the human spleen. It has been determined that active cells are in most populations of these cells and CD8 T cells are mostly in the cytotoxic CD27(-) CD45RA(+) memory cell phenotype [15]. It has been shown that the fact that the lymphocyte subgroups in the spleen differ according to the lymphocyte subgroups in the peripheral blood means that CD4 and CD8 T lymphocyte activation in the spleen has an important and distinct place [15]. It has been reported that CD4+ and CD8+ T cells play an important role in the cytokine response to virus infection, and the release of antiviral and regulatory cytokines is shared between these two-T lymphocyte subgroups [16]. In previous studies, the effects of Brodifacoum on the histological structure of the spleen have been observed. In this study, both structural and immunological changes in the membranes of rodent spleen cells, CD4 helper T lymphocytes, and CD8 cytotoxic T lymphocytes and how Brodifacoum affected the immunity of rats were determined. In this study, it was observed that Brodifacoum affected rat spleen structures and an immune response occurred in rats on the 7th and 14th days. It was thought that the results of the study would be a source and support for studies on the resistance of rats to rodenticides.

## 2. MATERIALS AND METHODS

### 2.1. Experimental animals

After the approval of the Ankara University Ethics Committee (Decision number: 2014-18-133), 30 male albino rats (*Rattus norvegicus*, 7–9 weeks old, 200–250 g) were used in the experiments, 24 of which were in the experimental group and 6 of them are in the control group (Table 1). Before starting the experiments, the experimental animals were quarantined for seven days. Each animal was kept in separate cages under appropriate laboratory conditions (22±2℃; 12h:12h photoperiod, 60% relative

humidity). Animals were fed ad libitum with solid food throughout the experiment.

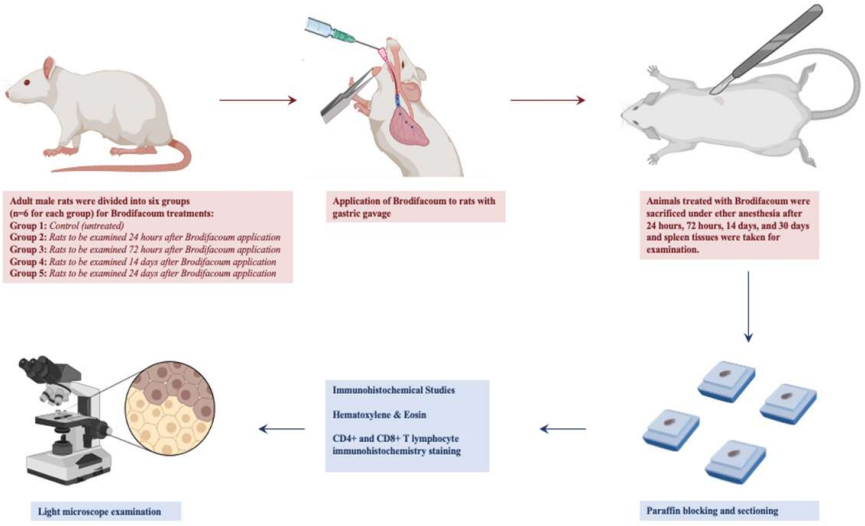


FIGURE 1. Schematic representation of the implementation stages of the experiment (Created with BioRender.com)

## 2.2. Brodifacoum Application

Experimental animals were divided into five groups (Table 1). Once groups of animals were determined, optimum drug doses (below LD50 dose to determine chronic cytotoxic effects) were calculated by measuring the weight of each rat.

TABLE 1. Experiment groups

| | | |
|---|---|---|
| Group 1 | *Control group rats* | n: 6 |
| Group 2 | *Rats to be examined 24 h after Brodifacoum application* | n: 6 |
| Group 3 | *Rats to be examined 72 h after Brodifacoum application* | n: 6 |
| Group 4 | *Rats to be examined 14 days after Brodifacoum application* | n: 6 |
| Group 5 | *Rats to be examined 30 days after Brodifacoum application* | n: 6 |

A dose of 0.2 mg/kg BW (Body Weight) Brodifacoum prepared with DMSO (Dimethyl sulfoxide)/polyethyleneglycol-400/water (0.02ml/1ml/1ml)

solution was given orally to rats by gastric gavage method [4]. 1ml/kg BW DMSO (Dimethyl sulfoxide)/polyethyleneglycol-400/water (0.02ml/1ml/1ml) solution was administered to the control group animals by gastric gavage. Spleen samples were taken from the rat groups under ether anesthesia 24 h, 72 h, 14 days, and 30 days after Brodifacoum was applied, while spleen samples were taken 14 days later from the control group [17].

### 2.3. Light Microscope Preparation

Spleen samples taken from rats in the control group (after 14 days) and Brodifacoum (0.5 mg/kg) administered (24 h, 72 h, 14 days, and 30 days) under ether anesthesia were prepared for examination under a light microscope. The removed spleen samples were weighed on a precision balance, the samples were washed with distilled water, and the samples were taken in 2.5% Formaldehyde (prepared in 0.1 M Sodium phosphate buffer) and their first determinations were made (2 h, +4 °C). After fixation, the samples were washed with the same buffer (washed with 3 solution changes in 1 h) and then incubated for 5 min in alcohol series (80, 90, 96, and 100%) to dehydrate the samples. The samples were embedded in paraffin blocks by passing through xylol and 5μ thick sections were taken using a microtome [18].

### 2.4. Immunohistochemical Analysis

Hematoxylin-eosin staining was performed to examine the general structure of the spleen. The sections taken were treated with hematoxylin and then washed under running water. After being treated with 95% ethyl alcohol, it was dyed with Eosin and the excess paint was removed by passing it through ethyl alcohol again. Special antibody staining kits (Santa Cruz Biotech, USA) were used to examine CD4 and CD8 T lymphocytes. The instruction manual in the kit was followed for CD4 and CD8 T lymphocytes immunohistochemical staining. The stained sections were examined under a light microscope (Olympus BX43, Tokyo, Japan).

## 3. RESULTS

This study investigated how Brodifacoum affected the spleen weights and histological structure of the spleen in rats. According to the spleen weight measurements (Figure 2.a.), although the spleen weight of the rats treated with Brodifacoum for 24 h, 72 h, and 14 days compared with the control

group decreased, a weight close to the control group was detected during the 30-day application period. The effect of Brodifacoum on CD8 and CD4 lymphocyte counts is given in Figure 2. b and 2. c. It was determined that CD4 and CD8 levels increased at the same rate in terms of the effect of brodifacoum on T lymphocyte types.

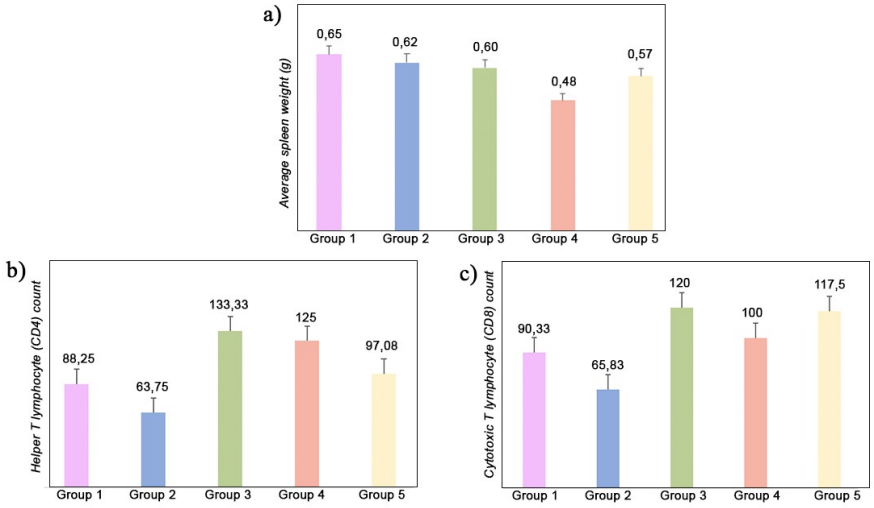


FIGURE 2. a) Spleen weights of rats treated with Brodifacoum b) Helper T lymphocyte (CD4) count of Brodifacoum-treated rat groups c) Cytotoxic T lymphocyte (CD8) count of rat groups treated with Brodifacoum

In this study, the effect of Brodifacoum on CD4 (helper T) lymphocytes, which stimulate other cells by secreting cytokines in the presence of immune antigen in the white pulp region of the spleen of rats, and on CD8 (cytotoxic T) lymphocytes, which eliminate foreign organisms and toxic substances in immunity with their enzymes, were examined by the histochemical method. When the control group was examined histologically, it was found that the capsule, white pulp, and red pulp regions in the spleen were in the normal structure and that the primary and secondary follicles (germinal center) were few in number as in their natural structures (Figure 3). A large number of normal erythrocytes, lymphocytes, and macrophage cells were found in the red pulp area. In terms of helper T lymphocytes, it was observed that the spleen of CD 4 Control group rats had a normal structure and white pulp, red pulp, and primary follicles were properly located. Additionally, it was noted

that there were a few primary follicles in the spleen samples taken from the control group of rats. In the spleen of the rats in the control group, CD4 T lymphocytes were found to have a spherical shape in the white pulp region where thymus-derived T lymphocytes were located. It was determined that CD4s were more numerous in this region compared to other cells. In the CD8 control group, white pulp and red pulp regions were prominent in the spleen structure, and there were hardly any primary and secondary follicles. It was observed that CD8 T lymphocytes in the control group were round or nearly angular and found in the same proportion as other cells.

In the spleen samples taken from rats treated with Brodifacoum for 24 h in the study, it was observed under the light microscope that large-scale germinal centers were formed and erythrocytes proliferated (Figure 4). In the spleen sample taken at the end of 24 h, it was determined that the lymphocyte count increased, and the erythrocytes were smaller than normal compared to the control group. As a sign of immunity, many newly developing primary follicles and a few secondary follicles (germinal centers) were found, in which primary follicles and germinal centers became prominent. It was observed that in the white pulp region, CD4 T lymphocyte cells also deteriorated and decreased in number compared to the control group, while in the red pulp region in the spleen of the CD8 group, the morphology of CD8 cytotoxic T lymphocytes changed from a round shape to a triangular or pentagonal shape compared to the control group and decreased compared with other cells.

The presence of primary and secondary follicles and the width of their diameters were noted in the spleen sample taken from the rat 72 h after the application of Brodifacoum, it was observed that erythrocytes were more numerous than lymphocytes and that they were smaller than their normal structure (Figure 5). While small-diameter primary follicles were seen in the areas close to the capsule, secondary follicles were not found and germinal center diameters were observed to be large. It was noted that the diameters of CD4s were smaller and in different shapes compared to the control group, and simultaneously, the number of cells other than CD4 was less. It was observed that CD8s, in which other cells were reduced, changed shape, were large and small, and macrophages were more numerous.

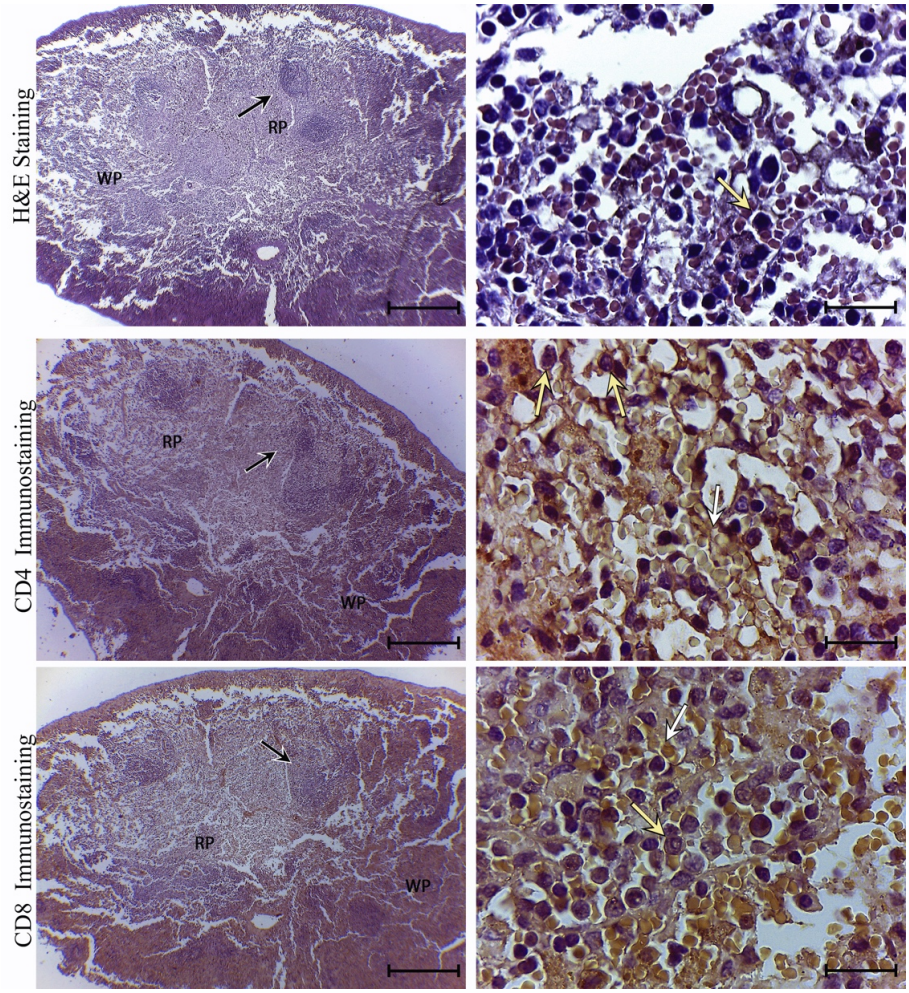


FIGURE 3. The general view of the control group rat spleen. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

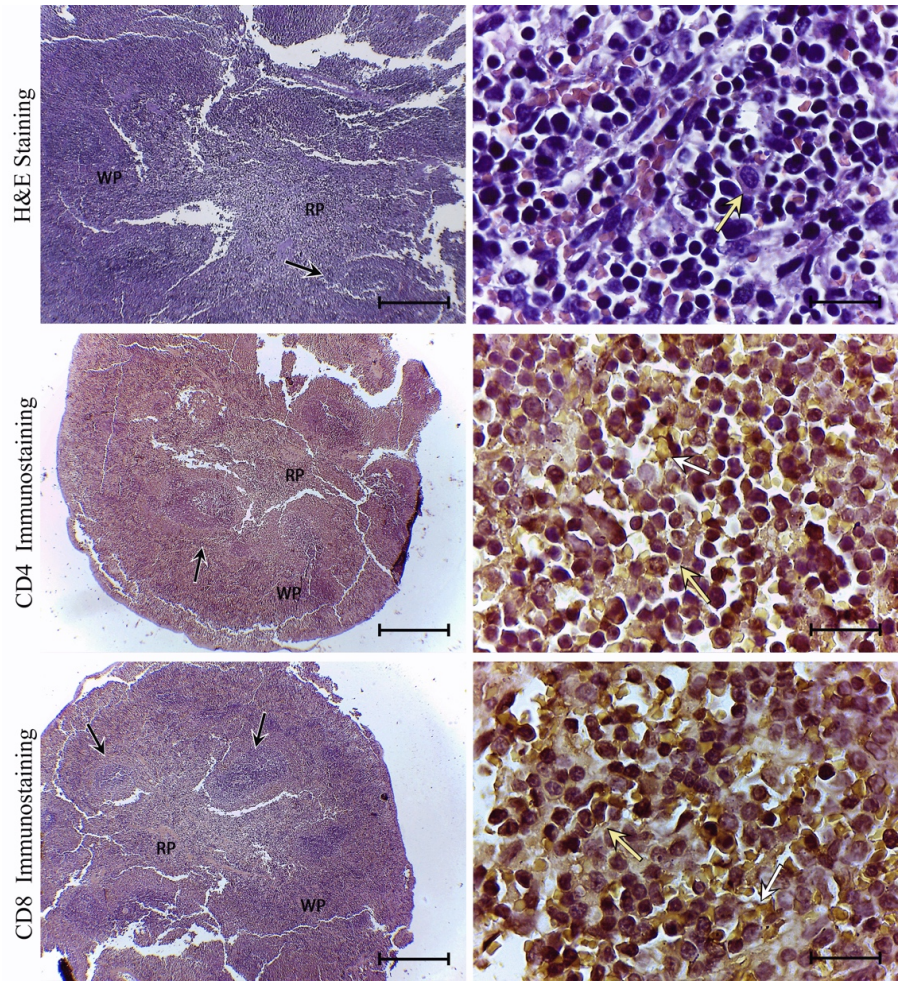


FIGURE 4. The general view of rat spleen taken 24 h after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

It was observed that the primary and secondary follicles in the spleen covered the entire organ on the day when the immunity was most intense 14 days after the application of Brodifacoum, which was the fourth experimental group (Figure 6). We observed that the erythrocytes, which should be of the same size as the lymphocytes, were gradually getting smaller compared to the control group. In some places, lysis and cell groups

were observed in the tissue, it was observed that CD4s changed into triangular or rectangular shapes in the white pulp region compared to the control group, and they fused in some regions. It was noted that CD8s were few, shrunk by changing their shape and formed groups, whereas cells other than CD8s were more numerous.

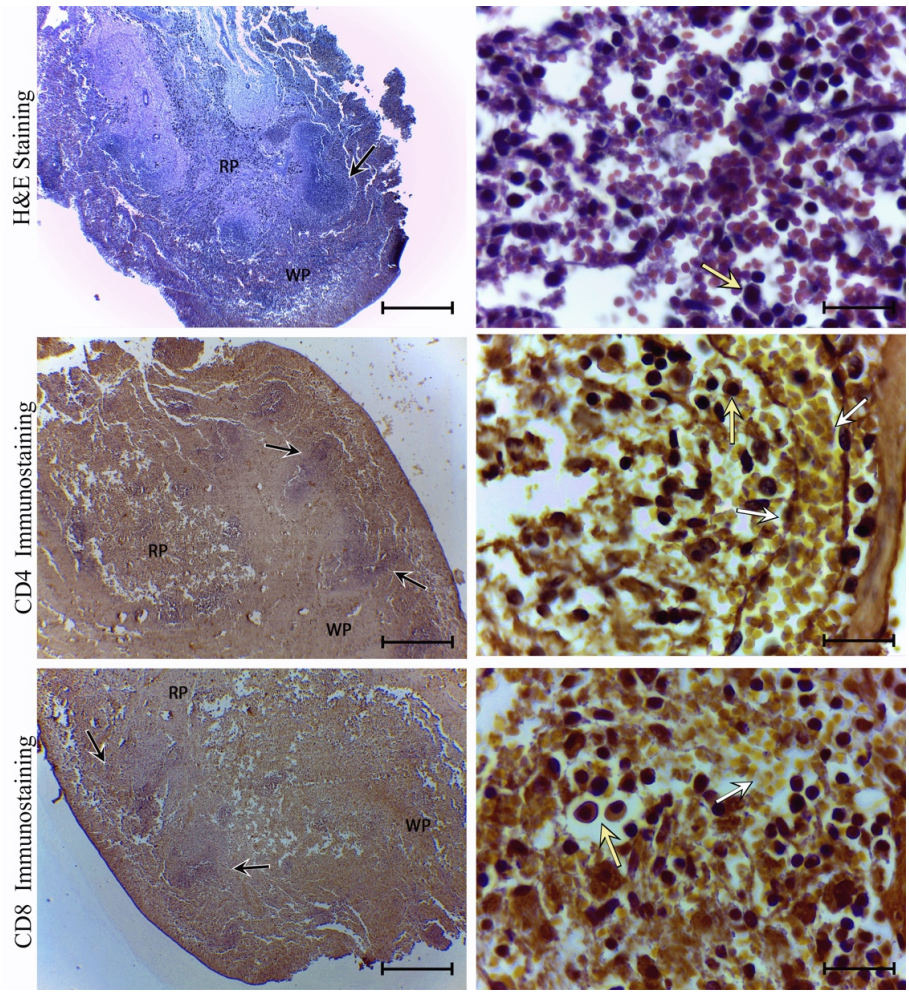


FIGURE 5. The general view of rat spleen taken 72 h after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 µm, right: 50 µm)

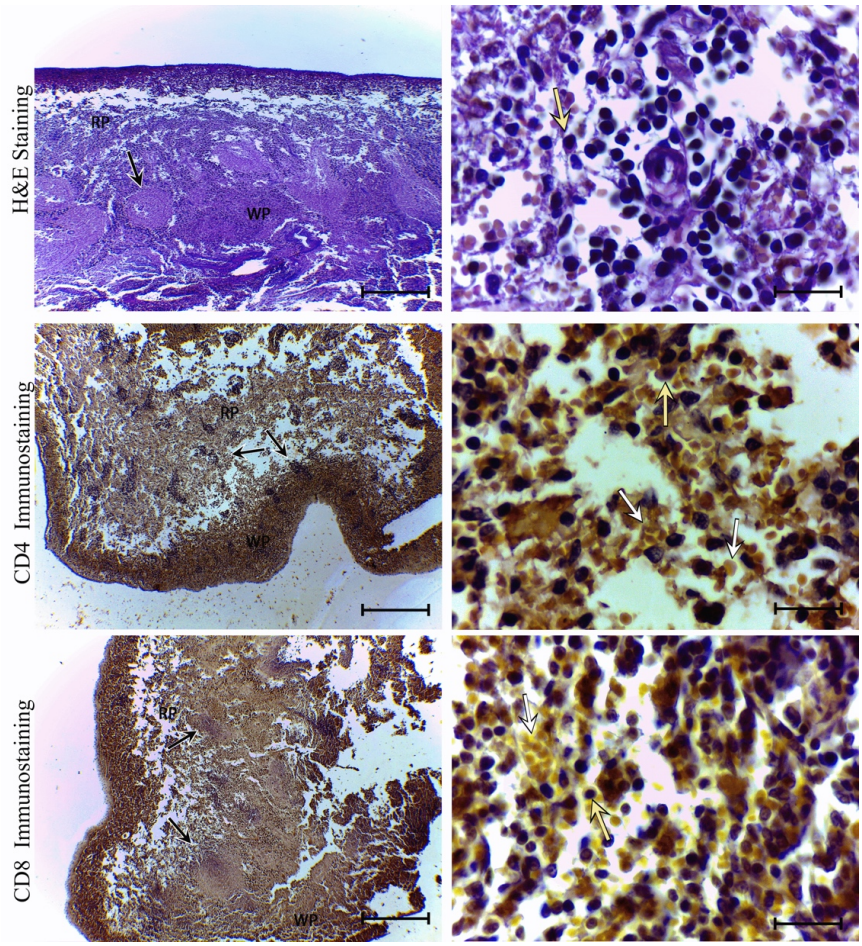


FIGURE 6. The general view of rat spleen taken 14 days after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

In the spleen samples taken 30 days after the application of Brodifacoum, which is the fifth and last of the experimental groups, it was observed that there were few primary and secondary follicles in the spleen like in the control group (Figure 7). A large number of lymphocytes and a smaller number of irregularly adhered erythrocytes compared with lymphocytes were observed. In the general structure of the spleen, the germinal centers

were almost non-existent, whereas the large and small diameter primary follicles were numerous, which was accepted as a sign of recovery. As in the control group, CD4 T lymphocytes were observed to be round in shape and all of approximately the same size. This situation was thought to be a sign of retrograde recovery, and few follicles were observed as another sign of recovery. It was observed that CD8 T lymphocytes were close in size to the control group and were in the same shape as the cells in the control group.

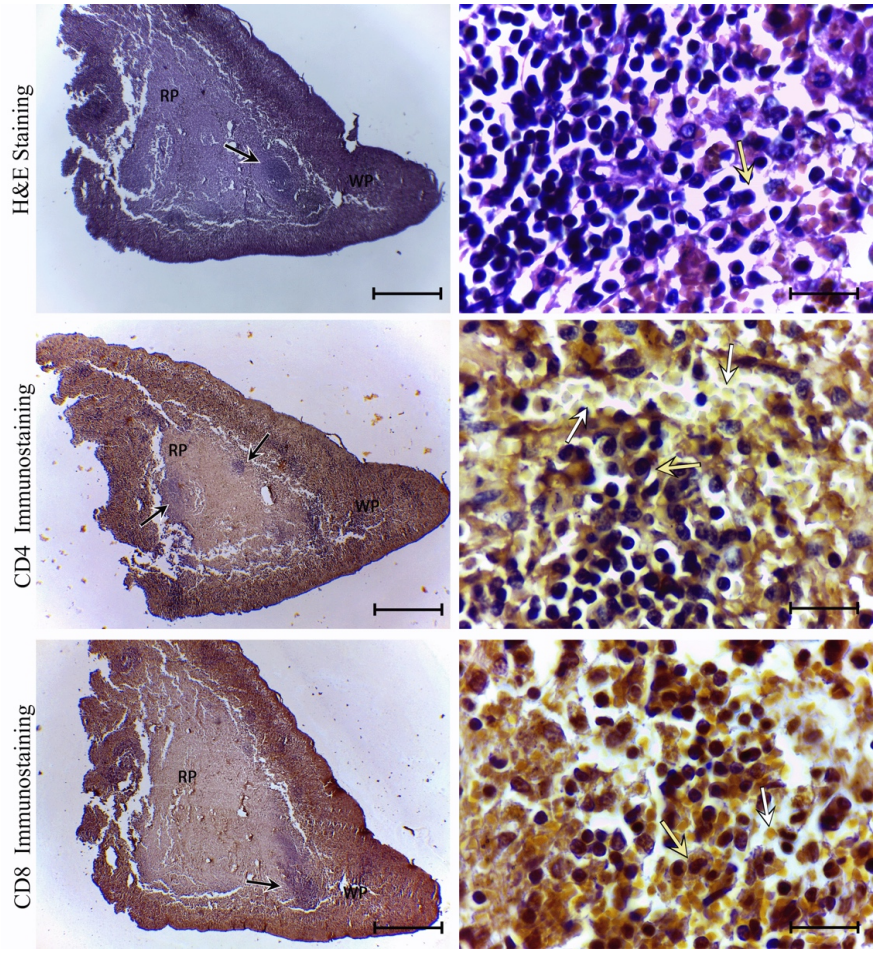


FIGURE 7. The general view of rat spleen taken 30 days after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

## 4. DISCUSSION

T cells are divided into 6 groups: helper T cells (CD4+ T cells), cytotoxic T cells (CD8+ T cells), effector T cells, suppressor T cells, memory T cells, and delayed-type hypersensitive (DTH) T cells. CD4+ T cells and CD8+ T cells are T cells involved in the immune system. CD4+ T cells assist cellular immune function, while CD8+ T cells can destroy target cells labeled with specific antigens [19]. Therefore, CD4 and CD8 T-cell counts are an important indicator of cellular immunity [20]. In a study on the effect of boron on spleen T lymphocytes in rats, the results obtained from immunohistochemical staining showed that the cytoplasm of CD4+ and CD8+ cells was yellowish brown, and the cells were scattered in and around the splenic periarteriolar lymphoid sheaths [21]. It is possible to interpret the immunohistochemical staining results of our study similarly. Likewise, immunohistochemical staining showed that the number of CD4 cells increased as the boron concentration increased [21]. Our study observed that Brodifacoum increased the number of CD4 cells on the 14th day, which is the most intense day of immunity. Because of immunohistochemical staining, deterioration in lymphocyte shapes was observed on the 72nd and 14th days. In the study examining the effect of Cyclosporine A on rats, structural deterioration was observed in lymphocytes in the spleen tissues, similar to our study [22]. The effect of ethanol on the immune system was investigated in a study on mice [23]. In this study, the ETOH diet was applied to mice and CD4 and CD8 cells in the thymus and spleen were examined by immunohistochemical staining at certain time points. According to the findings of the study, T cell loss was detected in mice on the ETOH diet. In our study, when the first 14 days are examined, the decrease in the numbers of CD4 and CD8 shows compatibility with this study. When lymphocytes in mouse spleens treated with ETOH were examined by immunohistochemical staining, it was revealed that T lymphocytes were structurally smaller, which is similar to our study.

Humoral and cellular immunity was investigated by administering Propoxur (PPX) insecticide to C57-bl/6 mice. Because of this study, it was reported that PPX at a dose of 10 mg/kg suppressed the delayed-type hypersensitivity response and increased the percentage of CD4(-)/CD8(+) T cells [24]. In another study, it was reported that cocaine increased cytokine-secreting CD4(+) T lymphocytes in the spleen in vitro [25]. In our study, it was observed that the CD4/CD8 ratio increased on the 14th day, which is the immune response was most intense.

It was administered to rats and the changes were observed to examine the changes in the spleen as a result of the toxicity of chlorophem. The spleen weights of the experimental rats increased significantly compared to the control group of rats, and fibrosis in the capsule and lesions in the parenchyma was observed [26]. In our study, it was observed that the weight decreased in groups 2, 3, and 4 in which Brodifacoum was applied and increased in group 5. It has been interpreted as a part of the healing process.

By the current research, Brodifacoum causes various clinical symptoms in rabbits and rats, including drowsiness, fatigue, anorexia, decreased movement, and rapid and easy exhaustion after administration [27]. Because of our experiments, male rats treated with Brodifacoum experienced fatigue, loss of appetite, etc., similar to previous studies. Weight loss was observed in Brodifacoum-administered rats because of anorexia. Likewise, studies have reported that Brodifacoum causes weight loss. Behavioral disorders and mouth and nose bleeding were observed in rats after Brodifacoum administration [28].

## 5. CONCLUSION

In this study, the damaging effect of Brodifacoum on the spleen of rats in the mammalian group, including humans, was demonstrated histochemically. It has been understood that Brodifacoum even disrupts the structure of CD4 and CD8, which shows an immune reaction, and therefore is effective against rats. It is thought that the results obtained from this study will contribute and be a source for chemical control studies against rodents. In addition, this study reveals that the spleen structure will histologically deteriorate in humans and non-target organisms that accidentally come into contact with Brodifacoum.

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**Author Contribution Statements** BBÖ - Conceptualization, Investigation, Data curation, Formal analysis, Writing. NG – supervising, resources, conceptualization, writing–review & editing. Both authors have read and approved the manuscript.

**Declaration of Competing Interests** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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