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Expression of HIF-1α, Caspase-3, p53, HSP70, HSP90, and NRF2 in the canine spleen with siderofibrotic nodules: an immunohistochemical and immunofluorescence perspective on apoptosis, ferroptosis, and oxidative stress

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Abstract: Splenic siderofibrotic nodules (SFNs) are benign tiny yellow-brownish foci found in dogs. Their pathogenesis is unclear whereas trauma and hemorrhage are assumed to be the possible causative factors. The spleen of 73 Iranian dogs of the mixed breed was examined for possible splenomegaly with SFNs. Ultrasonography, blood biochemistry, pathology, and immunohistochemistry revealed splenomegaly (n = 7), splenomegaly with SFNs (n = 6) and normal spleens (n = 60). There was no significant difference between the prevalence of splenomegaly with SFN among the examined dogs and their age or sex ($p > 0.05$). Ultrasonography represented a significant increase ($p \leq 0.01$) in spleen size, as well as serum Malondialdehyde (MDA) level, which increased significantly ($p < 0.05$) in dogs with splenomegaly compared to normal animals. Using Masson's trichrome and Pearl Prussian blue staining methods, SFNs' hallmarks of fibrosis (collagen deposition) and iron deposition were confirmed. Immunohistochemically, a significant increase of $S100^+$ dendritic cells, $CD3^+$ T cells, and $CD68^+$ macrophages, as well as a reduction of $CD20^+$ B cells representing chronic splenitis with splenomegaly and SFNs (n = 6), were revealed. Further, overexpression of vWF, HIF1-α, p53, caspase 3, and HSP70 and HSP90 were detected in the spleens with SFNs compared to normal animals. HSP70, HSP90, and p53 expressed at increased levels in spleens with SFNs mediate apoptosis and ferroptosis through interaction with factors like HIF1-α. Moreover, decreased immunofluorescence intensity for NRF2 expression in the SFN group represented the promotion of ferroptosis compared to normal spleens. Altogether, in canine splenomegaly with SFN, concurrent upregulation of p53, caspase-3 active, HSP70 and HSP90 as well as downregulation of NRF2, contribute to both apoptosis and ferroptosis, involving cellular injury through the interactive complicated network. Also, enhancement of MDA level and ferroptosis may reflect long-term exposure to ROS of dogs with splenomegaly and SFNs.

Key words: Apoptosis, canine spleen, ferroptosis, siderofibrotic nodules

1. Introduction

Splenic disorders in dogs mostly manifest as focal nodular changes or diffuse enlargements named splenomegaly [1]. Splenomegaly may be caused by congestion or some other infiltrative diseases. In line, various bacteria, viruses, fungi, and protozoa can cause splenitis in dogs, resulting in splenomegaly [2]. It has been reported that small-breed dogs were more susceptible to splenomegaly than larger breeds. However, in the veterinary literature, no greater risk of splenomegaly has been mentioned in these breeds [3]. Lymphoid hyperplasia and siderofibrotic nodules are two common changes in the canine spleen that may be associated with old hemorrhagic foci, aging, and disease recovery situations. There is an increase in the number of lymphoid foci in white pulps associated with lymphoid hyperplasia while siderofibrotic nodules have consisted of a dense accumulation of pigments and iron with collagen fibres, hemorrhage, and inflammatory immune cells infiltration with or without calcium deposits could be observed in the nodules [2,4]. Until now, few gross and pathological findings and necropsy observations reporting the presence of siderofibrotic nodules (SFNs) or Gamna-Gandy bodies (GGBs) in the canine spleen have been reported [5,6]. There are several types of regulated cell death (apoptosis, necrosis, necroptosis, and so on), but ferroptosis is unique from the rest due to its morphological, physiological, and biochemical differences. Although the defining patterns and the activation of ferroptosis have not yet been identified, it is generally characterized by an increased reactive oxygen species (ROS) accumulation, as one of the main products of elevated iron metabolism and lipid peroxidation, in various tissues [7]. A growing body of research suggests that pathological lipid peroxidation induces the overproduction of ROS that plays a crucial

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role in the process of cell death, including apoptosis, autophagy, and ferroptosis. Accordingly, the lipid peroxidation products interact with membrane receptors and transcription factors (or suppressors) that may end up with internal and external apoptotic signals [8]. Therefore, lipid peroxidation is a well-known mechanism for cellular injury that is used as a marker for oxidative stress of cells and tissues. In this respect, malondialdehyde (MDA) is considered a reliable biomarker to assess lipid peroxidation [9]. The nuclear factor erythroid 2-related factor 2 (NRF2) is a crucial nuclear transcription factor, which controls a battery of cellular defensive genes to maintain redox homeostasis and cell survival. Recently, NRF2 has been identified as a regulator of ferroptosis. In acute or chronic tissue/cell damage, NRF2 stabilization can inhibit ferroptosis and subsequently relieve injury [10]. In this study, therefore, we evaluated Iranian dogs of mixed breed for splenomegaly with SFNs by clinical signs, ultrasonography, hematology, biochemistry as well as pathology and immunohistochemistry (IHC). Indeed, we tried to differentiate and count the various splenic cells by immunohistochemical markers CD3, CD20, S100, and von Willebrand Factor (vWF) in the normal spleen and those had splenomegaly with SFNs. Moreover, the role of the possible factors involved in canine splenomegaly and SFNs- creation such as hypoxia, oxidative stress and lipid peroxidation were assessed by immunohistochemistry [Hypoxia Inducible Factor 1-α (HIF1-α), caspase 3, p53, Heat Shock Protein 70 (HSP70) and Heat Shock Protein 90 (HSP90)] and serum MDA level as well as immunofluorescence (IF) for NRF2. Finally, looking for a logical relationship of the interaction of the possible factors in the pathogenesis of cellular injury in the canine spleen, the interactive role of apoptosis and ferroptosis in regulating cell death was evaluated in the canine spleen with splenomegaly and SFNs.

2. Materials and methods

2.1. Animal groups and ultrasonography

The present research has been conducted under the supervision of Research Ethics Committee of Islamic Azad University-Urmia Branch with approval ID (IR.IAU.URMIA.REC.1401.027) dated (2022-04-24). From June 2018 to June 2021, 73 Iranian dogs of the mixed breed were evaluated for suspect splenomegaly. The examined male and female dogs were divided into three age groups based on tooth wear including under 3 years (n = 19); 3–6 years (n = 29) and over 6 years old (n = 25) (Table 1). Clinically, the dogs were evaluated by a physical examination and the case clinical signs such as anorexia, fever, vomiting, lethargy, anemia, or abdominal expansion were noted. To evaluate splenomegaly existence, abdominal ultrasonography with a 10 MHz linear probe was performed using a GE logiq e ultrasound machine (GE Healthcare Company, USA). For this purpose, the dogs were stabilized in a right lateral recumbency position without any sedation/anesthesia [11]. In this respect, three ultrasonographic indices of canine spleens including length in the sagittal section, width in the sagittal section, and height in the cross-section were measured. Next, to perform an ultrasound-guided biopsy, the animals were anesthetized with intravenous injectable propofol 6-10 mg/kg (PropoFloTM 28, Zoetis, USA). Then, the entry place of the needle (16G Tru-cut needle) on the skin was disinfected with chlorhexidine, and made a small cutaneous incision was performed to insert the needle. Subsequently, an ultrasound-guided biopsy was performed and the retrieved splenic tissue sample was placed in neutral 10% buffered formalin for fixation and histopathological procedures [12].

2.2. Hematology and MDA assessment

The collected blood samples from the cephalic vein of all examined dogs were divided into two parts. Sample a) for hematological analysis, the blood samples were collected in EDTA-containing tubes. Next, a complete blood count (CBC) analysis was conducted using an automated blood analyzer (BK 6200 hematology analyzer, China). Sample b) to evaluate serum MDA level, the blood samples were collected in anticoagulant free tubes and centrifuged at 1200 rpm for 5 min. The collected serums were kept at –70 °C for further biochemical analysis. Lipid peroxidation assessment of serum MDA concentration as reactive thiobarbituric acid (TBA) was performed by its absorbance measuring (at 523 nm). Indeed, MDA, as a secondary product, is produced during the heating TBA and polyunsaturated fatty acid in an acidic medium [9].

Table 1. Prevalence of splenomegaly and splenomegaly with SFNs based on the age and sex of the dogs.

Age (year)	Number	Mean (±SD) body weight (kg)	Sex		Normal spleen		Splenomegaly		Splenomegaly	
			M	F	M	F	M	F	M	F
<3	19	10.23 ± 2.12	11	8	9	8	1	-	1	-
3–6	29	16.41 ± 1.84	16	13	12	11	3	1	1	1
>6	25	22.11 ± 2.33	14	11	11	9	1	1	2	1

2.3. Pathology analysis

Out of 73 dogs, there was a suspicion of splenomegaly in ultrasonographic evaluations of 19 dogs. Therefore, for pathological definitive diagnosis, the collected spleen tissues [ultrasound-guided biopsy (n = 12), splenectomy (n = 4), and necropsy (n = 3)] were placed in neutral 10% buffered formalin for fixation. Out of 3 necropsied dogs, 1 dog with dirofilariasis was euthanized by a standard protocol (intramuscular injection of acepromazine followed by sodium pentobarbital intravenously) and 2 dogs died naturally. Of course, 10 µg/kg epinephrine was injected intravenously one min before barbiturate administration to prevent splenic congestion [13]. Following tissue processing procedures (dehydration, clearing, and infiltration) the 5 µm sections were prepared from paraffin-embedded tissue blocks (Leica RM2125 RTS, Germany). Then, all the tissue sections were stained by Hematoxylin and Eosin (H&E) staining method. Meanwhile, we used Perl's Prussian blue and Masson's trichrome staining methods for the tissue sections with SFNs to confirm iron and collagen fibers deposition. The histologic sections were blindly examined by a pathologist. On the other hand, to evaluate the positive reactions, the photomicrographs of cross-sections were analyzed with ImageJ software (Media version: 6.00; National institutes of health, USA). For this purpose, 20-megapixel photos were taken from cross-sections and thereafter, the pixel-based intensities of blue-stained (representing collagen in Masson's trichrome) and dark blue-stained (representing iron-positive reaction in Perl's Prussian blue) per total pixels were evaluated 2330 µm × 2330 µm of a photomicrograph.

2.4. Immunohistochemistry and immunofluorescence

Immunohistochemical labeling was performed by EnVision + Dual Link System-HRP system based on the manufacturer's suggested protocol. Briefly, the tissue sections with a thickness of 5 µm were dewaxed by xylene and rehydrated with descending gradients of ethanol. In addition, endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide (H2O2) in methanol for 30 min. The 0.01 M citrate buffer (pH 6.0) was used for antigen retrieval (25 min). Thereafter, to stop nonspecific antibody binding, the sections were incubated in 5% bovine serum albumin in Tris-buffered saline (TBS) for 30 min. Next, the sections were washed in water and TBS and thereafter incubated in a humid dark chamber. The primary antibodies that we used for immunohistochemical staining were CD3 (Dako, Glostrup, Denmark; 1: 200), CD20 (Dako, Glostrup, Denmark; 1:400), CD68 (Dako, Glostrup, Denmark; 1:100), S100 (Dako, Glostrup, Denmark, 1:800), vWF (Dako, Glostrup, Denmark; 1:1000), HIF1-α (Bethyl Laboratories, USA; 1:100), caspase 3 (Abcam, UK, 1:350), p53 (Elabsciences, USA, 1:300), HSP70 (Elabsciences, USA, 1:250), and HSP90 (Elabsciences, USA, 1:300). The slides were incubated with the primary antibodies (overnight, at 4 °C) and rinsed in phosphate-buffered saline. Subsequently, the sections were incubated with streptavidin-horseradish peroxidase for 20 min and rinsed in the buffer. Finally, the slides were incubated with diaminobenzidine at room temperature for 10 min to visualize the immunoreactions and counterstained with Harris's hematoxylin [14]. Appropriate positive controls were performed concurrently and negative controls were run by omitting the primary antibodies as well.

Spangler method was used for immunohistochemical scoring of the normal and SFN-positive splenomegaly spleens [15]. Accordingly, cytoplasmic or nuclear intensity of the immunolabeled splenic cells was scored as 0 (negative), 1 (nuclear or cytoplasmic reaction up to 15% of tissue cells), 2 (nuclear or cytoplasmic reaction in 15%–40% of tissue cells), 3 (nuclear or cytoplasmic reaction in 40%–70% of tissue cells), 4 (nuclear or cytoplasmic reaction in >70% of tissue cells). Moreover, positive immunolabeled cells were counted in 1 mm^2 of each tissue section. Indeed, 10 fields of each section were considered. In addition, similar to histopathologic analyses (Masson's trichrome and Perl's Prussian stains) the positive intensities of immunoreactions in the splenic cells were analyzed with the software. For this purpose, the mean (±SD) of positive immunoreactivities (brown/total pixels) were assessed in 2330 μm^2 of each photomicrograph from one section (6 random microscopic fields/each animal) [14].

Immunofluorescent staining was accomplished in the spleen tissue by using the primary antibody NRF2 (Abcam, Cambridge, UK). Also, the secondary antibody goat antirabbit IgG (Elabscience, USA) was used for one h after washing the slides in Tris Buffered Saline TBS plus 0.03% Triton X-100. Next, the counterstaining was performed with 4,6–diamidino-2-phenylindole (DAPI) to stain the cell nucleus blue (0.1 µL DAPI in 10 µL Phosphate Buffered Saline)/section for 1–10 min. After that, the slides were washed 3 times for 1 min in TBS plus 0.03% Triton X-100 (with gentle agitation). Finally, the slides were mounted and put the coverslip on them to evaluate under a fluorescence microscope (Olympus BX50, Japan) [16]. Similar to immunohistochemistry, the mean (±SD) of positive immunoreactivities (green/total pixels) were assessed in 2330 μm^2 of each photomicrograph from one section (6 random microscopic fields/each animal) [14].

2.5. Imaging of the microscopic tissue sections

The histological sections were examined by a light microscope (Nikon, Japan), equipped with an ApoTome optical sectioning device and SONY on-board camera (Zeiss, Cyber-Shot). The figures were compiled using Adobe Photoshop CS10 (Adobe, Mountain View, California, USA).

2.6. Statistical analysis

We used IBM SPSS Statistics 25 software (SPSS, Chicago, Illinois, USA) for data analysis. To examine the relationship between demographic variables (age and sex) on splenomegaly chi-square test and Fisher's exact test were used. Moreover, one-way analysis of variance (ANOVA), Tukey posthoc and T student tests were used for independent groups in ultrasonography, immunohistochemistry/immunofluorescence and MDA assessments. $P < 0.05$ was considered a significant value. All data are presented as mean ± SD.

3. Results

3.1. Clinical findings

Out of the 73 dogs tested, 19 dogs (26%) were <3 years old [mean (± SD) weight of 10.23 ± 2.12], 29 dogs (39.7%) were 3–6 years old [mean (± SD) weight of 16.41 ± 1.84] and 25 dogs (34.2%) were >6 years old [mean (± SD) weight of 22.11 ± 2.33]. According to clinical signs such as anorexia, fever, vomiting, lethargy, anemia, abdominal expansion, ultrasonography and histopathology, 60 (82.2%) animals examined had normal spleen while 7 (9.6%) and 6 (8.2%) canine spleens had splenomegaly and splenomegaly with SFNs respectively. Moreover, the prevalence of splenomegaly and splenomegaly with SFNs based on the age and sex of the dogs are presented in Table 1. Accordingly, no significant difference ($p = 0.833$) was detected between age and the prevalence of splenomegaly and splenomegaly with SFNs. On the other hand, no significant difference ($p = 0.596$) was demonstrated between sex and the prevalence of splenomegaly and splenomegaly with SFNs. The comparison of the measured indices (length in sagittal section, width in sagittal section and height in cross-section) of the canine spleen showed that all the measured indices had a significant ($p < 0.05$) difference in the splenomegaly group relative to the normal group in all age categories (Table 2). In addition, the comparison of the sonograms (normal spleen and splenomegaly with SFNs) revealed that there were the tiny hyperechoic foci (SFNs) on the spleen and splenic parenchyma were hypoechoic, enlarged, slightly nonhomogeneous, and irregular whereas, the normal spleen had homogenous parenchyma with a regular echogenicity (Figures 1A and 1B). Given the various examinations of the dogs, the main findings related to splenomegaly and splenomegaly with SFNs are presented in Table 3. Accordingly, our results show the age range of the found causes was as; 1 dog <3 years and 1 dog 3–6 years (Barbiturate with acepromazine utilization), 1 dog 3–6 years and 1 dog >6 years (hematoma), 1 dog >6 years (lymphoid hyperplasia), 1 dog <3 years and 2 dogs >6 years (trauma, road accident history in 2–5 months ago), 2 dogs <3 years (distemper), 2 dogs >6 years (dirofilariasis) and 1 dog 3–6 years (unknown).

3.2. Hematology

The changes in blood test of the samples taken from the examined dogs with splenomegaly or splenomegaly and SFNs which have been presented in Table 3, were as follows:

a) In two dogs with splenomegaly and splenomegaly with SFN nodules with distemper, there was anemia in both dogs, but it was more severe in the male dog (splenomegaly with SFNs). Moreover, leukocytosis and increased Seg. N were observed in both dogs. Other cells, platelets, lymphocytes, monocytes, and eosinophils, were in the normal range.

b) Two dogs with dirofilariasis (heartworm), which indeed had a cardiovascular failure. Both dogs were male, one with only splenomegaly and the other with splenomegaly with SFNs. Both dogs had a similar hematology panel as anemia, leukocytosis, and increased Seg. N. However, anemia, increased leukocytes, and Seg. N were milder than the cases with distemper. The lymphocytes and monocytes were relatively increased, but the increase for eosinophils was severe. The platelets were in the normal range.

c) Two dogs with splenomegaly or splenomegaly with SFNs had hematoma; there was relative anemia in these animals, although the anemia level was lower compared to cases with distemper. Other blood cells were in the normal range in both dogs.

d) Two dogs with splenomegaly due to barbiturate and acepromazine utilization; relative anemia was observed. Other blood cells were in the normal range in both dogs.

e) Three dogs with a road accident history (trauma) had splenomegaly or splenomegaly with SFNs. There was a relative anemia in all three cases. Other blood cells were in normal range.

f) Lymphoid hyperplasia in a female dog >6 splenomegaly; no anemia was observed in this dog and all blood cells were in the normal range.

g) One female dog aged 3–6 years with splenomegaly and SFNs; no notable alterations were observed in the hematology profile.

3.3. Blood MDA level

Data analysis by student's t-test for serum MDA level of the examined dogs showed there was a significant increase ($p < 0.01$) of the mean (±SD) of MDA level of male and female dogs with splenomegaly compared to dogs with the normal spleen (Table 4). Also, the increase in MDA level of dogs with splenomegaly was significant in all age range in comparison with the normal spleens ($p < 0.01$) (Table 4).

3.4. Histopathology

Pathological assessment of the 19 spleen tissues H&E-stained sections indicated that 6 spleens were normal and 13 had splenomegaly. Meanwhile, out of 13 cases with splenomegaly, 7 cases had only splenomegaly and

Table 2. Comparison of the ultrasonographic indices measured in normal canine spleens and with splenomegaly in the various age groups.

Index	Age (year)	Sex	Normal (cm) Mean ± SD	Splenomegaly (cm) Mean ± SD	p-value
Spleen length in Sagital section	<3	Male	15.70 ± 30	17.70 ± 0.28	0.001
		Female	14.93 ± 0.21	-	-
		Total	15.06 ± 0.28	17.70 ± 0.28	0.001
	3–6	Male	16.45 ± 0.36	19.17 ± 0.25	0.001
		Female	15.90 ± 0.40	18.30 ± 0.14	0.001
		Total	16.19 ± 0.46	18.88 ± 0.53	0.001
	>6	Male	17.07 ± 0.23	19.56 ± 0.75	0.001
		Female	16.35 ± 0.31	18.85 ± 0.21	0.001
		Total	16.75 ± 0.45	19.28 ± 0.66	0.001
Spleen width in Sagital section	<3	Male	4.42 ± 0.22	4.95 ± 0.07	0.01
		Female	4.10 ± 0.13	-	0.001
		Total	4.27 ± 0.24	4.95 ± 0.07	0.001
	3–6	Male	5.11 ± 0.14	5.90 ± 0.08	0.001
		Female	4.44 ± 0.18	5.35 ± 0.21	0.001
		Total	4.79 ± 0.37	5.71 ± 0.30	0.001
	>6	Male	5.36 ± 0.20	6.00 ± 0.10	0.001
		Female	5.05 ± 0.13	5.75 ± 0.07	0.001
		Total	5.22 ± 0.23	5.90 ± 0.16	0.001
Spleen height in ross-section	<3	Male	1.50 ± 0.12	1.95 ± 0.07	0.01
		Female	1.33 ± 0.13	-	-
		Total	1.42 ± 0.14	1.95 ± 0.07	0.001
	3–6	Male	2.02 ± 0.07	2.25 ± 0.05	0.001
		Female	1.80 ± 0.11	2.05 ± 0.07	0.001
		Total	1.91 ± 0.14	2.18 ± 0.11	0.001
	>6	Male	2.01 ± 0.09	2.33 ± 0.05	0.001
		Female	1.81 ± 0.07	2.15 ± 0.07	0.001
		Total	1.92 ± 0.13	2.26 ± 0.11	0.001

Note: $p < 0.05$ is significant.

6 ones with SFNs in addition to splenomegaly. Hence, in a necropsied dog >6 years old affected by dirofilariasis, SFNs were grossly observed on the dorsal surface of the spleen, which were as amber-yellow colored solitary or disseminated nodules and 2–5 mm in size. In addition, the rounded edges of the spleen with a length of about 20 cm were notable for splenomegaly (Figure 2). Evaluation of the (H&E)-stained spleen tissue sections showed that out of 13 spleen tissue specimens with splenomegaly, 6 specimens had SFNs. Furthermore, assessment of (H&E)-stained sections revealed that normal spleens had no nodular changes and an increase in the thickness of splenic capsular or subcapsular surface as well as histological changes such as inflammation, necrosis and hemorrhage in their parenchyma (Figure 3A). Whilst, in the spleens with SFNs, prominent nodules on capsular surface partly expanded to subcapsular parenchyma as solitary and scattered or diffused and integrated nodules, were observed (Figure 3A). In addition, increased connective tissue (collagen) and dark brown deposits, especially in the central areas of the nodules, as well as the hemorrhage in different parts of the nodules were also demonstrated by the H&E method (Figure 3A). On the other hand, Masson's trichrome and Pearl Prussian blue staining methods were used to confirm connective tissue (collagen fibers) and possible iron deposition in canine spleen tissue with SFNs, respectively.

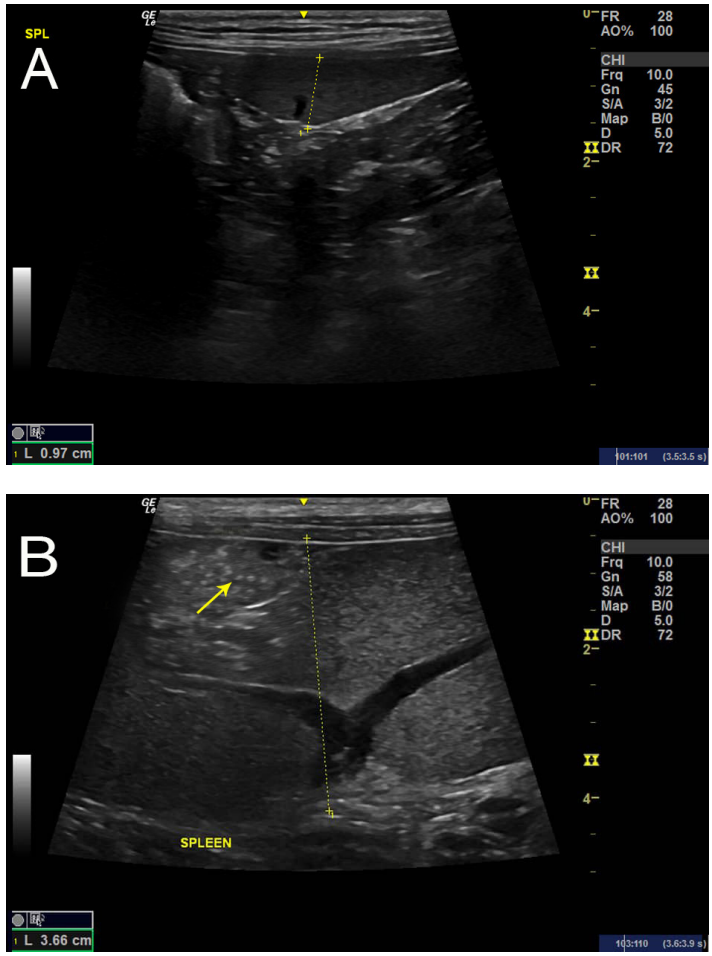

Figure 1. Sonograms related to dogs with splenomegaly and normal spleen. (A) Sagittal sonogram of the normal spleen with homogenous parenchyma and normal echogenicity. (B) Sagittal sonogram represents an obvious spleen enlargement (splenomegaly) and some hypoechoic nodules (arrow) with parenchymal heterogeneity.

In Masson's trichrome staining, the penetration and diffusion of collagen fibers were evident, especially in the peripheral areas as well as in the central areas of the nodules (Figure 3A). Due to the presence of hemorrhage and RBCs destruction, the iron deposits were quite evident in the nodules, especially in the central part of the nodules and inter collagenous spaces which could be confirmed by Prussian blue staining (Figure 3A). Other changes such as necrosis and parenchymal hemorrhage were not observed in the canine spleen with SFNs. Furthermore, pixel-based analysis by the software showed a notable increase of blue pixel intensity (collagen fibers) (Figure 3B) and dark blue intensity (iron deposits) (Figure 3C) in Masson's trichrome and Prussian blue-stained sections respectively in the spleen with SFNs compared to the normal one.

3.5. Immunohistochemistry and immunofluorescence

Immunohistochemical scoring results of the antibodies which were used are presented in Table 5. In this respect, the blind scoring was performed by a pathologist to differentiate the various cells as CD3 (T lymphocyte), CD20 (B lymphocyte), CD68 (Macrophage), S100 (dendritic cell) and vWF (endothelial and megakaryocyte cells) in 6 normal spleens and 6 spleens involved with splenomegaly and SFNs. The results showed that the increased expression of CD68, vWF and S100 was significant ($p < 0.05$) while increased expression of CD3 was not significant ($p > 0.05$) in the tissue sections with SFNs in comparison with the normal tissues. Also, reduced expression of CD20 has detected significance ($p < 0.05$) in the sections with SFNs compared to the normal

Table 3. Hematology profile of dogs with splenomegaly or splenomegaly with SFNs.

Cause		Parameter							
		HCT (%)	Hb (g/dL)	PLT ($\times 10^3$/μL)	WBC ($\times 10^6$/μL)	Seg. N ($\times 10^6$/μL)	Lymph. ($\times 10^3$/μL)	Mono. ($\times 10^3$/μL)	Eos. ($\times 10^3$/μL)
Barbiturate and acepromazine utilization	Case 1(M)	36.1	11.2	302	6.7	5.5	3.4	0.27	0.2
	Case 2 (M)	36.5	11.4	255	8.1	4.5	2.6	0.7	0.9
Hematoma	Case 1(M)	35.7	11.5	330	7.7	8.2	2.2	0.5	0.7
	Case 2 (F)	36.6	11.8	380	9.1	5.1	3.4	0.23	0.3
Lymphoid hyperplasia	Case 1(F)	40.6	13.3	290	8.2	4.6	2.9	0.65	0.6
Trauma (road accident history in 2-5 months ago)	Case 1(M)	35.2	11.3	260	6.8	4.9	1.9	0.8	0.2
	Case 2 (M)	35.8	11.7	330	8.8	7.2	2.4	0.33	0.2
	Case 3 (M)	35.5	11.3	410	6.2	3.6	3.1	0.4	0.6
Distemper	Case 1 (F)	31.2	9.1	280	33.9	23.23	2.6	0.83	0.5
	Case 2 (M)	35.6	11.2	310	28.2	18.31	2.1	1.03	0.2
Dirofilariasis	Case 1(M)	31.2	11.4	250	20.6	12.44	7.8	2.9	2.7
	Case 2 (M)	33.4	12.5	220	22.3	14.1	6.9	2.5	5.6
Unknown	Case 1(F)	42.1	13.7	310	7.4	5.6	3.3	0.3	0.2

HCT: Hematocrit. Hb: Hemoglobin. PLT: Platelets. WBC: White Blood Cells. Seg. N.: Segmented Neutrophil. Lymph.: Lymphocyte. Mono.: Monocyte. Eos.: Eosinophil. M: Male. F: Female.
Reference value: HCT: 37%–55%. Hb: 12–18 g/dL. PLT: 200–500 × 103/μL. WBC: 6–12 × 106/μL. Seg. N: 3–11.5 × 106/μL. Lymph: 1–4.8 × 103/μL. Mon: 0.15–1.35 × 103/μL. Eos: 0.1–1.25 × 103/μL.

Table 4. The level of serum malondialdehyde (MDA) in normal dogs and with splenomegaly

Parameter (μmol/L)	Age (year)	Sex	Normal (Mean ± SD)	Splenomegaly or splenomegaly with SFN (Mean ± SD)	p-value
Malondialdehyde (MDA)	<3	Male	2.21 ± 0.54	3.71 ± 0.23	0.005
		Female	1.23 ± 0.43	-	-
		Total	1.75 ± 0.69	3.71 ± 0.23	0.001
	3–6	Male	1.69 ± 0.62	3.69 ± 0.65	0.001
		Female	1.77 ± 0.51	3.33 ± 0.15	0.002
		Total	1.73 ± 0.56	3.57 ± 0.54	0.001
	>6	Male	2.13 ± 0.44	3.63 ± 0.24	0.001
		Female	1.50 ± 0.37	2.78 ± 0.12	0.001
		Total	1.84 ± 0.51	3.29 ± 0.50	0.001

Note: $p < 0.05$ is significant

ones (Figure 4A). On the other hand, to reduce errors, we counted the mean (± SD) number of the cells per one mm^2 in the spleen tissue with positive immunoreaction to the mentioned antibodies using the software (Figure 4B). Also, the immunoreaction intensity in the spleen tissue sections representing target proteins was analyzed in 2333 μm^2 of tissue. Accordingly, the expression intensity of the antibodies CD3, CD68, S100, and vWF in the cells of the spleen with SFNs was increased compared to the normal splenic cells. The intensity of CD20 in the cells of the spleens with SFNs was reduced compared to the normal cells as well (Figure 4C). CD3-positive cells (T lymphocytes) were more abundant around the trabeculae and interspaces of the red pulp. However, T cells also were

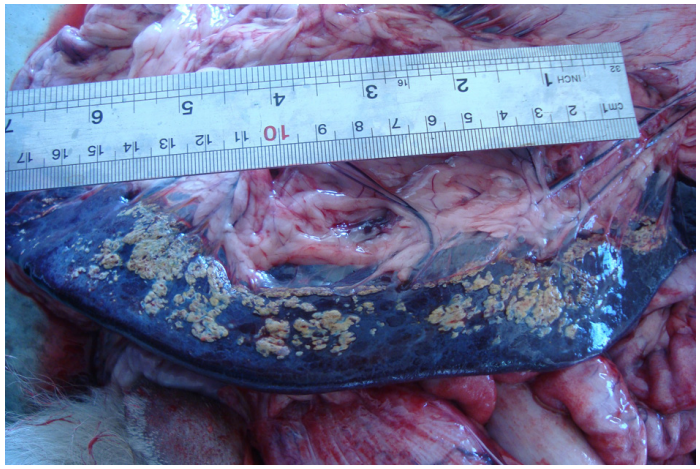

Figure 2. Gross appearance of the canine spleen with splenomegaly and SFN in a necropsied male dog older than 6 years affected by dirofilariasis. SFNs exist on the dorsal surface of the spleen, representing 2–5 mm amber-yellow colored solitary or diffused nodules. In addition, the rounded edges of the spleen with a length of about 20 cm are notable in this case with splenomegaly.

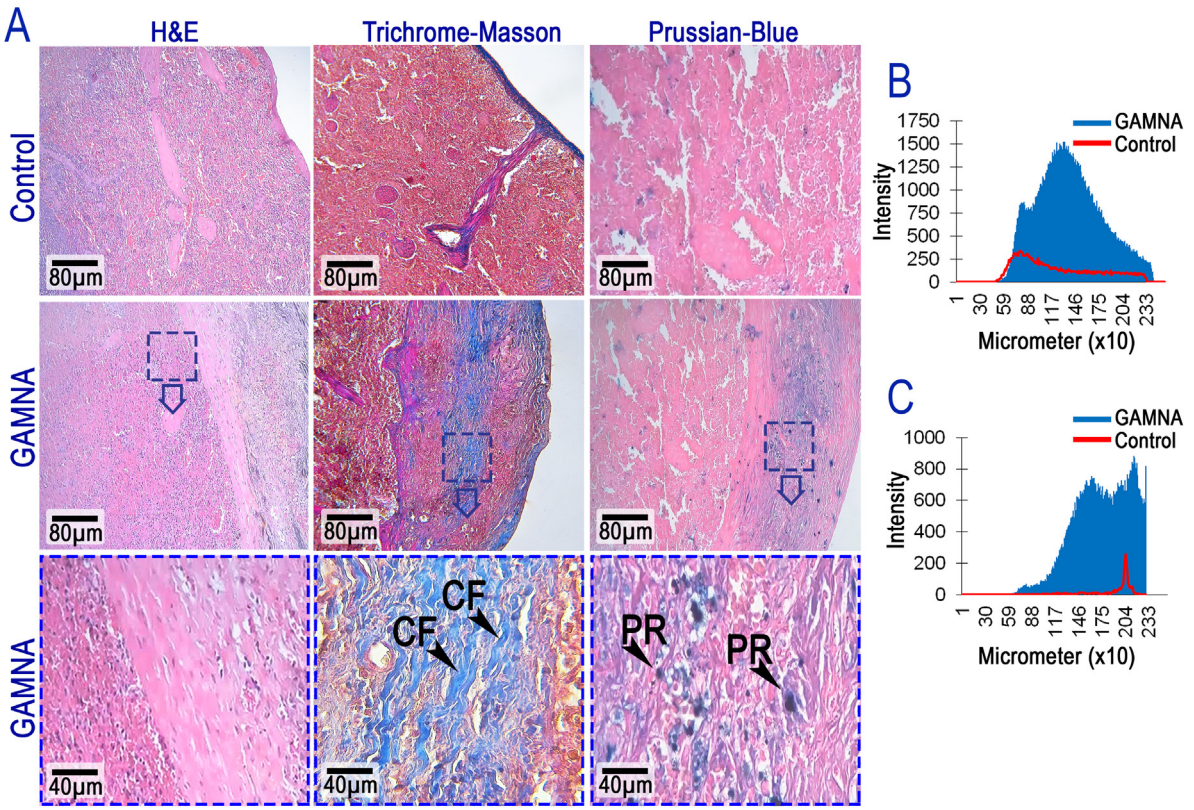

Figure 3. (A) Prominent siderofibrotic nodules on capsular surface (H&E), collagen (Masson's trichrome) and iron (Prussian-Blue) staining techniques, note the increased amounts of collagen (CF, fibrosis) and positive iron reaction (PR) in the sections from GAMNA (siderofibrotic nodules) group. (B) Software analysis for pixel-based intensity of blue reaction (collagen) in the sections stained with Masson's trichrome and (C) Software analysis for pixel-based intensity of black-stained reaction (iron-positive reaction) in Prussian blue-stained sections in 2330 µm × 2330 µm of tissue.

Table 5. Immunohistochemical scoring in the normal canine spleens and the spleens. with splenomegaly and SFN

Antibody	Normal spleen (n = 6)					Splenomegaly with SFN (n = 6)				
	0 (Negative)	1 (up to 15%)	2 (15%–40%)	3 (40%–70%)	4 (> 70%)	0 (Negative)	1 (up to 15%)	2 (15%–40%)	3 (40%–70%)	4 (> 70%)
CD3				2	4				1	5
CD20		2	4				6			
CD68		4	2				1	4	1	
S100		3	3						4	2
VWF		2	4						1	5
HSP70		1	5						6	
HSP90	1	4	1						5	1
HIF-1α		4	2				5	1		
P53		6						4	2	
Active caspase 3		4	2						1	5

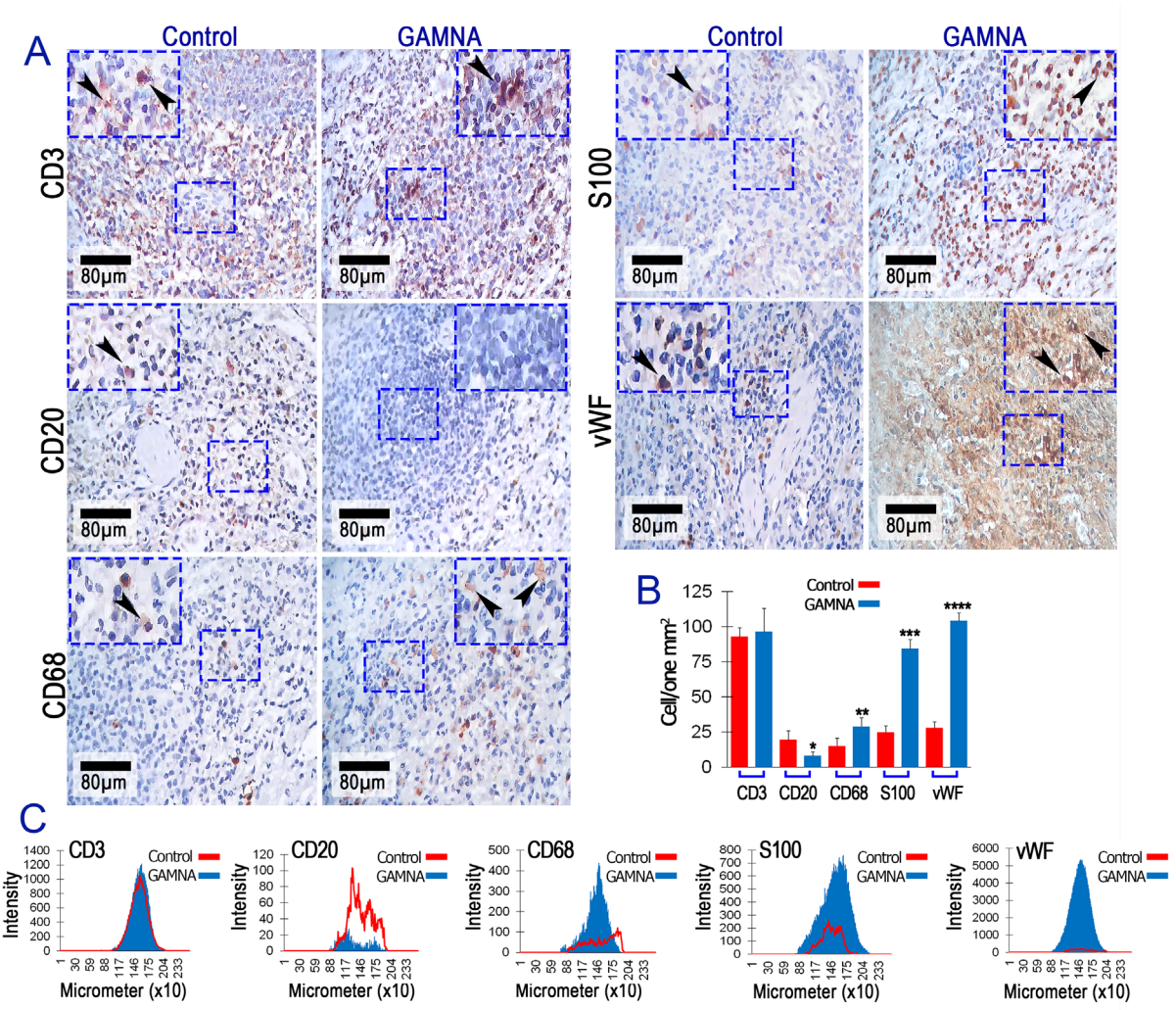

Figure 4. (A) Immunohistochemical staining of CD3, CD20, CD68, S100, and VWF: The positive reactions are marked with arrowheads, (B) Mean numbers of CD3+, CD20+, CD68+, S100+, and VWF+ cells per one mm² of tissue in different groups, and (C) software analysis for pixel-based intensity of brown-stained reactions (representing target proteins) in 2330 µm × 2330 µm of tissue, all data are presented in Mean ± SD, and different signs are representing significant ($p < 0.05$) difference between GAMNA (siderofibrotic nodules) and control groups.

present in the mantle zone of the lymph follicles. The CD20-positive cells were sparsely distributed in white pulp cells (follicles) in very small numbers, especially in the germinal zone, but were greater in other areas, such as interspaces of the red pulp. The density of S100-positive cells (dendritic cells) in spleens with SFNs was slightly different from that of healthy spleens in that dendritic cells were absent in the white pulp of healthy spleens, whereas in spleens with SFNs, in addition to a number of dendritic cells there were in different parts of the lymph follicles, many of which were present as a demarcation line around the white pulp and in the cortical areas of the lymph follicles. In both healthy spleens and spleens with SFNs, dendritic cells were also present in the interspaces of the red pulp. Vascular endothelial cells of intrafollicular vessels and intratrabecular vessels of the parenchyma in both healthy spleens and spleens with SFNs had a positive immunoreaction to vWF, but in spleens with SFNs, they were more numerous. In addition, although the number of megakaryocytes in the healthy spleen was very low, the number of these cells in the spleens with SFNs increased compared to the healthy spleens.

Increased dendritic cells, which are antigen-presenting cells, stimulate and increase T lymphocytes and the production of inflammatory cytokines by T lymphocytes and increased and activated macrophages. On the other hand, dendritic cells interact with B lymphocytes which is important in the initial stages of T cell-dependent

responses [17]. Therefore, the increase of dendritic cells, T lymphocytes and macrophages in spleens with SFN nodules indicates the presence of chronic splenitis in these spleens. In the current study, scoring of the immunoreaction, mean (±SD) number of the cells with positive immunoreactivity and expression intensity of the antibodies for oxidative stress (HSP70 and HSP90), apoptosis (p53 and caspase 3) as well as cellular hypoxia (HIF1-α) were simultaneously investigated in healthy splenic cells and spleen cells with SFN nodules. According to Figures 5A and 5B, the percentage and mean (±SD) number of immunolabeled cells with p53, caspase 3, HSP70 and HSP90 increased significantly ($p < 0.05$) in spleens with SFNs compared to the normal ones although this increase was not significant ($p > 0.05$) for HIF1-α. Moreover, it was found that the expression intensity of these antibodies was consistent with the percentage and mean (±SD) number of cells with a positive immune response in spleen tissue with SFN nodules (Figure 5C). Overall, results of immunohistochemical evaluations revealed the presence of chronic splenitis in dogs with splenomegaly and SFNs. Moreover, increased expression of HIF1-α, caspase 3, p53, HSP70 and HSP90 confirmed the role of hypoxia and oxidative stress (ROS and lipid peroxidation) to produce splenic cellular injures in dogs with splenomegaly and SFNs.

The results of immunofluorescent analysis for NRF2 indicated that immunofluorescence intensity notably decreased in the spleens with splenomegaly and SFNs compared to the normal ones (Figures 6A and 6B). The

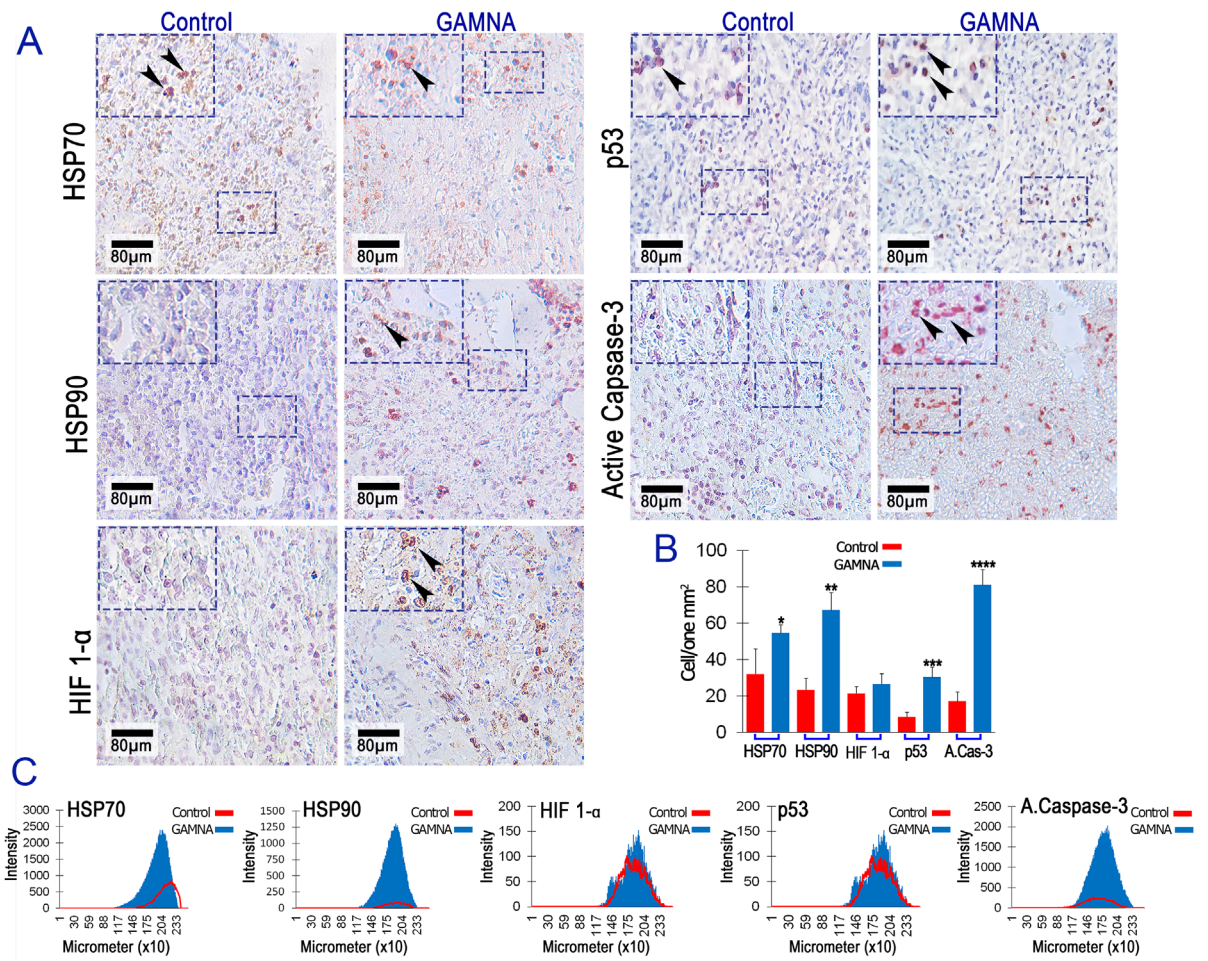

Figure 5. (A) Immunohistochemical staining of Hsp70, Hsp90, HIFα, p53, and caspase-3: the positive reactions are marked with arrowheads, (B) Mean numbers of Hsp70$^+$, Hsp90$^+$, HIF1-α$^+$, p53$^+$, and caspase-3$^+$ cells per one mm^2 of tissue in different groups, and (C) Software analysis for pixel-based intensity of brown-stained reactions (representing target proteins) in 2330 µm × 2330 µm of tissue, all data are presented in Mean ± SD, and different signs are representing significant ($p < 0.05$) difference between GAMNA (siderofibrotic nodules) and control groups.

decrease in NRF2 expression represented the promotion of ferroptosis in the spleen tissues with SFNs.

4. Discussion

In the present study, by clinical examinations, ultrasonography,hematology,biochemistry,histopathology and immunohistochemistry, 73 Iranian dogs of mixed breeds in different age groups under 3 years, 3–6 years and more than 6 years during 3 years were examined. Thirteen dogs (13/73; 17.80%) had splenomegaly and out of these cases, 6 dogs had splenomegaly with SFNs (8.9%). According to Table 1, the prevalence of splenomegaly was in the age group of 3–6 years (13.8%), more than 6 years (8%) and under 3 years (5.3%). The results of this study also showed that the prevalence of splenomegaly and splenomegaly with SFN nodules had no significant relationship with the sex ($p = 0.596$) and age ($p = 0.833$) of the examined dogs. Whilst, the prevalence of SFNS in the spleen of Beagle dogs has been reported in the age range 18–19 months old significantly more ($p < 0.01$) than the age range 7–9 months old [6]. Indeed, they reported spontaneous subclinical lesions in 86 beagle dogs that were utilized for toxicity studies. Since the exposure time of the experiment was longer in the older dogs (18 to 19 months old) compared to the younger ones (7 to 9 months old), possibly the longer handling period or forced handling could be responsible for more prevalence of SFNs in older animals. In our study, the clinical and experimental circumstances were similar for all dogs without the presence of force, long handling, or interventions. Given the results of the longitudinal and cross-sectional ultrasonographic evaluation in the current study (Table 2), it was found that the length, width and height of male canine spleen in all age groups with splenomegaly significantly increased ($p < 0.01$) compared to the spleen of healthy dogs. Ultrasonography is a useful imaging tool for assessing splenic abnormalities such as splenomegaly and changes in parenchymal echogenicity and its eco-texture. Focal changes in the spleen, especially in older dogs, are usually detected during routine ultrasonography. These are often random findings and challenge diagnosis. However, a definitive diagnosis of various spleen pathologies can only be obtained by histopathological analysis of samples collected through an ultrasound-guided biopsy or after splenectomy [11]. In the present study, healthy spleens had homogeneous eco-texture and uniform echogenicity without any lesions in the spleen as expected. In cases with SFN nodules, hyperechoic tiny nodules without focal or diffuse distal acoustic shadows were observed. In addition, the parenchyma of the spleen was hypoechoic, irregular and partially heterogeneous. It has been reported that the dogs had SFNs in their spleen, other disorders or diseases including distemper, pyometra, cardiovascular lesions, pancreatitis, and bacterial infections were the causes of their death or necropsy [5]. In the present research, the most found lesions or diseases for the presence of SFNs in canine spleens, were road accidents (trauma), hematoma (possibly caused by trauma), distemper and dirofilariasis. Moreover, we found that splenomegaly with SFNs is not confined to older dogs, but due to the causative agents may be observed in spleens of dogs of all ages. From a histopathological point of view, a notable increment in the collagen fibres and iron deposition was revealed in the spleen sections of dogs with SFNs. We did not perform any staining method to evaluate the presence of calcium deposits in SFNs while Yasuba et al. [6] reported no calcium deposits in canine splenic SFNs. In fact, our purpose of using the markers CD3, CD20, CD68, S100, and VWF was to separate the cell population in spleens had siderofibrotic nodules and compare the type and mean number of cells as well as the expression intensity of the mentioned antibodies with normal spleens. An increase in these markers in spleen indicates immune cell activation, which could lead to splenomegaly. Splenomegaly can occur as a result of a variety of conditions, including infections, autoimmune disorders, and hematological malignancies [18]. Ferroptosis affects immune cells by two major pathways; 1) impact on their number and function and 2) recognition of ferroptotic cells by immune cells and subsequently triggering variable inflammatory responses [19]. On the other hand, immune cells in the spleen are the key protectors because they recognize pathogens, cellular stress, remove dying cells and foreign materials, regulate tissue hemostasis and inflammatory responses as well as configure adaptive immunity. In this regard, splenic marginal zone macrophages have been raised as possible regulators of the function of other antigen-presenting cells that come in contact with the apoptotic materials [18]. Accordingly, it seems that the increase in CD3, CD20, CD68, S100, and vWF IHCs in the spleen does not necessarily have a direct relationship with apoptosis or ferroptosis. However, it is possible that the increased immune cell activation and subsequent inflammation could lead to oxidative stress and induce ferroptosis in some cells. The IHC results from SFN-positive revealed a significant increment in the T lymphocytes (CD3-positive cells) and dendritic cells (S100-positive cells) distribution compared to the normal spleen sections. Since dendritic cells are able to induce and suppress the primary immune response by activating Naïve T cells (precursor cells of memory T cells) they can play a crucial role in inducing and tolerating immunity under homeostatic conditions [20]. Therefore, we evaluated the T lymphocytes (CD3-positive cells) and dendritic cells distribution in histopathologic slides. On the other hand, in the SFNs-positive sections represented an increased $p53^{+}$ and caspase 3^{+} T lymphocytes

distribution versus normal spleens. It means that the removal of T cells through apoptosis may initiate chronic infection circumstance in SFNs-positive conditions [21] and even cause antiinflammatory effects by preventing the proinflammatory cytokines surge and activate the macrophages or dendritic cells [22]. As a result of these alterations, it can partially cause system homeostasis. Indeed, the removal of T cells through apoptosis may cause both inflammatory and antiinflammatory effects and act as an inflammatory regulator.

Further to unchanged numbers of HIF-α^+ cells in the sections from splenomegaly and SFNs-positive animals (versus normal dogs), one should not ignore the crucial role of HIF1-α in apoptosis and ferroptosis pathways. Moreover, HIF1-α plays an important role in activating Caspase 3, which in turn ends up with rotational apoptosis [23]. Therefore, based on our findings, there was a strong and significant enhancement in the distribution of the Caspase3$^+$ cells in the spleens with SFNs that may correlate with HIF1-α's role in initiating apoptosis. About ferroptosis, given the dual role of hypoxia in inhibiting and promoting ferroptosis, it regulates ferroptosis through a complex network in a context-dependent manner in different cell types and conditions. Accordingly, hypoxia usually inhibits ferroptosis in cancer cells, while hypoxia often induces or promotes ferroptosis in normal cells. Indeed, ferroptosis is mediated by HIFs, NRF2 signaling, and other mechanisms [24]. Therefore, in our study, it seems that hypoxia-induced expression of HIF-1α caused the downregulation of NRF2 expression thereby promoting ferroptosis. Considering our results of MDA level assessment (Table 4) as well as hematological results, because most dogs with splenomegaly and SFNs were anemic, this could be associated with lysis of RBCs and iron overload and lipid peroxidation [25]. But, regarding the mild anemia in dogs with splenomegaly with SFNs, the increase of lipid peroxidation-related ROS might be the most agent to induce ferroptosis in cooperation with other factors like hypoxia-induced HIF and NRF2.

In connection with vWF, under pathophysiological conditions such as inflammation, injury, infection and ischemia; hypoxia depending on the type of tissue and its severity can increase the expression of HIFs [26,27] and increased expression of HIFs, especially HIF-1α, activates and regulates angiogenesis-related factors [28,29]. Accordingly, angiogenesis-related factors regulate and induce the release and expression of vWF/WPBs (Weibel-Palade bodies) [30], it can be concluded that in the present study, in response to hypoxia, inflammation and increased lipid peroxidation (MDA), increase expression of HIF-1α causes increased vWF expression resulting in increased angiogenesis in spleens with SFNs. HSPs are known to play a role in both apoptosis and ferroptosis. In apoptosis, they regulate the expression of pro and antiapoptotic proteins, which can affect the balance between cell survival and death [31]. On the other hand, HSPs have different role in promoting or inhibiting ferroptosis. Accordingly, the family members of HSPs can affect ferroptosis as promotors such as HSP90 or inhibitors such as HSP70 [32]. Since ferroptosis can proceed even without apoptotic factors such as caspases, it has been suggested that both apoptosis and ferroptosis may concurrently occur during the pathological process of cell death [33]. In connection with p53, it has been approved this may be a key molecule in apoptosis-ferroptosis crosstalk. Accordingly, previous research has indicated due to the close relationship between apoptosis and ferroptosis, these processes can be switched from one to another [34]. Of course, unlike apoptosis, p53 itself alone cannot induce ferroptosis directly; instead, via its metabolic targets, p53 can modulate the ferroptosis response in the presence of ferroptosis inducers such as GPX4 inhibitors or high levels of ROS [35]. Also, P53 has been shown to regulate ferroptosis at transcriptional or posttranscriptional levels [36]. Altogether and regarding the dual role of p53 in ferroptosis (promotion and suppression) as well as the increase of MDA which is an indicator of overexpression of lipid peroxidation-related ROS, we can conclude simultaneous increase in the expression of caspase 3, p53, HSP70 and HSP90 in the current study may indicate the modulating role of these proteins in the generation of a balance between apoptosis and ferroptosis through a complicated interactive network.

Our immunofluorescent results indicated that the expression of NRF2 in the canine spleen with SFNs significantly decreased compared to the normal spleens (Figure 6). Accordingly, the downregulation of NRF2 represents an increase in ferroptosis in the spleens with SFNs compared to the normal spleens. It has been approved that ferroptosis plays an essential role in regulating oxidative stress and inflammatory responses [37]. However, given the chronic nature of SFNs, the expression of NRF2 gradually decreases when there is long-time resistance to oxidative stress. Indeed, this affair robustly correlates with the aggravation of tissue damage caused by oxidative stress [16].

Overall, our results indicated that the occurrence of splenomegaly with SFNs in Iranian dogs of mixed breeds had no significant relationship ($p < 0.05$) with the sex and age of the examined dogs. The indices studied for ultrasonographic evaluation represented a significant difference ($p \leq 0.01$) in the size of spleens with splenomegaly compared to healthy spleens. Moreover, increased lipid peroxidation (by evaluating serum MDA level) and iron overload indicated the role of lipid peroxidation and ROS in cellular ferroptosis in spleens with SFNs. Furthermore, it seems that in response to the effects of

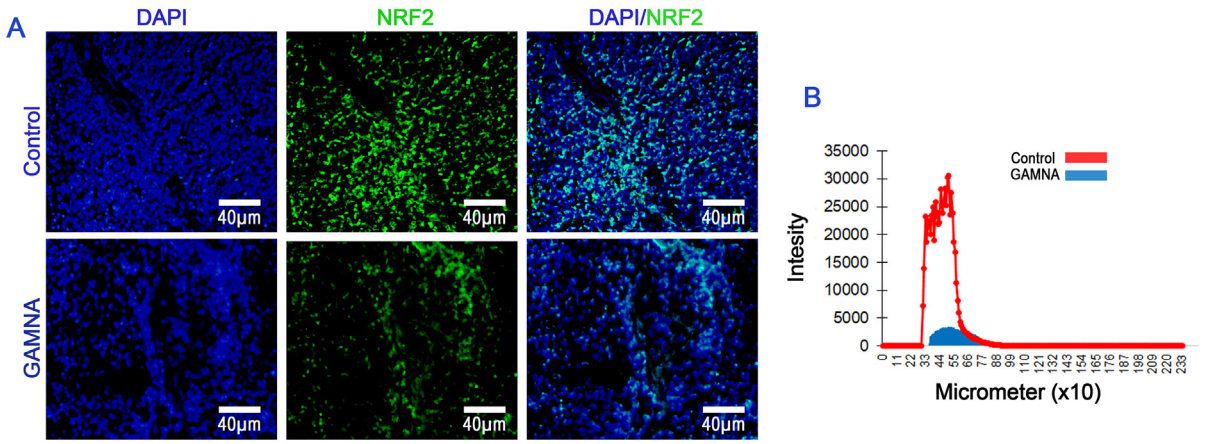

Figure 6. (A) Immunofluorescent analysis with NRF2 antibody (green) and nuclear staining with DAPI (blue) in control and GAMNA (siderofibrotic nodules) groups. (B) software analysis for pixel-based intensity of green-stained reactions (representing NRF2 expression) in 2330 µm × 2330 µm of tissue, data are presented in Mean ± SD, and there is a significant ($p < 0.05$) decrease of NRF2 expression intensity in GAMNA (siderofibrotic nodules) group compared to control.

oxidative stress and lipid peroxidation (MDA marker), hypoxia and inflammation (increased T lymphocytes and macrophages), HIF1-α expression is up-regulated by increasing vWF expression in vascular endothelial cells of spleens with SFNs. However, increased expression of HIF1-α was not significant, which may be related to the dependence of HIF1-α expression on tissue type and the time after hypoxia. On the other hand, with increasing expression of Caspase 3 and p53, especially in T cells, the rate of apoptosis in spleens with SFNs has significantly increased compared to healthy spleens. Regarding HSP70 and HSP90, it should be noted that due to the dual role of these proteins in apoptosis and ferroptosis heat shock proteins act as modulators between apoptosis and ferroptosis interacting with other factors such as HIF1-α and p53. Moreover, regarding a decrease in NRF2 expression in spleens with splenomegaly and SFNs which has led to the promotion of ferroptosis, simultaneous increases of apoptosis and ferroptosis were observed in our research. Certainly, an accurate understanding of interactions between apoptosis and ferroptosis and also all involving factors in these processes need to further complementary investigations. Finally, it should be noted that in this study we have focused only on the Iranian dogs of mixed breeds, so the small sample size could be a limitation. Moreover, due to the presence of splenomegaly in the animals with SFNs, we did not consider the cases with only splenomegaly for immunohistochemical evaluations.

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Conflict of interest

The authors have no conflict of interest to declare.

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