Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.

Complex diagnostics of fatty liver dystrophy in dogs

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Article info

Received 02.08.2025
Received in revised form 10.09.2025
Accepted 11.10.2025

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Slivinska, L., Gudyma, T., Shcherbatyi, A., Gutyj, B., Fedorovych, V., Leskiv, K., & Fedorovych, N. (2025). Complex diagnostics of fatty liver dystrophy in dogs. Regulatory Mechanisms in Biosystems, 16(4), e25202. doi:10.15421/0225202

Fatty hepatosis in dogs is a common pathological process accompanied by accumulation of lipids in hepatocytes, impairment of the liver's metabolic functions, and the development of inflammatory and dystrophic changes, which significantly affect the clinical course of the disease and prognosis. The objectives of the study were to evaluate the activity of cytolytic (alanine- and aspatate aminotransferase, glutamate dehydrogenase) and cholestatic (gamma-glutamyltransferase, alkaline phosphatase) enzymes, and also to determine the concentration of bile acids in blood serum of dogs of different breeds suffering fatty liver dystrophy, namely German shepherd, spaniel, Yorkshire terrier, and mixed-breed dogs. The animals underwent clinical examination, ultrasound diagnostics, liver biopsy, histological and electronic-microscope analysis of the bioptates, and their blood was gathered for laboratory analyses. We examined 140 dogs aged two to six years, weighing 2.5 to 46 kg. In the dogs with fatty liver dystrophy, the informative diagnostic criteria were concentration of bile acids in blood serum prior to feeding and two hours later, and also the activity of hepatoindicatory cytolytic (glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase) and cholestatic (gamma-glutamyltransferase, alkaline phosphatase) enzymes. Changes in the mentioned biochemical parameters were present in all the clinically ill dogs. We determined a high positive correlation between the concentration of bile acids prior to and two hours after feeding the dogs: German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs. In the clinically healthy German shepherds, the reference values of concentration of bile acids in blood serum before and two hours after meal measured 2.4–8.4 and 12.9–16.7 μmol/L, respectively. The concentration of bile acids in blood serum of the fatty-liver-dystrophy patients before and two hours after feeding was elevated, in particular 15.9% in the German shepherds, 33.3% in the spaniels. We also determined a strong positive correlation between the concentration of bile acids in blood serum of the ill German shepherds prior to and two hours after meal (r = 0.963). According to the ultrasound study, 100% of the dogs were observed to have uniform diffusive increase in echogenicity of the liver parenchyma. The liver contours were rounded, the vascular pattern was impoverished, the capsule had a dense echostructure, and the structure of parenchyma was coarse-granular. As revealed by the histological and histochemical study, the liver of the German shepherds had large foci of fatty dystrophy of hepatocytes, and also fine focal spots of dystrophy on the bile ducts' epithelium. In the liver bioptates from the German shepherds, we observed pronounced ultrastructural changes, including some hepatocytes containing average-sized lipid droplets with low electronic density and other hepatocytes having large lipoprotein inclusions. In the hepatocytes' cytoplasm, we detected an increase in the number of peroxisomes and autophagolysosomes against the background of an expansion of the spaces of Disse. In addition, we found sludges of red blood cell and electronically dense masses of blood plasma in the lumens of blood capillaries and an expansion of collagen fibers between hepatocytes we observed.

Keywords: hepatosis; cytolytic enzymes; cholestatic enzymes; bile acids; ultrasound diagnostics; histological analysis; electronic microscopy.

Introduction

The liver is the key organ of homeostasis that performs the central role in metabolism of proteins, carbohydrates, vitamins, lipids, fatty acids, amino acids, and micro- and macroelements involved in both anabolic and catabolic processes, as well as in bile formation and removal (Au et al., 2013; Chernushkin et al., 2020). Correspondingly, both primary and systemic liver pathologies can cause significant metabolic disorders that negatively affect the duration and quality of the dogs' life. Not only are dogs suffering from various congenital liver diseases, including congenital metabolic malfunctions and structural abnormalities, but are also commonly diagnosed with numerous acquired acute and chronic liver ailments that are infectious, inflammatory, degenerative, vascular, neoplastic, drug-toxic, or idiopathic in origin, accompanied by elevated activity of hepatic enzymes in blood serum (Webster et al., 2019).

Due to its multifunctionality, the liver is the first to react to the effects of external and internal unfavorable factors and often becomes involved in general pathological processes during development of avirulent, infectious, and parasitic diseases (DeMarle et al., 2021). Hepatoses in dogs are quite common, accounting for 30-40% of all avirulent pathologies. According to the dispensary studies, liver dystrophy was diagnosed in 50.8% of the animals (Hudyma & Slivinska, 2013), whereas other researchers report that liver diseases accounted for up to 25% of all avirulent diseases of the dogs (Levchenko et al., 2008).

The factors promoting the spread of liver pathologies in dogs include the use of low-quality fodders, deficiency of vitamins and essential amino acids, and also the use of hepatotoxic drugs. Due to the liver's high regenerative ability, diseases typically manifest clinically at late stages, when the liver function becomes hard to recover (Johnson & Sherding, 2006).

To diagnose liver diseases, modern studies intensively explore the activity of hepatoindicatory enzymes (aspartate- and alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, glutathione gamma transpeptidase), localized in cytoplasm and subcellular structures of hepatocytes, and also the parameters of total protein, its fractions, and total bilirubin (Kozat & Sepehrizadeh, 2017; Slivinska et al., 2022; Vlizlo et al., 2024). One of specific products of hepatocytes' functioning is bile, in particular bile acids – its most important component. Despite numerous domestic and foreign studies, the issue of the functional state of the liver remains underexplored. Especially little research has been done regarding bile formation and bile removal in dogs suffering liver dystrophy. Moreover, there are no reference values of bile acids during this pathology (Hudyma & Slivinska, 2014).

Measuring the level of bile acids prior and two hours after meal (postprandial level) in dogs allows for diagnosis of disorders of bile formation, bile stasis, and accumulation of fatty inclusions in hepatocytes, which cause liver encephalopathy with changes in laboratory parameters that are more sensitive than clinically pathoanatomic changes (Kashliak & Vlizlo, 2024).

Yet unsolved issue is the informativeness of bile acids in diagnostics of fatty liver dystrophy in dogs in the context of other, better studied, parameters of the liver's functional state.

In this regard, the study of informativeness of bile acids combined with other diagnostic criteria is a relevant task that would allow performing early diagnostics of this disease, as well as designing its complex treatment and prophylaxis.

The objective of this study was to explore the activity of cytolytic (alanine- and aspartate aminotransferase, glutamate dehydrogenase) and cholestatic (gamma-glutamyl transpeptidase, alkaline phosphatase) enzymes, concentration of bile acids in blood serum, and the informativeness of complex in vivo diagnostic of fatty liver dystrophy in dogs of different breeds using ultrasound survey, biopsy of the liver, histological, and electronic-microscope analysis of bioptates.

Materials and methods

Maintenance, feeding, monitoring, and all the procedures with the animals were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and the General Ethical Principles of Experiments on Animals, adopted by the First National Congress of Bioethics (Kyiv, 2001). The experiments were conducted adhering to the principles of humanity established in the Directive of the European community.

The material for the study were German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs. To diagnose the pathology, we performed clinical, instrumental (ultrasound), and laboratory blood studies. In the process of the work, we examined 140 dogs aged two to six years, weighing 2.5 to 46 kg. The blood analyses were conducted at the laboratory of the Department of Internal Diseases of Animals and Clinical Diagnostics, and histological, histochemical, and electronic microscopic studies were carried out at the laboratory of the Department of Normal and Pathological Morphology and Forensic Veterinary of S. Z. Gzhytskyi Lviv National University of Veterinary Medicine and Biotechnologies, and ultrasound studies were performed at the Clinic of the Department of Internal Diseases of Animals and Clinical Diagnostics of the Vetmed Veterinary Medical Center, the city of Lviv.

Blood for the studies was collected in the morning, prior to feeding, from the superficial vein of the forearm, v.saphena, or the jugular vein. Biochemical studies were performed on a Mindray BS-120 biochemical analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) using the reagents manufactured by PZ Cormay S.A. (Poland) according to the generally accepted methods (Vlizlo, 2012).

The concentration of bile acids in blood serum was determined using the enzymatic method with a test system manufactured by the Audit Diagnostic company prior to meal and the postprandial level of bile acids was measured two hours after meal.

For ultrasonographic studies, we used a Sono Scape A6Vet apparatus manufactured in China, equipped with the sensors C351 (2–6 mHz) and microconvex C612 (4–9 mHz).

The samples of the liver tissue from the animals were obtained by means of needle biopsy of the liver under ultrasonographic control. Using the method of thin-needle biopsy, we gathered bioptates from nine German shepherds, two clinically healthy and seven individuals with confirmed signs of fatty liver dystrophy.

The blood parameters were statistically analyzed using a personal computer and the Statistica 7 software (StatSoft Inc., USA). The graphs were developed using Statistica 7 according to the generally adopted algorithms. The paper presents mean and standard deviation (x ± SD). To compare the difference of average parameters between the control and experimental groups, we used the Tukey test, where the differences were considered statistically significant at $P < 0.05$ for all the data.

Results

The main parameters indicating the functional state of liver in the dogs were concentration of the indicatory enzymes alanine- and aspartate aminotransferase, glutamate dehydrogenase, and levels of total bilirubin and cholesterol, and the parameters reflecting the bile-forming and bile-removing functions of the liver were alkaline phosphatase, gamma-glutamyltransferase, and bile acids.

In the clinically healthy dogs, the average concentration of total bilirubin accounted for 2.84 ± 0.14 μmol/L (1.0–3.9), whereas in the animals with fatty liver dystrophy this parameter was elevated 1.5-fold, equaling on average 4.12 ± 0.19 μmol/L (1.0–7.5; $P < 0{,}001$; Fig. 1).

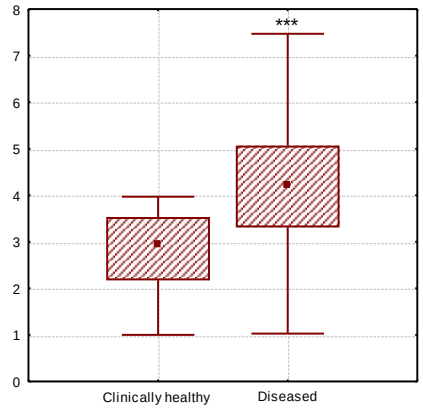


Fig. 1. Concentration of total bilirubin in blood serum of dogs (μmol/L): the abscissa axis indicates the groups of animals, the ordinate axis shows measurement units of the parameter; small square – median, upper and lower rectangle borders – 25% and 75% quartiles, vertical line – minimum and maximum values, circles – outliers; n = 40; $P < 0.001$***

For a more detailed study of the functional state of the liver in the dogs of different breeds, we performed a comparative analysis of the activities of the enzymes in blood serum of the clinically healthy and ill animals. In the dogs suffering fatty liver dystrophy, the activity of alanine aminotransferase was increased 2.4-fold and that of aspartate aminotransferase was higher 2.2-fold, compared with the healthy animals ($P < 0.001$; Fig. 2).

In particular, in the fatty-liver-dystrophy German shepherds, the activities of alanine aminotransferase and aspartate aminotransferase were higher by 2.7 and 2.1 times ($P < 0{,}001$); in the spaniels – by 2.2 and 2.1 times ($P < 0.001$); in the Yorkshire terriers – by 2.6 and 2.5 times ($P < 0.001$); and in the mixed-breed dogs – 2.4 and 2.2 times ($P < 0.001$), compared with the clinically healthy animals. The activity of glutamate dehydrogenase in the ill dogs was 2.3 times greater ($P < 0.001$) than in the control (Fig. 3a). As with cholestatic enzymes, the activities of alkaline phosphatase and gamma-glutamyl transpeptidase in the ill dogs were 2.5 and 2.3 times higher ($P < 0.001$) than in the clinically healthy dogs respectively (Fig. 3).

The concentration of cholesterol in the fatty-liver-dystrophy dogs was 1.8 lower ($P < 0.05$) than in the clinically healthy dogs (Fig. 4).

In the ill dogs, the concentration of bile acids before meal was significantly higher, ranging 11.1 to 44.1 μmol/L (18.83 ± 1.05), compared with the levels of the clinically healthy animals (5.0 ± 0.25 μmol/L; $P < 0.001$, Fig. 5). Two hours after meal, the level of bile acids in the ill animals measured 40.6 ± 1.79 μmol/L, i.e. 2.8 times greater ($P < 0.001$) than in the healthy dogs (14.31 ± 0.20 μmol/L, Fig. 5).

We found no differences in the levels of total bilirubin in blood serum among the dogs of different breeds. In particular, the content of total bilirubin ranged 2.1 to 3.4 μmol/L in the clinically healthy German shepherds, 1.0–3.8 μmol/L (2.64 ± 0.33) in the spaniels, and 1.0–3.9 μmol/L (2.71 ± 0.29) in the Yorkshire terriers. In the fatty-liver-dystrophy dogs of these breeds, we observed total bilirubin to increase respectively to 4.50 ± 0.25 (2.2–7.5; $P < 0.05$), 3.62 ± 0.49 (1.0–7.2), and 3.91 ± 0.45 μmol/L (2.1–6.4; $P < 0.05$). In the fatty-liver-dystrophy domestic mixed-breed dogs, the level of total bilirubin amounted to 4.0 ± 0.41 μmol/L (1.1–5.7), which was 1.4 times ($P < 0.05$) higher than in their clinically healthy counterparts, whose level ranged 1.1 to 3.8 μmol/L (2.90 ± 0.27).

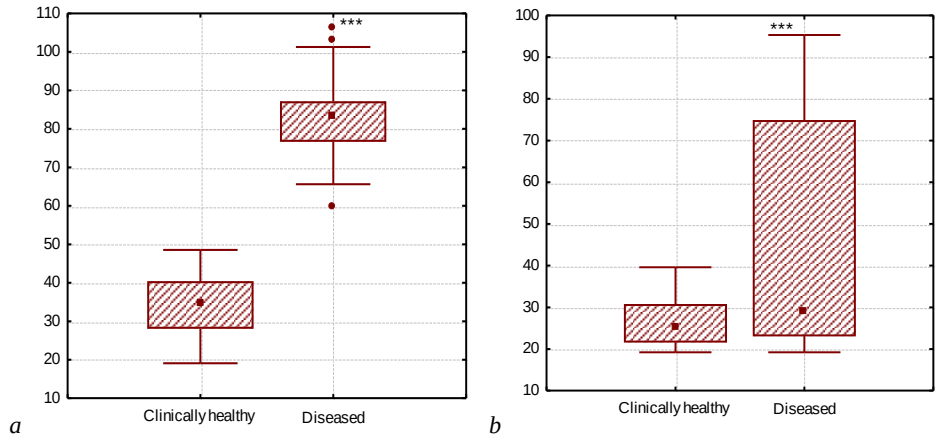


Fig. 2. Activity of amino transferases in blood serum in dogs: *a* – alanine aminotransferase; *b* – aspartate aminotrasferase (U/L); the abscissa axis indicates the groups of animals, the ordinate axis shows measurement units of the parameter; small square – median, upper and lower rectangle borders – 25% and 75% quartiles, vertical line – minimum and maximum values, circles – outliers; n = 40; P < 0.001***

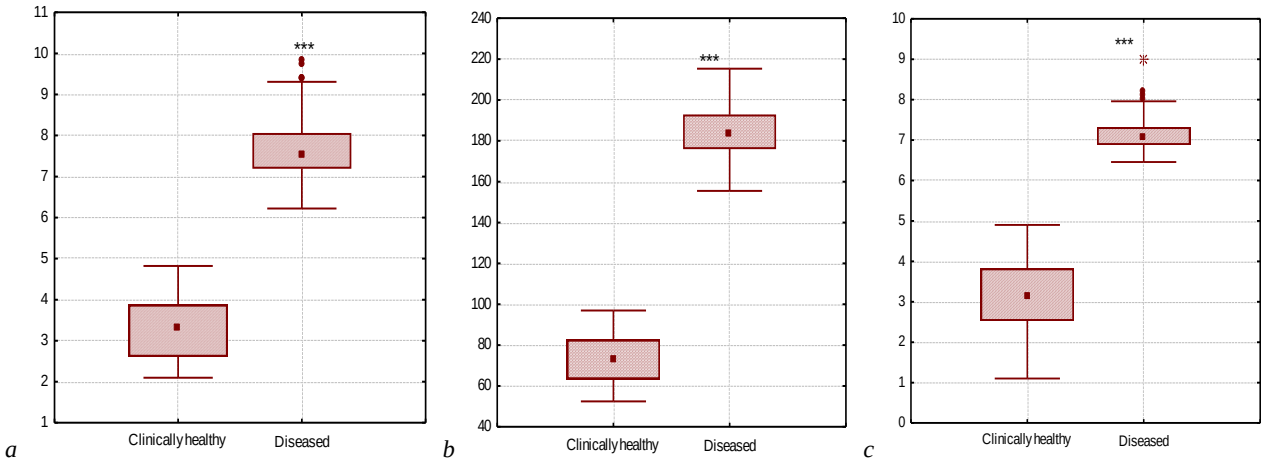


Fig. 3. Activity of cholestatic enzymes in blood serum of the dogs: *a* – glutamate dehydrogenase; *b* – alkaline phosphatase; *c* – gamma-glutamyl transpeptidase (U/L); the abscissa axis indicates the groups of animals, the ordinate axis shows measurement units of the parameter; small square – median, upper and lower rectangle borders – 25% and 75% quartiles, vertical line – minimum and maximum values, circles – outliers; n = 40; P < 0.001***

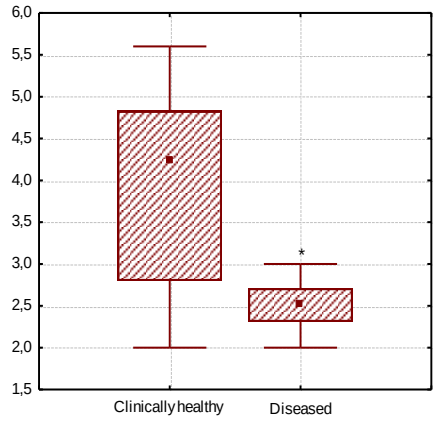


Fig. 4. Content of cholesterol in blood serum of the dogs (mmol/L): the abscissa axis indicates the groups of animals, the ordinate axis shows measurement units of the parameter; small square – median, upper and lower rectangle borders – 25% and 75% quartiles, line – minimum and maximum values, circles – outliers; n = 40; *P < 0.05

An informative marker of liver dystrophy is hyperenzemia. It has to be noted that the activity of cytolytic enzymes (alanine- and aspartate aminotransferase, and glutamate dehydrogenase) in the ill dogs of different breeds did not vary statistically (Table 1).

The content of glutamate dehydrogenase was 2.25 times greater in blood serum of the German shepherds, 2.32 times higher in the spaniels, 2.52 times higher in the Yorkshire terriers, and 2.15 times higher in the mixed-breed dogs, compared with their healthy counterparts (P < 0.001; Table 1).

A similar tendency was observed in the German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs regarding the activityes of alkaline phosphatase and gamma-glutamyl transpeptidase, which were higher (P < 0.001) by 2.5 and 2.3, 2.6 and 2.2, 2.6 and 2.3, and 2.4 and 2.5 times, respectively (Table 2). The activity of cholestatic enzymes in the ill dogs of different breeds was practically the same, except the mixed-breed dogs, in which the mean activity of gamma-glutamyl transpeptidase was greater (P < 0.05) than in the German shepherds, although the maximum values of these parameters were approximated (Table 2).

The mean values of cholesterol in the ill German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs were 2.52 ± 0.07, 2.51 ± 0.06, 2.59 ± 0.07, and 2.43 ± 0.07 μmol/L, respectively.

Due the literature sources lacking data pertaining to the condition of bile acids in blood serum of dogs, we aimed at determining their limits in clinically healthy German shepherds and interpret the results for diagnostic of fatty liver dystrophy.

Therefore, we determined the limits of concentration of bile acids in the German shepherds before and after meal and correlations between certain biochemical parameters, which not only indicate fatty liver dystrophy in German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs, but could also be used as early markers of this pathology.

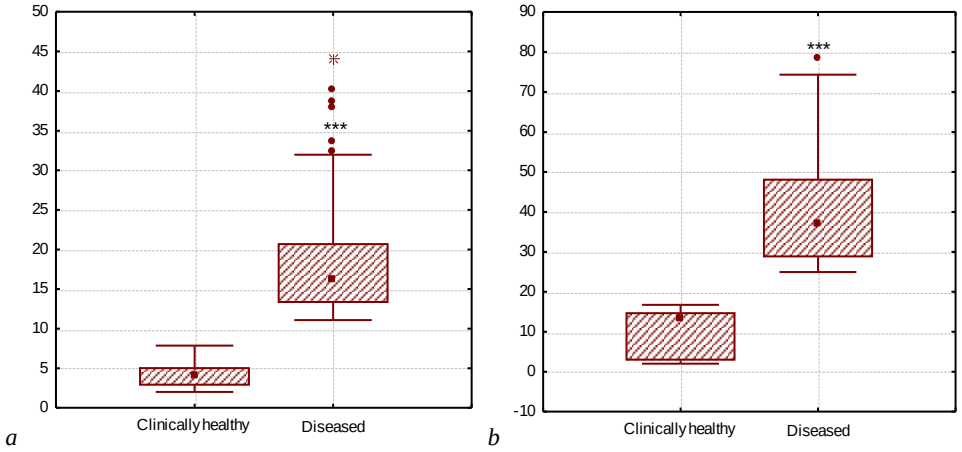


Fig. 5. Concentration of bile acids in blood serum of the dogs: *a* – prior to meal; *b* – two hours after meal (µmol/L); the abscissa axis indicates the groups of animals, the ordinate axis shows measurement units of the parameter; small square – median, upper and lower rectangle borders – 25% and 75% quartiles, vertical line – minimum and maximum values, circles – outliers; n = 40; *** P < 0.001

Table 1
Parameters of activities of cytolytic hepatic enzymes of the domestic animals across breeds (x ± SD)

Parameter	Breed	Clinically healthy dogs	Number of animals	Ill dogs	Number of animals
Alanine aminotransferase, U/L	German shepherd	31.9 ± 2.9^{a}	10	84.6 ± 1.9^{b}	24
	Spaniel	36.3 ± 2.7^{a}	10	80.0 ± 2.2^{b}	15
	Yorkshire terrier	33.9 ± 3.2^{a}	10	86.6 ± 3.0^{b}	9
	Mixed breed	34.4 ± 2.6^{a}	10	81.3 ± 3.9^{b}	12
Aspartate aminotransferase, U/L	German shepherd	26.8 ± 2.1^{ab}	10	56.4 ± 1.9^{c}	24
	Spaniel	25.8 ± 1.6^{a}	10	55.0 ± 2.3^{c}	15
	Yorkshire terrier	24.8 ± 1.7^{a}	10	63.1 ± 5.8^{d}	9
	Mixed breed	27.8 ± 1.9^{b}	10	59.8 ± 4.0^{cd}	12
Glutamate dehydrogenase, U/L	German shepherd	3.22 ± 0.28^{a}	10	7.23 ± 0.08^{c}	24
	Spaniel	3.14 ± 0.31^{a}	10	7.19 ± 0.12^{c}	15
	Yorkshire terrier	2.91 ± 0.32^{a}	10	7.32 ± 0.23^{c}	9
	Mixed breed	3.38 ± 0.26^{b}	10	7.29 ± 0.18^{c}	12

Note: different letters in column indicate that the sets of data are significantly (P < 0.05) different from one another according to the Tukey's Test.

Table 2
Activities of cholestatic hepatic enzymes of the dogs across breeds, x ± SD

Parameter	Breed	Clinically healthy dogs,	Number of animals	Ill dogs	Number of animals
Alkaline phosphatase, U/L	German shepherd	74.5 ± 4.5^{ab}	10	186.3 ± 2.7^{c}	24
	Spaniel	72.5 ± 4.0^{ab}	10	185.9 ± 4.3^{c}	15
	Yorkshire terrier	69.0 ± 4.7^{a}	10	180.4 ± 3.9^{c}	9
	Mixed breed	78.0 ± 3.2^{b}	10	184.4 ± 5.7^{c}	12
Gamma-glutamyl transpeptidase, U/L	German shepherd	3.21 ± 0.21^{a}	10	7.52 ± 0.11^{b}	24
	Spaniel	3.39 ± 0.28^{a}	10	7.61 ± 0.20^{b}	15
	Yorkshire terrier	3.42 ± 0.17^{a}	10	7.69 ± 0.35^{b}	9
	Mixed breed	3.23 ± 0.31^{a}	10	8.14 ± 0.26^{c}	12

The concentration of bile acids in blood serum of the clinically healthy German shepherds prior to meal on average measured 5.4 ± 0.51 µmol/L. According to mean quadratic deviation (σ±1.5), the concentration bile acids prior to meal was within a range of 3.9–6.9 µmol/L, which encompassed 66% of the obtained results. Because 34% of the results fell outside those limits, additional estimates were made with the use of double or triple σ (σ2 and σ3). At σ2 ±3.0, the concentration of bile acids accounted for 2.4–8.4 µmol/L, encompassing 100% of the results.

After meal, the limits of concentration of bile acids in blood serum of the German shepherds accounted for 13.3–16.1 µmol/L with a mean of 14.8 ± 0.31 µmol/L. Determining the thresholds at σ (±0.94) and σ2 (±1.88) provided the ranges of 13.86–15.74 and 12.92–16.68 µmol/L, respectively. During the estimates based on σ, only 48% of the data fell within the limits, whereas at σ2, 100% were included. Therefore, we recommend the limits of concentrations of bile acids for the clinically healthy German shepherds to be at a level of 2.4–8.4 prior to meal and 12.9–16.7 µmol/L two hours after meal.

In blood serum of the German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs with fatty liver dystrophy, the concentrations of bile acids prior to and two hours after meal were elevated (P < 0.001) by 3.5 and 2.8; 4.1 and 2.9; 4.5 and 3.3; 3.2 and 2.5 times, respectively (Table 3). It should be noted that in the ill dogs of different breeds, increase in the level of bile acids prior to meal was more pronounced than after meal, which is explained by the development of lipomobilization syndrome and intrahepatic cholestasis.

The concentrations of bile acids in blood serum of the ill German shepherds before and two hours after meal were found to be strongly positively correlated (r = 0.963). This correlation evidences the presence of intrahepatic cholestasis and cholemia during fatty liver dystrophy, and also can be an early diagnostic test for this pathology.

Additionally, positive correlations were detected between the activity of alkaline phosphatase and concentration of bile acids (r = 0.408), and also the activity of alkaline phosphatase and gamma glutamyltransferase (r = 0.423) prior to meal, confirming the cholestasis syndrome. Furthermore, we observed a positive correlation between the activity of alanine aminotransferase and lactate dehydrogenase (r = 0.395). In the spaniels, we found positive correlations between the concentration of bile acids in blood serum prior to and two hours after meal (r = 0.905), the activity of alkaline phosphatase and concentration of bile acids (r = 0.321), the activities of alkaline phosphatase and gamma glutamyl transpeptidase (r = 0.606), alanine aminotransferase and glutamate dehydrogenase (r = 0.635), and the concentrations of total bilirubin and bile acids (r = 0.494) prior to meal.

Table 3
Concentration of bile acids in blood serum of the domestic animals across breeds, x ± SD

Parameter	Breed	Clinically healthy dogs	Number of animals	Ill dogs	Number of animals
Bile acids prior to meal, µmol/L	German shepherd	5.4 ± 0.5^{b}	10	18.8 ± 1.4^{cd}	24
	Spaniel	4.6 ± 0.5^{a}	10	18.7 ± 5.6^{cd}	15
	Yorkshire terrier	4.6 ± 0.5^{a}	10	20.7 ± 3.1^{d}	9
	Mixed breed	5.4 ± 0.6^{b}	10	17.2 ± 2.1^{c}	12
Bile acids two hours after meal, µmol/L	German shepherd	14.8 ± 0.3^{b}	10	40.7 ± 2.5^{cd}	24
	Spaniel	13.8 ± 0.5^{a}	10	39.4 ± 4.0^{c}	15
	Yorkshire terrier	13.8 ± 0.4^{a}	10	46.2 ± 6.0^{d}	9
	Mixed breed	14.9 ± 0.4^{b}	10	37.5 ± 3.2^{c}	12

Furthermore, we determined positive correlations between the activity of gamma-glutamyl transpeptidase and concentration of bile acids (r = 0.625), alkaline phosphatase and bile acids (r = 0.458), the activities of alkaline phosphatase and gamma-glutamyl transpeptidase (r = 0.809), alanine aminotransferase and glutamate dehydrogenase (r = 0.770), and the concentrations of total bilirubin and bile acids (r = 0.758) prior to meal in the Yorkshire terriers, thus confirming the cytolysis and cholestasis syndrome occurring during the pathology.

The concentrations of bile acids in blood serum of the fatty-liver-dystrophy Yorkshire terriers both before and after meal were positively correlated (r = 0.909).

In the mixed-breed dogs, we observed positive relationships between bile acids prior to and after meal (r = 0.841, Fig. 6d), gamma-glutamyl transpeptidase and bile acids (r = 0.421), alkaline phosphatase and bile acids (r = 0.657), alkaline phosphatase and gamma-glutamyl transpeptidase (r = 0.344), aspartate aminotransferase and glutamate dehydrogenase (r = 0.839), and total bilirubin and bile acids (r = 0.361).

Although we found a weak negative correlation between cholesterol and bile acids concentrations in blood serum prior to meal in the Yorkshire terriers (r = –0.134), mixed-breed dogs (r = –0.108), German shepherds (r = 0.191), and spaniels (r = 0.098), it was low-positive, indicating a decrease in the synthesizing capacity of hepatocytes in the fatty-liver-dystrophy dogs.

Echographic survey of the liver is highly informative because of the liver's homogeneous structure and a high possibility of ultrasound waves penetrating it. The method allows estimating the sizes of the liver and the state of its margins, and detecting focal and diffusive lesions, and dense and large fluid build-ups. Valuable diagnostic criteria are sizes of liver, condition of the parenchyma and bladder, vascular pattern, thickness of the capsule, and the presence of fine- and coarse-grain inflammatory changes in the structure.

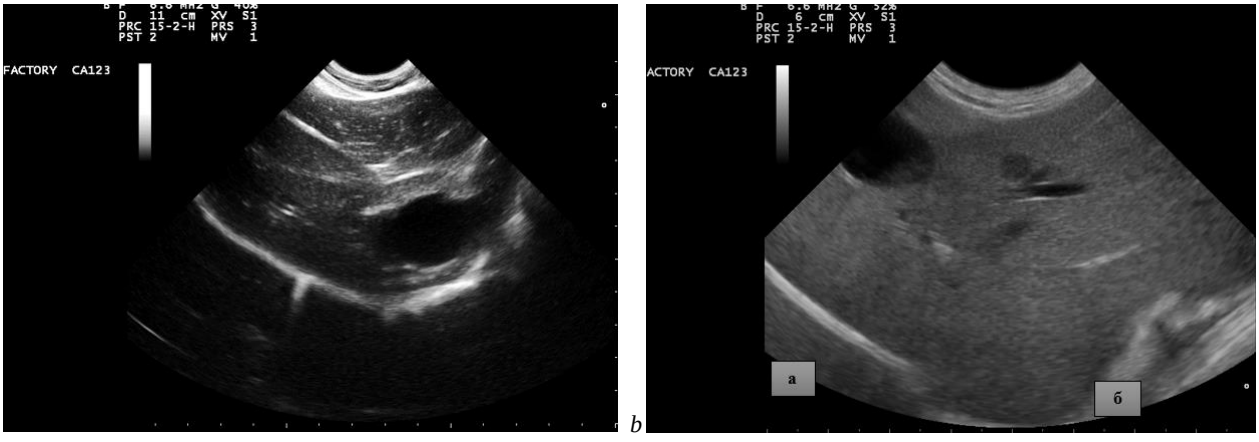
The ultrasonographic study was conducted for 100 dogs kept in private houses, including 40 clinically healthy (10 German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs) and 60 dogs with fatty liver dystrophy (24 German shepherds, 15 spaniels, 9 Yorkshire terriers, and 12 mixed-breed dogs).

The ultrasound examination of the dogs' liver was carried out through the ventral abdominal wall from the region of xiphoid process or the lateral rib cage in intercostal spaces. We started from the epigastral region. In the sensor-application region, we removed hair and applied ultrasound gel in necessary amount to ensure the absence of air layer between the sensor's operational surface and the animal's skin. For the examination through the ventral abdominal wall, we prepared the area from the xiphoid process to the navel area at a several centimeter distance from the central line along the costal margins.

The study through the intercostal spaces was carried out on the right side, because the examination of liver on the left side can be hindered by the stomach, especially when it is filled with gas or fodder.

For the study through the ventral abdominal wall, the sensor was installed just behind the xiphoid process, and ultrasound ray was directed craniodorsally, and afterward – to the left and right of the midline, until analyzing the entire parenchyma.

When studying the obese animals, we noted the presence of visceral fat, located between the abdominal wall and the liver in the region of the xiphoid process. The presence of this layer can affect the estimation of the liver's size, complicating the visualization of the organ because of fatty cells absorbing ultrasound. In the image, this layer of fat can be no different from that of the liver tissue, but is always immobile. In the clinically healthy dogs, the diaphragm was clearly visible as a thin, well expressed, echogenic line, oscillating in rhythm with breathing. The liver parenchyma looked rough – grained, with the same echostructure throughout, and moderately hypoechogenic.



a *b*

Fig. 6. The ultrasonography of the dog`s liver: *a* – within the norm; *b* – with fatty-liver-dystrophy: *1* – densification of the capsule; *2* – rounded liver margins

Limited regions, different according to echogenicity, were fibers of the connective tissue – falciform ligament (ligamentum falciforme), ligament of head of femur (ligamentum teres), left triangular ligament (ligamentum triangulare sinistrum), and other ligaments, interlobular spaces, etc. The liver margins were within the norm, distinct, and the hepatic lobes usually were well separated one from another. The intrahepatic ducts in the norm are practically untraceable, but diagnostically valuable signs are their expansions. The vascular pattern in the norm was well expressed (Fig. 6). In all of the fatty-liver-dystrophy dogs, we found even, diffusive increase in echogenicity of the liver parenchyma, paired with a notable weakening of the signal in remote zones. The liver margins, especially of the right and square lobes, were rounded, and the capsule echostructure was dense (Fig. 6). The vascular pattern was impoverished. The liver echostruc-

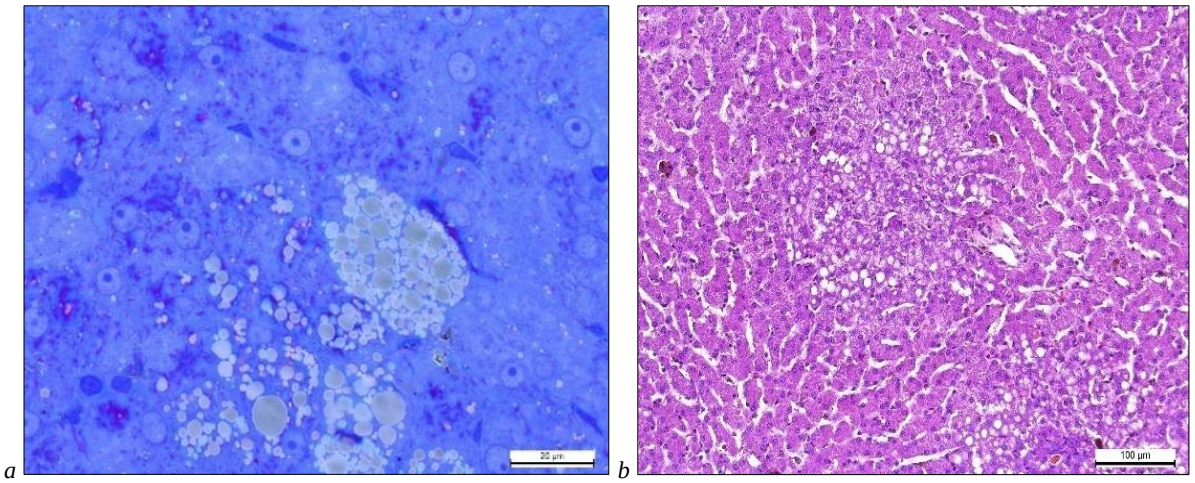
ture was coarse-grained. In 44 fatty-liver-dystrophy patients (60.3%), we detected hepatomegaly – the contours of the organ were beyond the costal margin. The parallel studies of respective morphological and laboratory parameters of blood of these animals confirmed the presence of pathological process in the liver.

During the histological and histochemical studies of the liver, we found notable changes in hepatocytes, characterized by the development of parenchymatous fatty dystrophy. In particular, the study of the histopreparations, made using freezing microtome, revealed accumulations of neutral fats, uniformly stained into orange-red color by Sudan-III and into red with Nile Blue. In semithin sections, stained with basic fuchsine, we observed that the cytoplasm of many hepatocytes contained large vacuoles, which were visualized, filled with homogeneous light-gray content. Deposition of lipids in hepatocyte cytoplasm in the preparations made using sledge microtome and stained with hematoxylin eosin optically looked as empty droplets.

Hepatocytes that underwent fatty dystrophy were increased, with their nucleus (or two nuclei in binucleated hepatocytes) shifted by the fatty droplet to the peripheral areas of the cell (Fig. 7). Fatty droplets located in the cytoplasm of hepatocytes were rounded or oval, with distinct contours, and mostly quite large (large-droplet fatty dystrophy). Individual hepatocytes were subject to necrotic changes. In the areas where hepatocytes were undergoing necrosis, fatty droplets accumulated in massive conglomerates (Fig. 8).

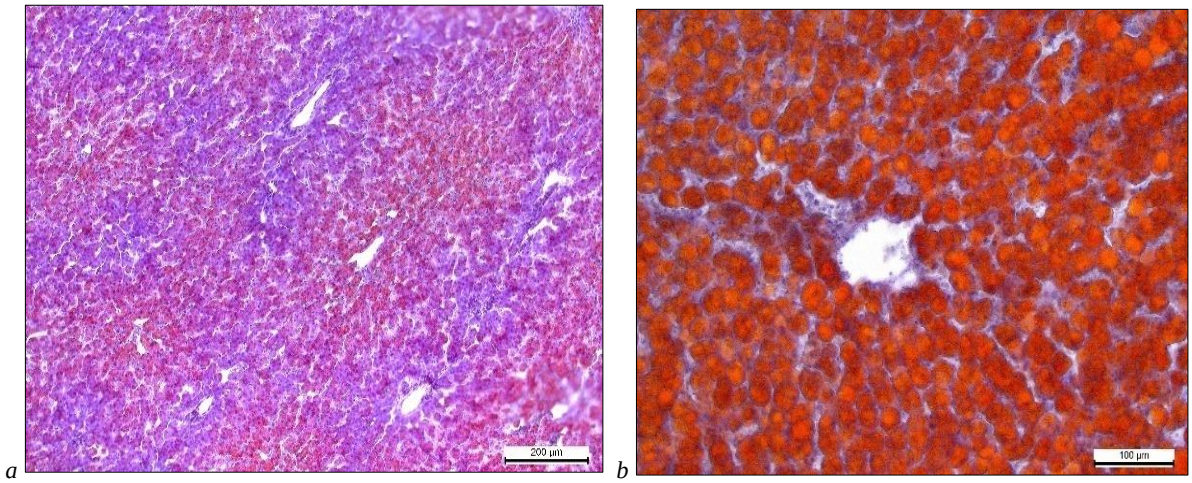
It has to be noted that in large amounts, neutral fats concentrated in hepatocytes located in the central areas of hepatic lobules and to a lesser degree in the peripheral zones. Individual hepatic lobules were quite massively, densely, and uniformly infiltrated by triglycerides. At the same time, the cytoplasm of the hepatic lobules nearby contained an insignificant amount of small dust-like fatty inclusions (dust-like fatty dystrophy).

The central veins of many hepatic lobules were enlarged and overfilled with red blood cells. In some places, we observed red blood cells sticking together. Individual sinusoids and interlobular veins were also insignificantly enlarged and overfilled with erythrocytes. The epithelium of interlobular bile ducts was swollen, and small droplets filled with neutral fats were visualized in its cytoplasm.



a *b*

Fig. 7. The liver of a German shepherd: massive conglomerates of neutral fats:
a – semithin section methylene blue and fuchsine; *b* – focal necrosis of hepatocytes; hematoxylin and eosin



a *b*

Fig. 8. The liver of a German shepherd: massive infiltration and accumulations of neutral fats around the central vein:
a – hematoxylin and Sudan-III; *b* – Sudan-III

In some places, a proliferation of the bile ducts' epithelium was seen. Individual epitheliocytes of the interlobular bile ducts were subject to dystrophic and necrotic changes and were desquamated into their lumen.

Therefore, during histological and histochemical studies, we determined that the liver of the German shepherds was undergoing a development of fine-focal and large-focal fatty dystrophy of hepatocytes and fine-focal dystrophy of the bile ducts' epithelium, accompanied by necrotic changes progressing into micronecroses.

When using transmission electronic microscopy on ultrathin sections of liver bioptates from the healthy German shepherds, we noted that at the ultrastructural level, the main mass was represented by hepatic cells and sinusoid blood capillaries adjacent to them. The hepatocytes were of polygonal shape and of average electronic density. Such hepatocytes mostly contained a large nucleus or two nuclei and cytoplasm filled with mitochondria, single peroxisomes, granular and agranular endoplasmic reticulum, Golgi complex, fine-granular hyaloplasm, single lipid droplets, and glycogen granules. Cytoplasm

of the hepatocytes was surrounded by plasmatic membrane, which at the biliary pole formed microvilli, included in bile capillaries. At the sinusoidal pole, the plasmatic membrane formed a labyrinth of finger-like projections, located in the space of Disse. The hepatocytes' nuclei were mostly rounded, mainly filled with euchromatin and a small amount of heterochromatin, localized in peripheral regions. The central part of the nuclei was occupied by one or two nucleoli, in which we clearly saw fibrillar centers, fibrillar and granular components. The nuclei were surrounded by nuclear membrane, in which nuclear pores could be seen. The outer layer of the nuclear membrane was in some places connected to the channels of granular endoplasmic reticulum and was their extension. The sinusoidal blood capillaries were densely adjacent to the hepatic laminae. Their walls were formed of the basal membrane, endothelial cells, and Kupffer cells. The lumen of the sinusoidal blood capillaries was filled with blood plasma of average electronic density and with red blood cells.

The animals with laboratory confirmed fatty liver dystrophy presented with the most appreciable ultrasound changes in the regions of damaged sinusoidal blood capillaries. Thus, in the capillaries' lumen, there were well-seen sludges of red blood cells and electronically dense masses of blood plasma. Red blood cells in the sludges were mostly of irregular shape, with their plasmatic membranes loosened. The luminal surfaces of endotheliocytes formed a large amount of microvilli and protrusions, some of which were shortened and desquamated into the lumen of such capillaries. The regions of the spaces of Disse adjacent to the damaged capillaries were enlarged and filled with detritus masses. Between the hepatocytes, an expansion of collagenic fibers was observed, indicating a strengthening of sclerotization processes. The changes detected in the liver bioptates suggested a long circulatory hypoxia. On the sinusoid pole of cytoplasm of many hepatocytes, there were concentrations of large lipoprotein droplets of high electronic density and highly dense autophagolysosomes. This pole of cytoplasm formed no labyrinth of microprotrusions and villi. The nuclei of the described hepatocytes (under the influence of lipoprotein droplets) acquired irregular, bean-like shape and were eccentrically located in the hepatocyte cytoplasm. In such nuclei, we mostly found heterochromatin and a highly electronically dense nucleolus. On the biliary pole of cytoplasm of individual hepatocytes, we observed a large accumulation of average-sized lipid droplets of low electronic density. Such lipid droplets were surrounded by channels formed by agranular plasmatic network, with a significantly enlarged lumen. In the peripheral regions of the agranular endoplasmatic reticulum, we detected single zones of accumulation of α-glycogen. In the mytochondria adjacent to those areas, we noted a well-developed system of cristae, which provided a high electronic density of the matrix.

As known, increased electronic density of granules of the mitochondrial matrix indicates an accumulation of calcium and other cations, which may be a sign of impaired ion homeostasis and functional loading on the organelle. In the cytoplasm, we also found numerous peroxisomes of different sizes and with high electronic density, along with a high number of autophagolysosomes, which contained lipoprotein inclusions, indicating an activation of autophagous pathway of breakdown of excessive lipids. In some hepatocytes, we detected a small number of lipoprotein droplets of very low electronic density, located in the light, electron-light hyaloplasm, which was saturated with enlarged channels of agranular endoplasmic reticulum. Between the cisterns of agranular endoplasmic reticulum, we registered a considerable accumulation of α-glycogen granules arranged in rosette-like pattern, indicating a compensatory restructuring of the carbohydrate metabolism against the background of impairments in lipid homeostasis. In some hepatocytes, we found large spherical lipoprotein inclusions, which filled the most part of cytoplasm and were surrounded by mytochondria that had been substantially reduced in size. The mytochondria were characterized by widely spread, in some places disorganized, cristae, suggesting deep energy malfunctions. Single fatty droplets were found in the spaces of Disse, where we also observed tissue detritus.

The ultrastructural changes in the liver bioptates from the German shepherds were as follows: Some hepatocytes exhibited formations of lipid droplets, which were of average sizes and with low electronic density, while other hepatocytes showed massive lipoprotein inclusions, increase in the number of peroximes and autophagolysis in cytoplasm, expansion of the spaces of Disse, the presence of erythrocyte sludges and electronically dense protein-lipid masses in capillaries, and expansion of collagen fibers between hepatocytes. The overall detected changes suggest the presence of a long circulatory hypoxia, which promotes the progression of fatty dystrophy and formation of sclerotic processes in the liver parenchyma.

Discussion

Fatty liver dystrophy in dogs is a complex pathological process that stems from metabolic disorders, in particular a disbalance between the influx and use of energy, and also deficit of nutrients and biologically active compounds in diet. In addition, development of this pathology oftentimes owes to the use of poor-quality fodders and complications of different diseases that lead to metabolic disorders. Dogs' diet varies depending on the physiological state and functional loading. During an average loading, it is recommended to elevate the content of proteins approximately by 30%, with necessary inclusion of no less than 30% of animal protein. Deficit of nutrients in diet entails mobilization of lipids, exhaustion of the organism, and development of chronic stress and fatigue, whereas excess of nutrients in dogs with low physical activity promotes obesity (Assawarachan et al., 2019; Buckley et all., 2025). The analysis of dog nutrition demonstrated an imbalance: Mixed feeding with excess of proteins and fats led to impaired metabolism and deterioration of the liver's functional state.

The main biochemical markers of fatty liver dystrophy are considered to be hyperenzymemia, increased total bilirubin, reduced cholesterol, and elevated concentration of bile acids in blood serum.

The diagnostic biochemical markers of fatty liver dystrophy in dogs is hyperenzymemia of specific hepatoindicatory enzymes, content of total bilirubin, and cholesterol. Fatty infiltration of the liver causes damage to the structure and permeability of hepatocyte membranes, with further release of indicatory enzymes into blood. An early diagnostic criterion of the pathology is concentration of bile acids in blood before and two hours after meal.

Impairment of the functional state of the organ caused a significant ($P < 0.001$) increase in the concentration of total bilirubin in blood serum of dogs suffering fatty liver dystrophy. Impaired capacity of hepatocytes to absorb, conjugate, and release bilirubin is due to decrease of their energy resources, which is characteristic for parenchymatous jaundice. Besides, concentration of total bilirubin depends not only on damage to hepatocytes and impairment of their functional ability, but also on the degree to which red blood cells have been ruined (Hange & Abdelkader, 1986).

With the purpose of determining the damage to hepatobiliary system, we measured the activity of hepatoindicatory enzymes that indicate the main syndromes of liver diseases, in particular cytolysis and cholestasis syndromes. The level and duration of hyperenzymemia depend on the enzymes' activity in the tissues, subcellular localization, rates of their elimination into blood, their release from blood by macrophagocytes, and also on the duration and nature of the disease (Alvarez & Whittemore, 2009).

We determined the activity of three enzymes: aminotransferases – alanine and asparagin (ALT and AST) and glutamate dehydrogenase (GLDH), which are indicative of liver function and early informative parameters of cytolysis syndrome. In the ill dogs, the activities of ALT and AST significantly ($P < 0.001$) increased compared with those of the healthy dogs. In the fatty-liver-dystrophy German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs, the activity of indicatory enzymes was significantly ($P < 0.001$) higher than in their healthy counterparts.

In the ill dogs, the GLDH activity was also significantly higher ($P < 0.001$) by 2.3 times than in the clinically healthy dogs. Therefore, despite the fact that GLDH is a mitochondrial enzyme, its activity in blood serum notably increases, suggesting damage to hepatocytes and their

organelles – mytochondria. Our data are consistent with the results of DeMarle et al., (2021), Giannetto et al. (2022), and Vlizlo et al. (2024).

The activities of serum alkaline phosphatase and GGT in the ill dogs increased 2.5 and 2.3 times ($P < 0.001$) respectively. Hyperenzymemia of AP and GGT was registered in 100% of the ill animals. Increase in the activity of both enzymes indicates ruination of canalicular membranes of hepatocytes, their biliary pole, and endothelial cells of the intrahepatic bile ducts, which leads to impaired bile flow and to cholestasis syndrome. Our data align with the studies by other researchers who recorded increased AP and GGT in blood serum of dogs and rats with experimentally modeled liver pathology. Increase in the activity of those enzymes in blood serum of dogs suffering fatty liver dystrophy was also observed by other researchers (Kruitwagen et al., 2014; Imbery et al., 2022).

The concentration of cholesterol in blood serum from the dogs with fatty liver dystrophy decreased 1.8-fold ($P < 0.001$), indicating reduced synthesis function of hepatocytes. Hypercholesterolemia in all of the dogs with fatty liver dystrophy was not confirmed by any of our studies and experimental modeling of this pathology in rats (Vlizlo et al., 2024).

During the biochemical study of blood serum of the fatty-liver-dystrophy dogs, we determined that the fatty acid concentration prior to meal was significantly ($P < 0.001$) higher by 3.8 times and two hours after meal it was higher by 2.8 times. This may be explained by a disorder in the secretion of bile into bile capillaries, the emergence of the cholestasis syndrome, and its subsequent absorption back into blood. In the literature sources, there are no data regarding the concentration of bile in blood serum of dogs suffering fatty liver dystrophy. Therefore, studies of the concentration of bile acids in blood serum are relevant and those we performed revealed that they are an informative marker of early diagnostics of the pathology in dogs. Our conclusion is evidenced by a direct positive correlation ($r = 0.963$, $r = 0.905$, $r = 0.909$, $r = 0.841$) between the FA concentration in blood serum of the German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs, respectively, prior to and two hours after meal.

When analyzing the results of our studies, it is noteworthy that cytolysis and cholestasis syndromes were found in all the dogs suffering fatty liver dystrophy. The most pronounced cytolysis syndrome was seen in the Yorkshire terriers and mixed-breed dogs ($r = 0.770$ for ALT and GLDH and $r = 0.839$ for ALT and GLDH, respectively), and cholestasis syndrome was the most notable in the Yorkshire terriers ($r = 0.625$ for GGT and FAs, $r = 0.458$ for AP and FA, and $r = 0.809$ for AP and GGT), less pronounced in the spaniels ($r = 0.321$ for AP and FA and $r = 0.606$ for AP and GGT) and the mixed-breed dogs ($r = 0.421$ for GGT and FA, $r = 0.657$ for AP and FA, and $r = 0.344$ for AP and GGT), and the least expressed in the German shepherds ($r = 0.408$ for AP and FA and $r = 0.423$ for AP and GGT). In our opinion, this was associated with breed specifics, body mass, and sizes of the animals, because the digestive system of large dogs is 2.5-fold less effective than in small dogs (the mass of the digestive tract in small dogs accounts for 7% of their body mass, while in large dogs for only 2.7%), and accordingly the food consumption varies.

To interpret the results of the diagnostic of fatty liver dystrophy in the dogs, we determined the limits of the concentration of bile in blood serum of the clinically healthy German shepherds, because the dogs of this breed in the experiment were most numerous ($n = 50$). According to the estimates of quadratic deviation ($\sigma 2 \pm 3$), the concentration of FAs in blood serum prior to meal was 2.4 – 8.4 µmol/L and these limits ($\sigma 2$) encompassed 100% of the results. After meal, the FA concentration limits in blood serum of the dogs (according to the mean quadratic deviation ($\sigma 2 \pm 1.88$)) accounted for 12.92–16.68 µmol/L, and these limits included 100% of the results as well. Because according to $\sigma 2$ estimate prior to meal and two hours after it, the limits encompassed 100% of the animals, we consider the reference values for FA concentration in blood serum of the clinically healthy German shepherds to be 2.4–8.4 prior to meal and 12.9–16.7 µmol/L two hours after meal.

Informative results for confirmation of liver dystrophy were those of ultrasonographic study: an even increase in the liver with nonhomogeneous echogenicity of its parenchyma and rounded margins of the organ, impoverishment of the vascular pattern, and overfilled bladder. The detected ultrasound changes point to the development of dystrophic processes in the liver of ill dogs, which is consistent with the data (Pelligra et al., 2024).

The clinical symptoms and changes in the laboratory parameters were compared with the results of histological study of the liver bioptates. The histology revealed enlarged hepatocytes with fatty inclusions in the form of vacuole in cytoplasm. Some cells were observed to undergo necrotic changes and contain large concentrations of neutral fats. Certain hepatocytes included large fatty droplets, which completely filled the cell, displacing the nucleus with atrophied cytoplasm to the periphery, forming signet ring cells. Those morphological changes reflected the depth of lesion on the hepatic parenchyma and were confirmed by electronic-microscopic data.

The ultrastructural damages were most notable in the zones of affected sinusoid capillaries. The luminal surface of endothelyocytes formed numerous microvilli and protrusions, and some of them were desquamated into the lumen of capillaries. The adjacent spaces of Disse were enlarged and filled with detritus and collagen fibers. On the sinusoidal pole of the hepatocyte cytoplasm, we found large lipoprotein droplets of high electronic density and numerous autophagolysosomes with very high density. At the same time, this pole had no characteristic labyrinth of microvilli. The cell nuclei were irregular, bean-shaped, eccentrically located under the influence of lipid inclusions, and contained heterochromatin and dense nucleoli. On the biliary pole of individual hepatocytes, we found accumulations of average-sized lipid droplets with low electronic density, surrounded by channels of agranular endoplasmic reticulum. The peripheral regions of the reticulum contained single zones of α-glycogen. The adjacent mytochondria exhibited a developed network of cristae and high electronic density of the matrix. We observed a high number of peroxisomes of different sizes and autophagolysosomes with lipoprotein inclusions.

The electronic-microscope data provided a detailed understanding of the course of biochemical processes in the liver parenchyma, confirming the role of etiological factors in the disease's pathogenesis.

Based on the literature and our own results, we formulated the main links of pathogenesis of fatty liver dystrophy in working dogs. The main pathogenetic effect is caused by etiological factor, which impairs the functional state of the liver, inducing notable structural divergencies. Fatty infiltration of the liver is due to enhanced transport of lipids from fat depots against the background of energy deficit, caused by breakdown of carbohydrates, which initiates the development of compensatory lipomobilization syndrome. During the influx of excess fatty acids from the gastrointestinal tract or the tissues during active lyposis, neutral fats accumulate in hepatocytes, leading to the formation of fatty liver dystrophy. Because the liver is the key organ of lipid metabolism, particularly esterification of cholesterol and synthesis of bile acids, those processes reflect the synthetic functions of hepatocytes (Kakimoto et al., 2017).

During the development of cytolysis syndrome, the permeability of the membranes increases as a result of damage to hepatocytes, which rapidly increases the activity of enzymes that are significant diagnostic markers. Hyperenzymemia —according to the parameters of alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase—is often observed earlier than the disease's clinical symptoms appear. Measuring the activity of those enzymes that circulate in blood allows not only diagnosing damage to hepatocytes (cytolysis syndrome) but also evaluating impairments in bile removal – cholestasis syndrome (Oikonomidis & Milne, 2023).

Cholestasis is accompanied not only by disorders in bile formation, but also by delay and malfunctioning of its release. This process starts at the level of membranes of bile ducts of hepatocytes and is called intrahepatic cholestasis. Increase in the concentration of bile acids during cholestasis can cause additional damage to hepatocytes, forming a pathophysiological vicious circle: Increase in the concentration of bile acids in the hepatic tissue enhances the intrahepatic cholestasis, which in turn drives further accumulation of bile acids in the tissues (Vlizlo et al., 2023).

The cholestasis syndrome is accompanied by rise in the activity of gamma-glutamyl transpeptidase in blood serum. This enzyme is lo-

calized in epithelial cells of bile ducts, canalicular membranes of hepatocytes near the biliary pole, and also in the walls of bile ducts. Increase in the level of gamma-glutamyl transpepidase is a sensitive marker of pathological processes in the hepatobiliary system, an important parameter of cholestasis, and is used as informative test for malfunctioning of bile release with prognostic value (Rahman et al., 2020).

During cholestasis, dogs with fatty liver dystrophy were also observed to have considerably elevated activity of alkaline phosphatase, which is associated with impaired secretion of this enzyme into bile due to damage to extrahepatic bile ducts.

Biliruin, formed as a result of breakdown of hemoglobin in macrophages of the spleen and bone marrow, conjugates with glucuronic acid in the liver. Hyperbilirubinemia in this context is an indicator of cholestasis owing to fatty liver dystrophy (Vlizlo et al., 2021; Pena-Ramos et al., 2021).

Conclusion

In the dogs with fatty liver disease, the informative diagnostic markers included concentration of bile acids in blood serum prior to meal and two hours after meal, and also the activity of hepatocindicatory enzymes of cytolytic (glutamate dehydrogenase, aspartate- and alanine aminotransferase,) and cholestatic (gamma-glutamyltransferase and alkaline phosphatase) actions, changes in which were observed in all of the examined animals. For the German shepherds, spaniels, Yorkshire terriers, and mixed-breed dogs, we found a high positive correlation between concentrations of bile acids prior to and after meal. The data of ultrasonography indicated the presence of even diffusive increase in echogenicity of the liver parenchyma in all the dogs, rounding of its contours, impoverishment of the vascular pattern, densification of the echostructure of the capsule, and coarse-grained structure of the parenchyma. Histological and histochemical studies of the liver of the German shepherds confirmed the development of small- and large-focal fatty dystrophy of hepatocytes, and also dystrophic changes in the epithelium of bile ducts, accompanied by local necroses and the formation of micronecroses. In the liver bioptates from these dogs, we found ultrastructural abnormalities: some hepatocytes exhibiting formation of averaged-sized lipid droplets with low electronic density, while other hepatocytes contained large lipoprotein inclusions; increase in the amount of peroxisomes and autophagolysosomes in cytoplasm; expansion of the spaces of Disse; the presence of sludges of red blood cells and electronically dense masses in capillaries; and expansion of collagen fibers between hepatocytes. The changes indicated a long circulatory hypoxia, which causes progression of fatty dystrophy and development of sclerotic processes in the liver tissue.

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