博士論文

Analysis of serum alkaline phosphatase isoenzymes and hepatic lipid accumulation in dogs with hepatobiliary diseases

(肝胆道系疾患のイヌにおける血中 ALP アイソザイムと肝脂質蓄積に関する解析)

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General Introduction

In veterinary clinical practice, alkaline phosphatase (ALP) is very important in blood biochemical examinations as an indicator of canine hepatobiliary diseases. ALP is a membrane-associated enzyme present in various tissues (Hoffmann and Dorner, 1975). In dogs, only 3 ALP isoenzymes, liver, bone, and corticosteroid-induced ALP can be detected in the serum because the other ALP isoenzymes have very short half-lives (Hoffmann and Dorner, 1977). The liver ALP (LALP) is produced in the liver and accounts for the highest isoenzyme percentage in normal canine serum (Ruegnitz and Schwartz, 1971). Bone ALP (BALP) is produced in actively growing bone (Fishman, 1990) while the corticosteroid-induced ALP (CALP) is produced in the liver and induced by endogenous and exogenous corticoid hormone stimulation (Eckersall and Nash, 1983; Wellman et al., 1982). CALP is a unique isoenzyme of dogs, and has little or no activity in the serum of clinically healthy dogs (Dorner et al., 1974). However, its function and mechanism of production is not well known. It was confirmed that experimental administration of corticosteroid to dogs increased the serum CALP activity and hepatocyte vacuolation secondary to glycogen accumulation (Syakalima et al., 1997a; Wiedmeyer et al., 2002). Although a few studies have reported that serum CALP was detected in dogs with hepatobiliary diseases, its actual activity or percentage was not reported (Syakalima et al., 1997b; Wilson et al., 1992). Therefore, the relationship between serum CALP levels and hepatobiliary diseases is still unclear, and studies on the

clinical utility of serum CALP levels are inadequate.

In dogs, hepatocyte vacuolation is believed to occur as a result of excess accumulation of glycogen, lipid, water, or intracellular edema (Guilford W.G. Center, 1996; Rothuizen, 2006) and frequently associated with high serum ALP activity (Dorner et al., 1974; Rogers and Ruebner, 1977; Sanecki et al., 1987). Canine hepatocyte vacuolation caused by excess glycogen accumulation, which is induced by endogenous or exogenous glucocorticoid (Fittschen and Bellamy, 1984; Syakalima et al., 1997a). In Scottish Terriers, various endogenous steroid hormones were reported to be associated with vacuolar hepatopathy and high serum ALP activity (Zimmerman et al., 2010) and hepatocellular carcinoma associated with vacuolar hepatopathy has been reported (Cortright et al., 2014).

On the other hand, hepatocyte vacuolation caused by excess lipid accumulation was reported in dogs with congenital portosystemic shunt (Hunt et al., 2013; Lee et al., 2011) and nodular hyperplasia (Fabry et al., 1982). Hepatocyte vacuolation caused by lipid and glycogen accumulation accompanied by hyperlipidemia and increased plasma ALP activity was also reported in Schnauzers (Guilford W.G. Center, 1996). Although canine hepatocyte vacuolation is induced by multiple factors, the relationship between ALP isoenzyme activity and causative substances of hepatic vacuolation has not been thoroughly investigated. Excess hepatic lipid accumulation is referred to as fatty liver in

other species (Anstee and Goldin, 2006; Arslan, 2014; Musso et al., 2009). In humans, fatty liver is a confirmed clinical condition, which progresses to hepatitis, cirrhosis, and hepatocellular carcinoma (Dietrich and Hellerbrand, 2014; Musso et al., 2009). In addition, gene expression associated with hepatic lipid metabolism and serum biochemical characterization have been well-studied in this condition (Arslan, 2014; Dietrich and Hellerbrand, 2014; Kikuchi et al., 2014). Therefore, canine hepatic lipid accumulation is considered a useful fatty liver animal model; however, little is known about the mechanism and clinicopathological effect of hepatic lipid accumulation in dogs.

Therefore, the studies conducted for this thesis were carried out to investigate the relationship between the activity of serum ALP isoenzymes and the pathological features of hepatic lipid accumulation in dogs with hepatobiliary diseases. The study described in Chapter 1 of this thesis was aimed at clarifying the relationship between serum ALP isoenzyme activities and hepatobiliary diseases in dogs without a history of glucocorticoid drug administration. In chapter 2, the actual lipid or glycogen amount in the liver was measured and compared to serum ALP isoenzyme activity and hepatic vacuolation severity in dogs with hepatobiliary diseases. In addition, the expression of lipid metabolism-associated genes in the liver tissue was analyzed. In Chapter 3, hepatic lipid accumulation was experimentally induced in dogs by intravenous administration of fat emulsion, and the change in the serum activity of each ALP isoenzyme was evaluated.

In addition, the expression of lipid metabolism-associated genes and each ALP isoenzyme gene in the liver was analyzed. These studies were aimed at assessing the clinical significance of serum CALP levels and hepatic lipid accumulation.

Chapter 1

Analysis of alkaline phosphatase isoenzymes in dogs with hepatobiliary deseases

ABSTRACT

In dogs, 3 alkalinephosphatase (ALP) isoenzymes, liver ALP (LALP), bone ALP (BALP) and corticosteroid-induced ALP (CALP), can be detected in serum. CALP is unique ALP isoenzyme of dogs and confirmed to be increased in dogs with hyperadrenocorticalism or glucocorticoid drug administration. Although few reports showed serum CALP activity was detected in dogs with hepatobiliary diseases, the actual CALP activity was not shown. The objective of this study is to reveal the relationship between canine CALP activity and hepatobiliary diseases, together with other factor in dogs without glucocorticoid administration.

Serum ALP isoenzymes in dogs with hepatobiliary diseases was evaluated retrospectively. Dogs had history of glucocorticoid administration was excluded. Serum CALP percentage and activity were compared with diagnoses, serum cortisol concentration, age, sex, breed and ALT activity.

Seventy-two dogs with hepatobiliary diseases were analyzed in this study. Serum CALP level was increased in dogs with hepatobiliary diseases and serum CALP activitiy was significantly increased in chronic hepatitis, hepatocellular adenoma and nodular hyperplasia group. No significant correlation was observed between serum cortisol concentration and serum CALP percentage or activity.

Dogs with hepatobiliary disease could show high serum ALP activity because of

increasing serum CALP level, even if the dogs have not been administrated glucocorticoid drug and the serum cortisol consentration is normal.

INTRODUCTION

In dogs, alkaline phosphatase (ALP) isoenzyme is produced in various tissues (liver, bone, kidney, intestine, and placenta) (Dorner et al., 1974; Eckersall and Nash, 1983; Hoffmann and Dorner, 1975; Wellman et al., 1982). Only three ALP isoenzymes, liver ALP (LALP), bone ALP (BALP), and corticosteroid-induced ALP (CALP), can be detected in canine serum because of the very short half-lives of other ALP isoenzymes (Hoffmann and Dorner, 1977). LALP, which is produced in the liver, is present in the highest concentration in normal canine serum (Hoffmann and Dorner, 1975). BALP is an isoenzyme produced in actively dividing bone tissue. Usually, it is not detected in canine serum except in puppies, dogs with bone fractures, or bone neoplasms, such as osteosarcoma (Ehrhart et al., 1998; Fishman, 1990; Syakalima et al., 1997b). CALP, which is produced in the liver and is induced by endogenous or exogenous corticoid hormone stimulation (Eckersall and Nash, 1983; Hoffmann and Dorner, 1975; Wellman et al., 1982) is unique to dogs (Dorner et al., 1974). However, little is known about the detailed mechanism of CALP production.

The activities of each ALP isoenzyme in canine serum had been calculated by measuring the differences in sensitivity against heat (Farley et al., 1994; Hoffmann and Dorner, 1975; Saini and Saini, 1978; Teske et al., 1986) or chemical inactivation (Mahaffey and Lago, 1991; Ruegnitz and Schwartz, 1971) between each isoenzyme.

Although these biochemical methods provide direct result of ALP isoenzyme activities, the effect of these inactivation is overlapped between each ALP isoenzymes (Dorner et al., 1974; Syakalima et al., 1997b; Teske et al., 1986). The overlap makes it difficult to distinguish clearly between the ALP isoenzyme activities by biochemical methods. Alternatively, electrophoresis has been widely used to discriminate between different ALP isoenzymes (Hatayama et al., 2011; Itoh et al., 2002).

CALP activity was increased in dogs that received experimental administration of corticosteroid (Syakalima et al., 1997a; Wiedmeyer et al., 2002) and dogs with spontaneous hyperadrenocorticism (Teske et al., 1989; Wellman et al., 1982). In Scottish Terriers, endogenous hormones (cortisol, estradiol, androstenedioneandrostendione, progesterone, aldosterone, and 17α-hydroxyprogesterone) and aging have also been reported to relate with high serum ALP activity (Cortright et al., 2014; Gallagher et al., 2006; Nestor et al., 2006; Zimmerman et al., 2010).

In clinical practice, serum hepatic enzymes such as ALP and ALT are widely evaluated as part of diagnostic evaluation of hepatobiliary diseases. Few studies have measured canine serum CALP levels in hepatobiliary cases. In these reports, a certain number of dogs with hepatobiliary diseases including cirrhosis, liver tumor (Syakalima et al., 1997b), portosystemic shunt, chronic hepatitis, hepatocellular carcinoma, hepatoma, and phenobarbital-related vacuolar hepatopathy (Wilson et al., 1992) had increased

CALP activities. However, these studies used the chemical inactivation method to determine CALP activities and the numbers of dogs used were too small to determine the differences between each hepatobiliary disease. Another report showed that serum CALP was detected in dogs with hepatocellular injury, but the number of dogs in the study was small and CALP activity in each case was not shown (Kidney and Jackson, 1988).

The purpose of the present study is to clarify the relationship between canine ALP isoenzyme activities and hepatobiliary diseases, as well as serum cortisol concentration, age, sex, breed, and ALT activity.

MATERIALS AND METHODS

Clinical Cases

The Medical records of dogs referred to the Veterinary Medical Center of the University of Tokyo from April 2012 to March 2014 were reviewed retrospectively. The following inclusion criteria were used: (1) confirmed diagnoses of hepatobiliary disease and (2) complete history, signalment (age, sex, and breed), and clinicopathological data (ALP isoenzymes and ALT activity, percentage of each ALP isoenzymes). Dogs treated with a glucocorticoid drug within 6 months before their first visit were excluded from this study. Seven clinically healthy laboratory beagles were assigned as normal controls for assessment of serum ALP isoenzyme and ALT activities.

Analyses of ALP isoenzymes, ALT, and Cortisol

The activity of total ALP was measured using the p-nitrophenylphosphate assay (reference range: 47-237 U/L). Agarose gel electrophoresis was performed for serum to separate each ALP isoenzyme. ALP staining was performed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) and the density of each band was measured (Itoh et al., 2002). Activity of each ALP isoenzyme was calculated from the ratio of densitometry and total ALP activity (LSI Medience, Tokyo, Japan). Serum cortisol concentration was measured (Monolis, Inc. Tokyo, Japan)

without ACTH stimulation test. The plasma ALT activity was measured by automatic blood chemistry measurement equipment (DRI-CHEM, Fujifilm Medical Co., Japan, reference range: 17-78).

Statistical Analysis

Statistical analyses were conducted using the JMP Pro 11 software (SAS Institute, Cary, North Carolina, United States). Statistical analyses were performed for groups that include more than 2 dogs. The Steel test was used to test for significant differences in total ALP activity, CALP percentage, and CALP activity between the control dogs and dogs with hepatobiliary diseases. Spearman's rank correlation was used for correlation analysis between cortisol concentration and CALP activity or CALP percentage, age, and CALP activity or CALP percentage. Wilcoxon rank sum test was used to test for significant differences in age between the portosystemic shunt group and other hepatobiliary disease groups. The Steel-Dwass test was used to test for significant differences in CALP percentage and CALP activity in sex or breeds groups. $P < 0.05$ was considered significant for the remaining statistical analysis in the present study.

RESULTS

Signalment

According to the inclusion criteria, 103 dogs were recruited in the present study. Thirty-one dogs were excluded because of a history of glucocorticoid administration, thus, 72 dogs were included in this study. The population of each diagnosis was as follows: portosystemic shunt (PSS, n=26), chronic hepatitis (CH, n=17), hepatocellular carcinoma (HCC, n=7), hepatocellular adenoma (HCA, n=6), nodular hyperplasia (NH, n=5), vacuolar hepatopathy (VH, n=3), hepatic abscess (HA, n=1), and gallbladder mucocele (GM, n=7). The summary of clinicopathological data in each diagnostic group is shown in Table1. Dogs with hepatobiliary diseases included 30 male (neutered, 15) and 42 female (neutered, 28). The median age of the dogs with hepatobiliary disease was 8.3 years (range 0.4-14.1 years). Dogs with PSS (median age 2.4 years, range 0.4-8.9 years) were significantly younger than dogs with other hepatobiliary diseases (median age 9.63 years, range 3.0-14.1 years) ($P < 0.001$). A significant difference in total ALP activity was observed between control group and dogs with CH ($P = 0.0012$), HCC ($P = 0.0128$), HCA ($P = 0.0196$) and NH ($P = 0.0321$) group, respectively. There was a significant difference of ALT activity between the control group and dogs with CH ($P = 0.0145$) and HCA ($P = 0.413$) groups. Breeds of the dogs with hepatobiliary diseases were as follows: Toy poodle (n=10), Chihuahua (n = 6), Yorkshire Terrier (n = 5), Miniature Schnauzer

(n=5), Labrador Retriever (n=4), Shih Tzu (n=3), Italian Greyhound (n=3), and others (n= 34).

Analysis of ALP isoenzymes

Activity of total ALP, LALP, CALP and BALP were calculated in 79 dogs (hepatobiliary diseases group n=72, control group n=7). None of 79 dogs had BALP. Both LALP and CALP were detected in 77 of 79 dogs in various proportions. CALP was not detected in 2 dogs.

The median of CALP percentage in the control group was 6.8% (range, 0-12.1%) (Figure 1A). Sixteen of 72 (22.2%) cases had CALP percentage > 50%. The number of dogs with CALP percentage higher than 50% were as follows: PSS (1/26 [3.8%]), CH (2/17 [11.8%]), HCC (1/7 [14.3%]), HCA (3/6 [50%]), NH (2/5 [40%]), VH (3/3 [100%]), HA (0/1 [0%]) and GM (3/7 [42.9%]). There was no significant difference in CALP percentage between controls and any diagnosis group. The median of CALP activity in the control group was 18 U/L (range, 0-28 U/L) (Figure 1B). There was a significant difference in CALP activity between control group and CH (P = 0.0038), HCA (P = 0.0198) and NH (P = 0.0332) group.

Serum cortisol concentration was measured in 42 dogs with hepatobiliary diseases. Of these, 33 of 42 dogs (78.6%) showed a lower serum cortisol concentration than the reference value (7.8μg/dL), while 8 (19.0%) had concentrations above the reference value (Figure 2). The number of dogs with high serum cortisol concentrations was as follows: PSS (1/18 [5.2%]), CH (4/6 [66.7%]), HCC (1/7 [14.2%]), HCA (1/6 [16.7%]), NH (1/2 [50%]), and GM (0/3 [0%]). Serum cortisol concentration was not measured in the HA and VH groups. Serum cortisol concentration was not significantly correlated with CALP percentage (r = 0.17, P = 0.25) and CALP activity (r = 0.04, P = 0.58).

The relationships between age and CALP percentage and activity are shown in Figure 3. There was a weak correlation between age and CALP percentage (r = 0.32, P = 0.008). A moderate correlation between age and CALP activity was found (r = 0.54, P < 0.001). There non-significant correlation between ALT activity and CALP percentage (r = -0.10, P = 0.34) or CALP activity (r = 0.19, P = 0.12) (data not shown). No significant difference in CALP percentage or CALP activity was observed in any breed or sex group (data not shown).

DISCUSSION

This is the first report in which serum CALP isoenzyme was analysed with serum cortisol concentration, signalment, and ALT activity in canine hepatobiliary disease groups. In this study, I reviewed 72 dogs with hepatobiliary diseases that had not been administered corticosteroid drugs. CALP levels were higher in dogs with various hepatobiliary diagnoses, especially those with CH, HCA, and NH.

In the present study, CALP percentage and CALP activity did not show a significant correlation with serum cortisol concentrations. This result suggests that cortisol is not the only potential underlying mechanism for high serum CALP percentage and activity. In Scottish Terriers, various serum corticosteroid hormones (cortisol, androstenedione, estradiol, progesterone, 17α-hydroxyprogesterone, and aldosterone) were reported to be increased in cases with high serum ALP activity (Cortright et al., 2014; Zimmerman et al., 2010). Other studies showed that dogs suspected as hyperadenocorticism had a normal cortisol concentration in serum after an ACTH stimulation test. The dogs also showed elevated sex hormone concentrations (androstenedione, estradiol, progesterone (Syme et al., 2001), and 17α-hydroxyprogesterone (Ristic et al., 2002; Syme et al., 2001)) after an ACTH stimulation test. Some of these dogs had higher sex hormone concentrations than the reference range at baseline even before the ACTH stimulation test. In this study, I did not

carry out measurement of the serum sex hormone concentrations with an ACTH stimulation test, so endogenous sex hormones and potential hyperadrenocorticism might be a candidate to induce serum CALP in dogs with hepatobiliary diseases. Because serum cortisol concentration was measured only once in this study, it is possible that a circadian fluctuation of cortisol (Murase et al., 1988; Palazzolo and Quadri, 1987) would influence serum CALP level.

In this study, dogs with CH showed significantly higher serum CALP activity. Serum cortisol of 4 of 6 dogs with CH exceeded the reference range. In a previous report, 20 of 59 dogs with non-adrenal disease showed higher baseline cortisol concentrations than the reference range and while only 8 of the 59 dogs with non-adrenal disease had serum cortisol concentration higher than the reference range after an ACTH stimulation test (Kaplan et al., 1995). Dogs with CH in this study also might have a high baseline serum cortisol concentration, and CALP activity may be increased in response to this serum cortisol increase. The number of dogs in the CH group in which serum cortisol was measured was small in this study, so a larger scale study would be needed to reveal the relationship between CALP and cortisol in CH dogs.

Dogs with HCA and NH also showed a significantly higher serum CALP activity in this study. One dog with each disease had serum cortisol concentrations that exceeded the reference range, suggesting that the increase of CALP activity in dogs with

HCA and NH are related to serum cortisol. Another possible explanation for the CALP increase is that CALP may be produced in tumours and hyperplasia of liver since CALP isoenzyme is produced in liver tissue (Sanecki et al., 1987). To clarify the relationship between CALP increase and HCA and NH, studies are needed to investigate whether there is a difference of CALP isoenzyme activity or expression between affected and non-affected areas of the liver in HCA and NH dogs.

There was a moderate correlation between CALP activity and age in dogs with hepatobiliary diseases. However, dogs in PSS group were significantly younger than dogs in other hepatobiliary groups and the increase of serum CALP activity in the PSS group was not significant (data not shown). There was no significant difference between CALP levels and age when dogs with PSS were excluded from the hepatobiliary disease group. Therefore, differences in age in each disease group may have influenced the relationship between age and CALP activity.

In conclusion, the present study suggests that specific hepatobiliary diseases relate to high serum CALP percentage and activity without the administration of a glucocorticoid drug. Since I did not study the mechanism of the relationship between CALP increase and each factor in this study, additional investigations including the measurement of serum corticosteroid hormones are necessary to determine the direct cause of high CALP activity in dogs with hepatobiliary diseases.

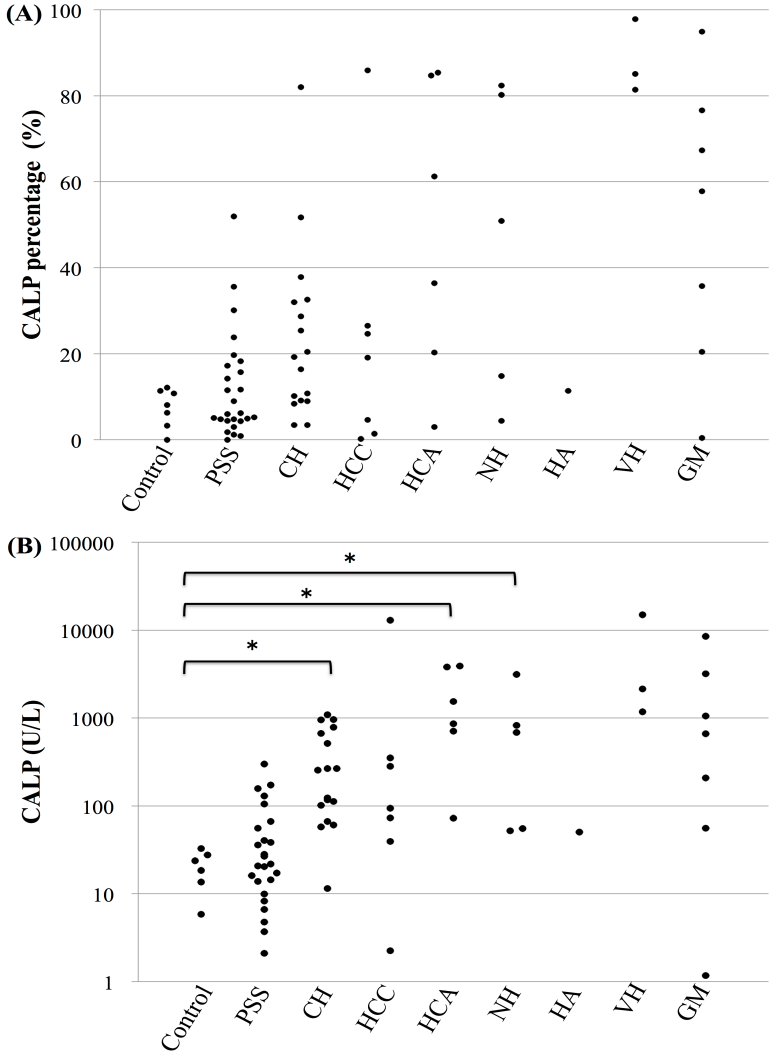
Table 1. Summary of diagnoses and clinical data of dogs with hepatobiliary disease

Category	n	male (neutured) / female(neutured)	Median age (y) (range)	Total ALP (U/L)	ALT (U/L)
Control	7	5(3) / 2(2)	4.8 (3.3-7.2)	177 (106-230)	56 (22-74)
PSS	26	10(2) / 16(8)	2.4 (0.4-8.9)	304 (70-1816)	131 (43-1743)
CH	17	8(3) / 9(5)	8.8 (3.0-14.1)	1143 (336-7416)*	488 (65-1495)*
HCC	7	3(3) / 4(4)	11.5 (6.6-12.9)	1581 (383-15070)*	417 (62-1025)
HCA	6	4(4) / 2(1)	8.9 (7.5-12.0)	2464 (1017-19281)*	494 (287-562)*
NH	5	1(0) / 4(4)	9.2 (8.0-13.8)	1182 (374-3918)*	143 (36-284)
HA	1	0(0) / 1(1)	16.8	440	148
VH	3	1(1) / 2(1)	9.0 (6.3-10.3)	2635 (1381-15322)	156 (98-215)
GM	7	3(2) / 4 (4)	10.4 (4.1-12.8)	2948 (117-11089)	194 (45-1156)

PSS: portosystemic shunt, CH: chronic hepatitis, HCC: hepatocellular carcinoma, HCA: hepatocellular adenoma, NH: nodular hyperplasia,HA; hepatic abscess, VH: vacuolar vepatopathy, GM: gallbladder mucocele

*:significant difference between control group

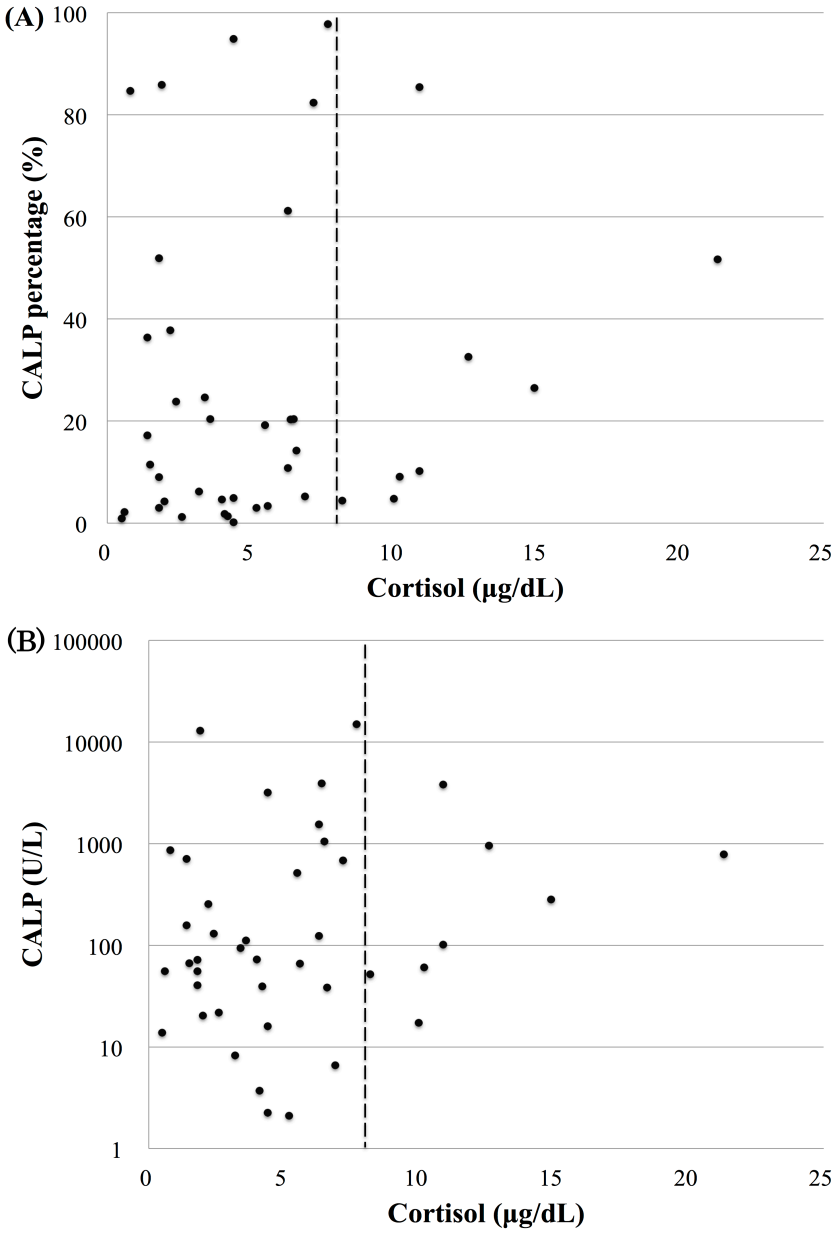
Figure 1



Dot plot of (A) serum CALP percentage and (B) serum CALP activity of each diagnosis. PSS: portsystemic shunt, CH: chronic hepatitis, HCC: hepatocellular carcinoma, HCA: hepatocellular adenoma, NH: nodular hyperplasia, VH: vacuolar hepatopathy, GM: gallbladder mucocele. One dog in control group and one dog in PSS group showed 0 (U/L) in CALP activity. So, the results of these 2 dogs are not drawn in Figure 1B.

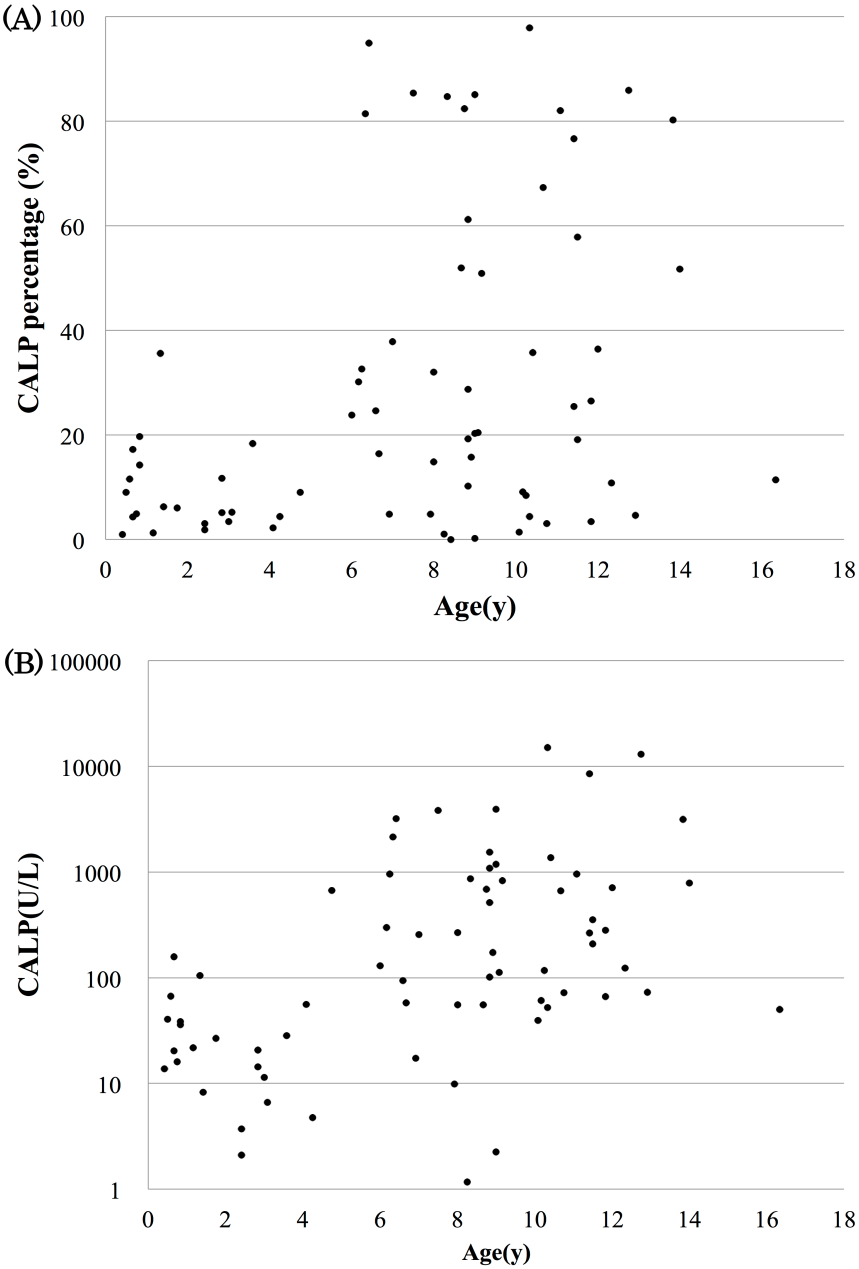
*CALP activity in dogs with hepatobiliary disease is significantly ($P < 0.05$) increased, compared with CALP activity in control dogs. Cc

Figure 2



Scatter plot of serum CALP percentage (A) and CALP activity (B) and cortisol concentration in 43 dogs with hepatobiliary disease. Dot line shows upper limit of reference range in serum cortisol.

Figure 3



Scatter plot of (A) CALP percentage and age, (B) CALP activity and age in 72 dogs with hepatobiliary disease.

Chapter 2

Excess lipid accumulation in the liver of dogs with hepatobiliary diseases

ABSTRACT

Vacuolation is a common hepatic histological change in dogs. Accumulation of lipid and glycogen is believed to be the cause of hepatocyte vacuolation, but the relationship between vacuolation and the actual amount of glycogen and lipid in the canine liver has not been clarified yet. The aim of this study is to quantify lipid and glycogen accumulation in canine liver and compare them with vacuolation severity, blood biochemical parameters, and body conditioning score.

Liver tissue samples were taken prospectively from dogs with suspected hepatobiliary diseases. Lipid accumulation of the liver was analyzed by calculating the percentage of lipid area in Oil red O stained frozen sections. Glycogen amount was quantified by a colorimetric method. Lipid and glycogen accumulation in the liver was compared between each diagnosis, histological vacuolar severity score blood chemistry profile and ALP isoenzyme, body conditioning score and history of glucocorticoid drug administration. Relative expression of 8 lipid metabolism-associated genes (*SREBP1c, PPARα, PPARγ, ChREBP1, LPL, LFABP, DGAT1* and *DGAT2*) in liver tissue was quantified by real time RT-PCR.

Percentage of lipid area was evaluated in 58 dogs and glycogen weight was measured in 54 dogs in this study. The percentage of lipid area was significantly higher in the liver of chronic hepatitis group and non-neoplastic area of hepatocellular carcinoma

group than healthy control group. In dogs with non-neoplastic diseases, dogs with more lipid accumulation than control showed higher corticosteroid-induced ALP (CALP) percentage and activity than dogs with normal lipid accumulation groups. Glycogen weight in liver was not significantly elevated in any of the disease group and was unrelated to history of glucocorticoid drug administration. No relation was observed between lipid or glycogen accumulation and the respective vacuolation severity scores. Plasma concentration of triglyceride had significant and moderate correlation with percentage of lipid area in dogs with non-neoplastic liver diseases, while other blood biochemical parameters were not related to liver accumulation of lipid nor glycogen. Expression of lipoprotein lipase (LPL), which induce lipolytic pathway, was elevated in dogs with high level of hepatic lipid accumulation, but not in dogs with normal level of hepatic lipid accumulation. Both groups of dogs with high and normal level of lipid accumulation showed high expression of PPARγ and DGAT1, which are lipid synthesis associated genes, when compared to normal control dogs.

This study revealed that there is a number of excess lipid accumulation in dogs with hepatobiliary diseases. The result of this study suggests that excess hepatic lipid accumulation would be a new cause of increased serum CALP level and increased lipogenesis would cause excess lipid accumulation in liver of dogs with hepatobiliary diseases.

INTRODUCTION

Hepatocyte vacuolation is a common hepatic histopathological finding in dog with various diseases (Sepesy et al., 2006). In the HE stained liver section with hepatocyte vacuolation, enlarged hepatocytes with formation of cytoplasmic spaces are observed (Guilford W.G. Center, 1996; Sepesy et al., 2006), which is considered to be the result of excess accumulation of glycogen, lipid and water in the cytoplasm, or intracellular edema (Guilford W.G. Center, 1996; Rothuizen, 2006). Even though hepatocyte vacuoleation has been widely known, the clinical importance has not been clarified yet.

Hepatocyte vacuolation in dogs has been considered to relate with steroid hormones and often accompanied with high serum ALP activity, though the mechanism is not clear. Dogs with hyperadrenocorticism, which is characterized by endogenous glucocorticoid excess, often have vacuolar hepatopathy (VH) (Rogers and Ruebner, 1977; Sepesy et al., 2006). Scottish Terriers are known to have a predilection to develop VH associated with endogenous sex hormones, high production of steroid hormones in adrenal grand, together with characteristic high serum ALP activity (Cortright et al., 2014; Zimmerman et al., 2010). Experimental glucocorticoid administration also induced glycogen accumulation in canine liver (Fittschen and Bellamy, 1984), dose-dependent hepatic vacuolation and elevation of corticosteroid-induced ALP isoenzyme activity

(Syakalima et al., 1997a). On the other hand, moderate or severe VH was observed in dogs without history of exogenous glucocorticoid exposure or excess endogenous glucocorticoid production (Sepesy et al., 2006). And, although glycogen is believed to be the major component of hepatic vacuolation, quantitative analysis on glycogen accumulation has not been reported so far.

The relation between hepatocyte vacuolation and lipid accumulation has not been studied in detail at present, however, several reports have shown the hepatic lipid accumulation in dogs. Hepatic vacuolation observed in nodular hyperplasia was stained with Oil Red O (ORO) indicating the accumulation of lipid (Fabry et al., 1982). In Schnauzer, VH associated with hyperlipidemia were shown to contain both lipid and glycogen accompanied with high serum ALP activity (Guilford W.G. Center, 1996). Furthermore, excess lipid droplet in hepatocytes was reported in dogs with congenital portosystemic shunt (cPSS) evaluated by imaging analysis of ORO stained frozen section (Hunt et al., 2014; Hunt et al., 2013). However, hepatic lipid accumulation in dog with other hepatobiliary diseases has not been evaluated at present. The relation of lipid accumulation and serum ALP activity has also not been clarified yet.

In other species, fatty liver is caused by hepatic de novo lipogenesis (Anstee and Goldin, 2006; Arslan, 2014; Assy et al., 2000). Four transcription factors regulating hepatic lipid metabolism include sterol response element binding protein-1c (SREBP-1c),

carbohydrate response element binding protein (ChREBP), the peroxisome proliferator-activated receptor α (PPAR α) and PPAR γ. These transcription factors regulate lipid metabolism-associated genes including lipoprotein lipase (LPL) which hydrolysis lipid for uptake in hepatocyte, liver fatty acid binding protein (LFABP) which delivery fatty acids in hepatocyte, diacylglycerol acyltransferase (DGAT)1 and DGAT2, which catalyzes the final step of triglyceride synthesis.

The aim of this study is to determine the relationship of lipid and glycogen accumulation in the liver with clinical or biochemical features in canine hepatobiliary diseases, and to investigate the mechanism of canine hepatic excess lipid accumulation with gene expression analysis.

MATERIALS AND METHODS

Cases

Seventy-two dogs that underwent liver biopsy in the Veterinary Medical Center of the University of Tokyo (VMC-UT) between April 2012 and December 2014 were included in this prospective study. Seven clinically healthy beagles were assigned as control dogs and biopsied the liver. The procedure was conducted in compliance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo (approval number P14-924; approved on 2014).

Clinical data collection

Clinical records including body conditioning score (BCS: 1-5), age, history of glucocorticoid drug administration, and blood biochemical data were reviewed. Blood biochemical data included serum ALP isoenzyme activity and percentages (LSI Medience, Tokyo, Japan), plasma ALT activity, concentration of glucose, albumin, ammonia, total cholesterol and triglyceride (DRI-CHEM, Fujifilm Medical Co., Japan).

Sample collection

All liver biopsy samples were taken by laparotomy. Both neoplastic and non-neoplastic samples were taken from dogs with suspected liver tumors. All liver

samples were treated and stored in 4 procedures; 10% formalin fixed sections for HE staining, frozen with compound gel for ORO staining, frozen directly for glycogen assay and frozen in RNAlater solution (Ambion, Austin, TX) for RNA extraction. HE stained sections were subjected to histological diagnosis according to the WSAVA's criteria (Rothuizen, 2006). Congenital portosystemic shunt (cPSS) was diagnosed by contrast enhanced CT and portography. Gallbladder mucocele (GM) was diagnosed macroscopically on surgery. Vacuolar hepatopathy was diagnosed when there was no histopathological disorder other than hepatocyte vacuolation. Dogs diagnosed as non-hepatobiliary disease were excluded from this study.

Histopathologic evaluation of hepatocyte vacuolation

Single pathologist who was unaware of the diagnosis of patients evaluated hepatocyte vacuolation. Lipid accumulation was determined as discrete clear vacuolation (Figure 4A). Glycogen accumulation was identified on the basis of the finding of wispy cytosolic vacuolation with central location of cell nuclei (Figure 4B) (Cortright et al., 2014). The severity of hepatocyte vacuolation was scored from G0 to G3 (G0, or absent = 0% of hepatocyte vacuolated, G1, or mild <33% of hepatocyte vacuolated, G2 or moderate 34-66% of hepatocyte vacuolated, G3, or severe >67% of hepatocyte

vacuolated), based on the human non-alcoholic fatty liver disease grading score (Kleiner et al., 2005b). Zonal location of hepatocyte vacuolation was evaluated and the classification of vacuolar location was as follows: all zone, zone 1 (periportal), zone 2 (midzornal), zone 3 (perivenous), zone 1~2, zone 2~3 (Sepesy et al., 2006). Zonal location was not evaluated in samples of neoplastic area of hepatic tumors.

Quantification of lipid and glycogen accumulation in liver

The biopsy sample was placed into a Tissue tek container, which was then filled with Tissue tek OTC compound gel (Sakura Finetek, Torrance, CA). The samples were frozen rapidly, prior to being cut into 6μm slices, and stained for lipid using a standard Oil Red O protocol (Hunt et al., 2014; Hunt et al., 2013). The percentage of the area occupied by Oil Red O-stained lipid droplets in the liver tissue was calculated using Image J software (http://rsbweb.nih.gov/ij/), for 5 separate, randomly selected ×200 fields (Liu et al., 2014). Dogs with lipoglanunomas were excluded from this study because the lipid accumulation exists other than hepatic parenchymal cell. Glycogen amounts in the liver tissue were analyzed by Glycogen Assay Kit (BioVision, Mountain View, CA) using OxiRed probe according to manufacturer's suggestion.

Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted from the liver samples by using RNAspin Mini RNA Isolation Kit (GE Healthcare). The extracted RNA was then treated with DNase I (Invitrogen, Carlsbad, CA) and reverse-transcribed with a PrimeScript RT reagent kit (Takara Bioscience, Tokyo, Japan) according to the manufacturer's protocol. Eight lipid metabolism associated genes were selected for quantitative analysis: *SREBP1c, PPARα, PPARγ, ChREBP1, LPL, LFABP, DGAT1* and *DGAT2*. Three genes, *HMBS*, *RPL13A* and *RPL32*, were included as internal controls. Gene expression was quantified in triplicate samples using the Thermal Cycler Dice Real-Time System (Takara Bio Inc, Tokyo, Japan) with THUNDERBIRD SYBR qPCR Mix (Toyobo Co. Ltd, Osaka, Japan) according to the manufacturer's instructions. Primers were designed using Primer 3 (http://primer3.sourceforge.net/) or were selected by referring to previous publications(Peters et al., 2007). The sequences of the primer pairs used are shown in Table 2.

The threshold cycle (Ct value) was determined using the second derivative maximum method. Before the samples were quantified, standard curves of the relative starting amount versus the Ct value were constructed using pooled liver cDNA. The amplification efficiency of each reaction was within 80% to 120%. The dissociation curve was confirmed to have a single peak in each experiment. Moreover, the PCR

products were confirmed to be in agreement with their theoretic sizes by using agarose gel electrophoresis. The quantity of each transcript was determined by comparing to the mean expression level of *HMBS*, *RPL13A*, and *RPL32* and represented as relative value assuming the mean expression level of healthy control dogs as 1.

Statistical analysis

Statistical analyses were conducted using the JMP Pro 11 software (SAS Institute, Cary, North Carolina, United States). Statistical analyses were performed for groups that include more than 2 dogs. Steel test was used to test significant differences in percentage of lipid area and glycogen weight between control dogs and dogs with hepatobiliary diseases. Steel-Dwass test was used to test for significant differences in percentage of lipid area and glycogen weight between each group of vacuolation severity score. Spearman's rank correlation was used for correlation analysis between lipid area percentage or glycogen weight and ALP isoenzyme activity, ALP isoenzyme percentage, plasma ALT activety, plasma concentration of glucose, albumin, ammonia total cholesterol and triglyceride. Wilcoxon rank sum test was used to test for significant differences in glycogen weight of the liver between dogs with or without history of glucocorticoid drug groups, expression of lipid metabolism associated genes between

each group of diagnosis, normal and excess lipid accumulation groups and serum CALP activity and percentage between normal and excess lipid accumulation groups. $P < 0.05$ was considered significant in the present study.

RESULTS

Clinical cases with heaptobiliary diseases

Seventy-two dogs underwent liver biopsy in the VMC-UT between April 2012 and December 2014. Six dogs were excluded because the diagnoses were cancer or adenoma that derived from non-hepatic tissue. Eight dogs were excluded because lipogranulomas were found in ORO staining sections. These exclusions left 58 dogs in the study, and the dogs were grouped according to diagnoses: congenital portosystemic shunt (cPSS, n=19), chronic hepatitis and cholangiohepatitis (CH, n=14), vacuolar hepatopathy (VH, n=3), hepatocellular carcinoma (HCC, n=9), hepatocellular adenoma (HCA, n=8), cholangiocarcinoma (CC, n=1) and gallbladder mucocele (GM,n=4). All of dogs with cPSS were also histopathologically diagnosed as intercurrent primary portal vein hypoplasia (PHPV). Three of four dogs with GM were also diagnosed as chronic hepatitis from the histopathological observation. In two dogs with HCA, non-neoplastic liver samples could not be taken. Fifty-eight of dogs with hepatobiliary disease in this study included 27 male (neutered, 17) and 31 female (neutered, 15) and the median age was 9.5 (range; 0.4 - 13.2) years. Breeds of the dogs were as follows: Toy poodle (n=10), Yorkshire Terrier, Mixed breed (n = 5 each), Chihuahua, Maltese, Miniature Dachshund, Shih Tzu (n = 3 each), American Cocker Spaniel, Pekingese, Pomeranian, Pug, Shetland Sheepdog, Shiba (n=2 each) and other 14 breeds (n=1 each).

Measurement of lipid and glycogen in the liver

Lipid accumulation in hepatocyte was examined by ORO staining observed in 58 dogs with various hepatobiliary diseases. In control dogs, small lipid droplets were observed in hepatocytes (Figure 5A). Lipid droplets in the hepatocytes of hepatobiliary cases were much larger in size and number than control dogs (Figure 5B), and some hepatocytes were ballooned because of lipid accumulation (Figure 5C). The median percentage of lipid area was 11.2% (range, 5.6-17.8%) in healthy control group, and a number of dogs with hepatobiliary diseases showed high percentage of lipid area. The number of dogs with higher percentage of lipid area than maximum value of control dogs were as follows: cPSS (9/19 [47.3%]), CH (11/14 [78.5%]), VH (2/3 [66.7%]), neoplastic area of HCC (1/9 [11.1%]), non- neoplastic area of HCC (8/9 [88.9%]), neoplastic area of HCA (6/8 [75.0%]), non- neoplastic area of HCA (5/6 [83.3%]), neoplastic area of CC (1/1 [100.0%]), non- neoplastic area of CC (1/1 [100.0%]) and GM (3/4 [75.0%]). The highest percentage of lipid area in dogs with hepatobiliary diseases was observed in a dog diagnosed as cPSS in this study (78.6%), The percentage of lipid area was significantly larger in CH (P=0.0168) and non- neoplastic area of HCC (P=0.0180) groups than control dogs (Figure 6A).

Since tissue sample for measurement of glycogen weight could not be obtained

in six dogs (CH: n=3, non-neoplastic area of HCA: n=2, cPSS: n=1), glycogen weight was measured in 54 dogs. The median glycogen weight of control dogs was 25.3 mg/g (range, 7.7-37.8 mg/g). The number of dogs with glycogen amount larger than maximum value of control dogs (37.8mg/g) was as follows: cPSS (6/18 [33.3%]), CH (2/11 [18.1%]), VH (3/3 [100.0%]) neoplastic area of HCC (2/9 [22.2%]), non-neoplastic area of HCC (2/8 [25.0%]), neoplastic area of HCA (2/8 [25.0%]), non- neoplastic area of HCA (2/6 [33.3%]), neoplastic area of CC (0/1 [0%]), non- neoplastic area of CC (1/1 [100.0%]), GM (2/4 [50.0%]). No significant difference of glycogen weight was found between control and any diagnosis group.

Evaluation of hepatocyte vacuolation

Hepatocyte vacuolation of lipid and glycogen (Figure 4) were evaluated in 74 samples from 58 dogs with hepatobiliary diseases and 7 samples of 7 control dogs (Table 3) by HE staining. Only 1 sample of a control dogs showed mild glycogen and lipid vacuolation in all zones. Lipid accumulation was not observed in 35 of 74 samples (47.3%) from dogs with hepatobiliary disease. The number of samples that showed lipid vacuolation in each severity score was as follows: G1 (24/74, 32.4%), G2 (12/74, 16.2%), G3 (3/74, 4.1%). Zonal location of vacuolation was evaluated in the samples that showed vacuolation severity score of glycogen or lipid more than G1. There were 26

non-neoplastic samples except for samples of neoplastic area of hepatic tumors, and lipid vacuolation were observed in all zone in 21 of 26 samples (80.7%), localized to zone 1 (periportal vacuolar changes) in 1 (3.8%) sample, localized to zone 3 (perivenular vacuolar changes) in 3 (11.5 %) samples, and in zone 2 to 3 in 1 (3.8%) sample. Glycogen vacuolation was not observed in 5 of 74 samples (6.8%) from the dogs with hepatobiliary disease. The number of samples that showed lipid vacuolation was as follows: G1 (26/74, 35.1%), G2 (30/74, 40.5%), G3 (13/74, 17.8%). There were 53 non-neoplastic samples with glycogen vacuolation, and diffuse glycogen vacuolation was observed in all zone in 48 samples (90.6%), in zone 1 to 2 in 3 (5.6%) samples and involving zone 2 to 3 in 2 (3.8%) samples.

The distribution of percentage of lipid area and glycogen weight in each vacuolation severity score was shown in Figure 7. There are some cases that showed high vacuolation severity score despite the actual lipid area percentage or glycogen amount was comparable with control dogs (5.6-17.8%). No significant difference of percentage of lipid area or glycogen weight was observed between any vacuolation severity score groups.

Analysis between lipid/glycogen accumulation and clinical data in non-tumor diseases

In 40 dogs with non-hepatic tumor diseases, percentage of lipid area was

compared to BCS (Figure 8). The percentage of lipid area tended to be higher when the dogs showed higher BCS, but no significant difference in percentage of lipid area was found between any BCS groups. (r =0.30, P=0.0712)

Scatter plot of percentage of lipid area and concentration of triglyceride (reference range, 30-133 mg/dL) and total cholesterol (reference range, 111-312 mg/dL) was shown in Figure 9. A moderate correlation between percentage of lipid area and plasma triglyceride concentration was found (r =0.41, P=0.0141) and no significant correlation between percentage of lipid area and plasma total cholesterol concentration was found (r = 0.12, P = 0.134) (Figure 9B). There was no significant difference between percentage of lipid area or glycogen weight and serum total ALP activity, percentage and activity of each ALP isoenzyme, plasma ALT activity and plasma concentration of glucose, albumin and ammonia (data not shown). The glycogen weight of the liver was not significantly different between dogs with or without history of glucocorticoid drug administration (data not shown). Dogs with excess lipid accumulation showed higher serum CALP percentage and activity than dogs with normal lipid accumulation (Figure 10A and B), however no significant difference was observed. There was no significant difference in serum CALP percentage and activity (Figure 10C and D)

Expression of lipid metabolism-associated gene in liver

The relative expression of lipid metabolism-associated genes was measured in 8 healthy control dogs and 53 hepatobiliary diseases dogs. The number of each diagnosis groups was as follows; cPSS (n=16), CH (n=14), VH (n=3), HCC (n=9), HCA (n=7) and GM (n=4). Gene expressions of non-neoplastic area was not examined in one dog in HCA group because tissue sample could not be obtained.

The relative expression of lipid metabolism-associated gene in each diagnoses groups was shown in Figure 11. There were significantly higher relative expression of *LPL* in CH (P=0.0072) group, *DGAT1* in CH (P=0.0072), neoplastic (P=0.0120) and non-neoplastic (P=0.0069) area of HCC groups. No significant difference was observed in relative expression of *SREBP1c*, *PPARα*, *PPARγ*, *ChREBP1*, *LFABP*, and *DGAT2* between healthy control group and any of the heaptobiliary diseases group.

The non-neoplastic liver samples of hepatobiliary diseases were classified to normal lipid accumulation group (n=20) and high lipid accumulation group (n=32) compared to the upper limit of lipid area percentage in control group. Expression of lipid metabolism-associated genes in each groups and control group was shown in Figure 12. Dogs with excessed lipid accumulation showed significantly higher expression of

PPARα(*P*=0.0443), *PPARγ* (*P*=0.0027), *LPL* (*P*=0.0029) and *DGAT1* (*P*=0.0012) than healthy control group. Normal lipid accumulation group showed significantly higher expression of *PPARγ* (*P*=0.0249) and *DGAT1* (*P*=0.0240). No significant difference was observed in relative expression of *SREBP1c*, *ChREBP1*, *LFABP*, and *DGAT2* between any groups.

DISCUSSION

In this study, I showed excess lipid accumulation in the liver of dogs with hepatobiliary diseases. The liver tissues of CH and non-neoplastic area of HCC groups contained significantly more lipid than that of control groups. Considerable numbers of dogs in other disease groups also showed high lipid accumulation though no significant increase was observed. This result suggests that various hepatobiliary diseases would associate with alteration of lipid metabolism in canine hepatocyte.

The condition of excess lipid accumulation in the liver is called fatty liver and is considered as risk factor for progressive fibrosis, cirrhosis and liver failure or hepatocellular carcinoma in human (Kikuchi et al., 2014; Takahashi and Fukusato, 2014). In this study, dogs in CH group showed significantly higher lipid accumulation than control dogs. It is conceivable that inflammatory stress induced lipid accumulation as previously reported in mouse (Ma et al., 2008) hepatic lipid accumulation relate to hepatitis development in dogs as well as in other species (Anstee and Goldin, 2006; Kikuchi et al., 2014; Takahashi and Fukusato, 2014). Liver of non-neoplastic area of HCC also showed significantly higher lipid accumulation in this study. HCC associated with vacuolar hepatopathy had been reported in Scottish Terriers (C. PEYRON, 2014; Cortright et al., 2014), though the content of the vacuole was unknown. From our result,

it can be hypothesized that vacuolar hepatopathy containing excess lipid might be associated with HCC development not only in Scottish terriers but also in other breeds, and therefore, higher lipid accumulation were observed around HCC tissue.

The difference of lipid area percentages in liver tissue between cPSS group and control group was not significant. However, some cases showed markedly high percentage of lipid area. It has been reported that considerable percentage of dogs with cPSS accompanied liver steatosis (Baade et al., 2006; Lee et al., 2011), which is correspondent to our present result. Decrease of portal vein flow might induce canine hepatic steatosis in dogs with cPSS, because the steatosis was decreased after surgical attenuation of cPSS (Lee et al., 2011). The same mechanism might also induce lipid accumulation in the non-neoplastic liver area of tumor in this study since regional blood flow around tumor tissue tends to be decreased. In the present study, the percentage of dogs with lipid or glycogen vacuolation in all zones was higher than previous study (Sepesy et al., 2006), which investigates the hepatic vacuolation in hepatobiliary and non-hepatobiliary diseases. Diffuse hepatic vacuolation was observed in this study because the vacuolation was evaluated in only hepatobiliary diseases.

Obesity is one of the risk factor of fatty liver in human (Arslan, 2014; Dietrich and Hellerbrand, 2014), and dogs with higher BCS also tend to show higher percentage

of lipid area in this study. However, the percentage of lipid area was not significantly different between any BCS groups. Further study with more objective evaluation of canine obesity, such as measurement of body fat mass, would provide more accurate relation between obesity and hepatic lipid accumulation in dogs. Significant and moderate correlation between percentage of lipid area and plasma triglyceride concentration was observed in this study. Fatty liver associated with hyperlipidemia had also been reported in human (Assy et al., 2000). In Schnauzer, vacuolar hepatopathy associated with hyperlipidemia had been reported (Guilford W.G. Center, 1996), though most of the dogs with hepatobiliary diseases used in this study showed normal concentration of triglyceride (Guilford W.G. Center, 1996). Further study using dogs with both hepatocyte vacuolation and high concentrations of plasma triglyceride would be needed. Serum CALP level of excess lipid accumulation group tented to be higher than normal lipid accumulation group in this study. The relationship between hepatic lipid accumulation and increase of serum CALP level was unclear and canine CALP was influenced by various factors, such as glucocorticoid drugs and endogenous hormones (Eckersall and Nash, 1983; Hoffmann and Dorner, 1975; Wellman et al., 1982). Further study is needed to reveal the mechanism of CALP increasing secondary to lipid accumulation in liver.

I also evaluated the expression of lipid metabolism-associated genes in liver.

Expressions of *PPARα*, *PPARγ*, *LPL*, *DGAT1* were elevated in dogs with excess lipid accumulation in the liver. Increased expression of *PPARα* and *LPL* were not observed in dogs with normal level of lipid accumulation. LPL is an enzyme contribute to hepatocyte incorporation of lipid, ant the expression is regulated by *PPARγ* (Schoonjans et al., 1996). High expression of *LPL* might be one of the reasons of excess lipid accumulation in liver. In contrast, *PPARα* is a transcription factor inducing gene expression related to beta-oxidation of fatty acids (Reddy, 2001). Since the pathway is accelerated when lipolysis is active, high expression of *PPARα* might be a result of lipid accumulation in the liver. Expression of *PPARγ* and *DGAT1* were elevated in dogs with or without lipid accumulation in the liver. The reason for this result is unclear, however, both genes have stimulating effect on lipid lipogenesis in liver (Inoue et al., 2005; Nakamuta et al., 2005). Consistent expression of these genes might be related to induction of lipid accumulation in canine liver.

No significant difference was observed in glycogen weight between any hepatobiliary disease groups in this study. It has been indicated that endogenous or exogenous glucocorticoid exposure would induce canine hepatic glycogen accumulation in the previous report(Fittschen and Bellamy, 1984; Rogers and Ruebner, 1977; Sepesy et al., 2006). Since serum glucocorticoid hormones were not measured in this study, it is not

clear whether high glycogen accumulation in this study were the result in increase of serum cortisol induced by stress associated with chronic illness.

In this study, the vacuolation score determined by histology did not have correspondence with glycogen weight or percentage of lipid area percentage. Therefore, it would be difficult to estimate actual amount of glycogen or lipid in the liver from the observation of HE stained section. The discrepancy between the observation of HE staining and the actual amount of glycogen and lipid might be attributed to the intracellular edema or water, which is also a suspected component of hepatocyte vacuolation (Guilford W.G. Center, 1996; Rothuizen, 2006).

In conclusion of this chapter, dogs with hepatobilially diseases tended to showed excess lipid accumulation in the liver and it was suggested that up-regulation of lipogensis would relate to canine excess lipid accumulation in liver. Moreover, dogs with excess lipid accumulation tended to show higher serum CALP level than dogs with normal lipid accumulation. The cause and effect of lipid accumulation in the liver for dogs is still unclear, so it is necessary to clarify the mechanism and effect of lipid accumulation for canine liver in further study.

Table 2. Primers used for quantitative polymerase chain reaction analysis

Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size(bp)
SREBP1c	TGGATTGCACATTCGAAGAC	AGCTCATTGTGGCAGGAGAT	168
PPAR α	CACAGACACGCTCTCACCAG	AAGAAACCCTTGCAGCCTTC	174
PPAR γ	GAAGGATGCAAGGGCTTCTT	CCAAACCTGATGGCATTATGA	162
ChREBP1	GATGCCTATGTGGGCAATG	GGTAGCTGGTGAAGAAATCTGA	132
LFABP	TCAAGGCAGTGGTTCAGATG	TGGTATTGGTGATCACATCG	108
LPL	CTACCGGTGCAACTCAAAGG	GGAAGACTTTGTAAGGCATCTGA	161
DGAT1	TGGTGATGCTGATCTTGAGC	TGGTGATGCTGATCTTGAGC	150
DGAT2	CTTCCTTGTGCTTGGAGTGG	GACCTCCTGCCACCTTTCTT	135
HMBS	TCACCATCGGAGCCATCT	GTTCCCACCACGCTCTTCT	112
RPL13A	GCCGGAAGGTTGTAGTCGT	GGAGGAAGGCCAGGTAATTC	87
RPL32	TGGTTACAGGAGCAACAAGAAA	GCACATCAGCAGCACTTCA	100

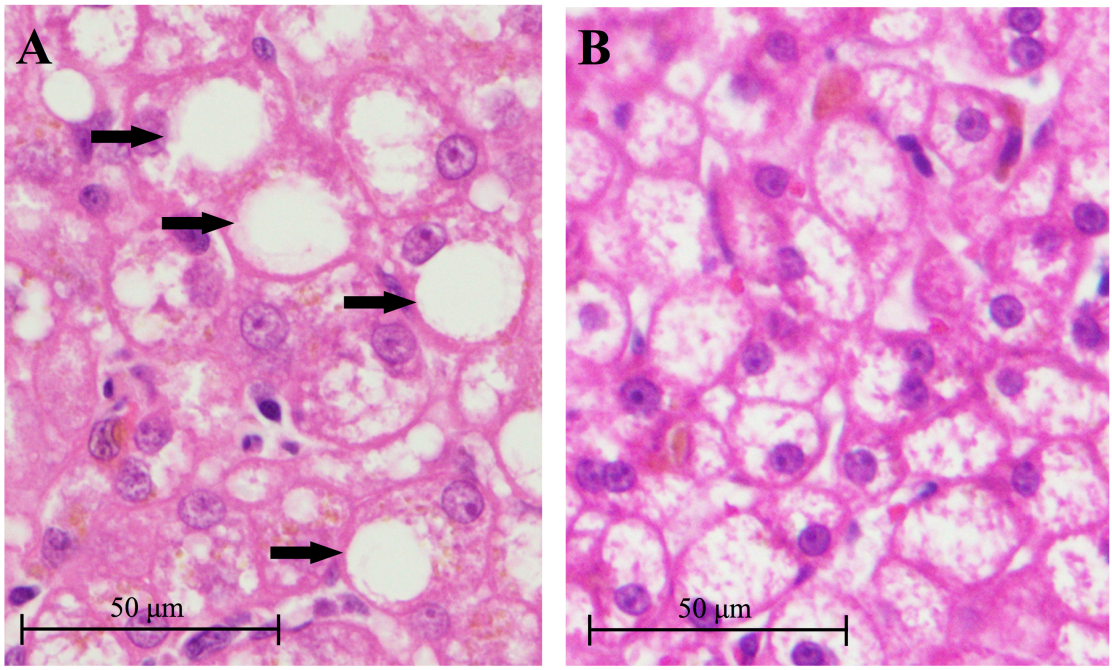
Table 3. Summary of severity score of hepatic vacuoles change

		Lipid				Glycogen			
		Severity score of vacuoles change				Severity score of vacuoles change			
Category	n	G0	G1	G2	G3	G0	G1	G2	G3
Healthy control	7	6	1	0	0	6	1	0	0
PSS	19	10	8	1	0	1	9	8	1
CH	14	7	5	1	1	1	4	7	2
VH	3	3	0	0	0	0	1	2	0
HCC (neoplastic area)	9	3	4	1	1	1	2	3	3
HCC (non-neoplastic area)	9	6	0	3	0	0	5	3	1
HCA (neoplastic area)	8	2	2	3	1	1	2	3	2
HCA (non-neoplastic area)	6	2	2	2	0	1	2	0	3
CC (neoplastic area)	1	0	0	1	0	0	0	1	0
CC (non-neoplastic area)	1	0	1	0	0	0	0	1	0
GM	4	2	2	0	0	0	1	2	1

PSS: portosystemic shunt, CH: chronic hepatitis, VH: vacuolar hepatopathy, HCC: hepatocellular carcinoma, HCA: hepatocellular adenoma,CC: cholangiocarcinoma, GM: gallbladder mucocele.

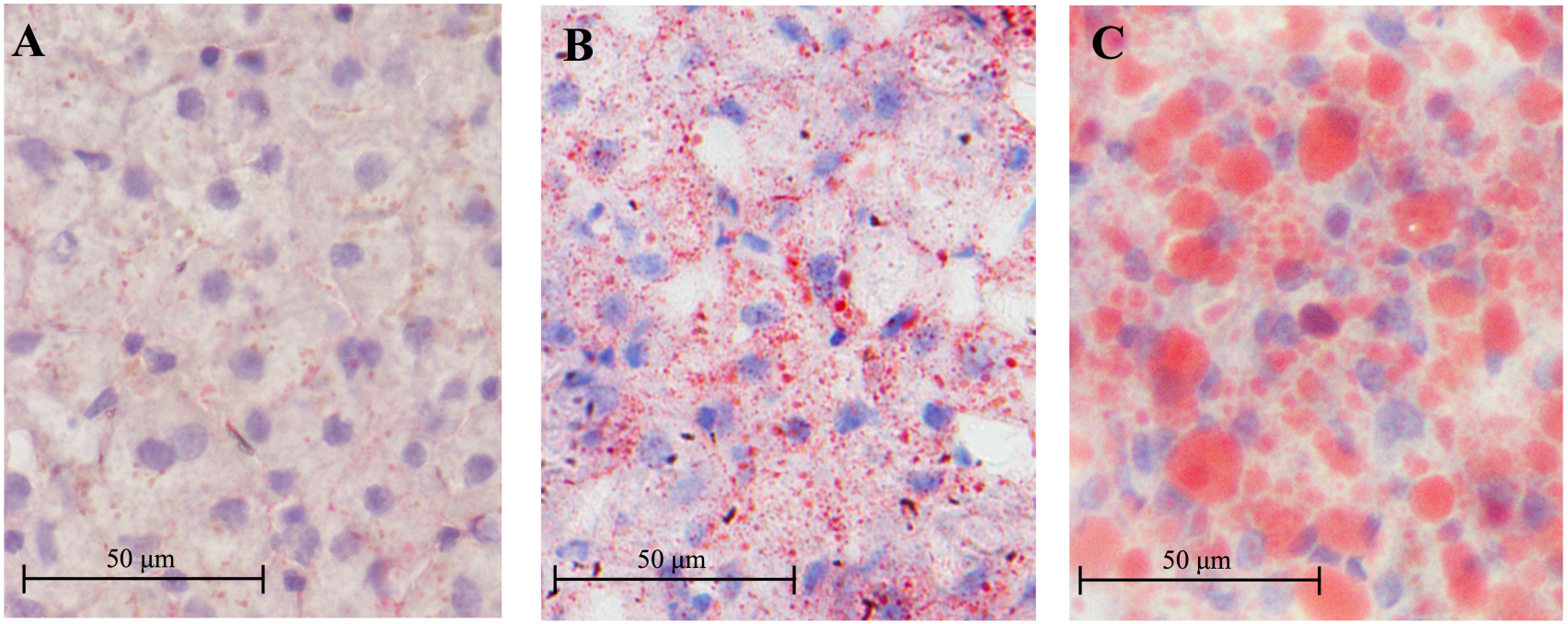
G0:0%, G1:1~33%, G2:34~66%, G3:66%~ of hepatocyte vacuolated

Figure 4



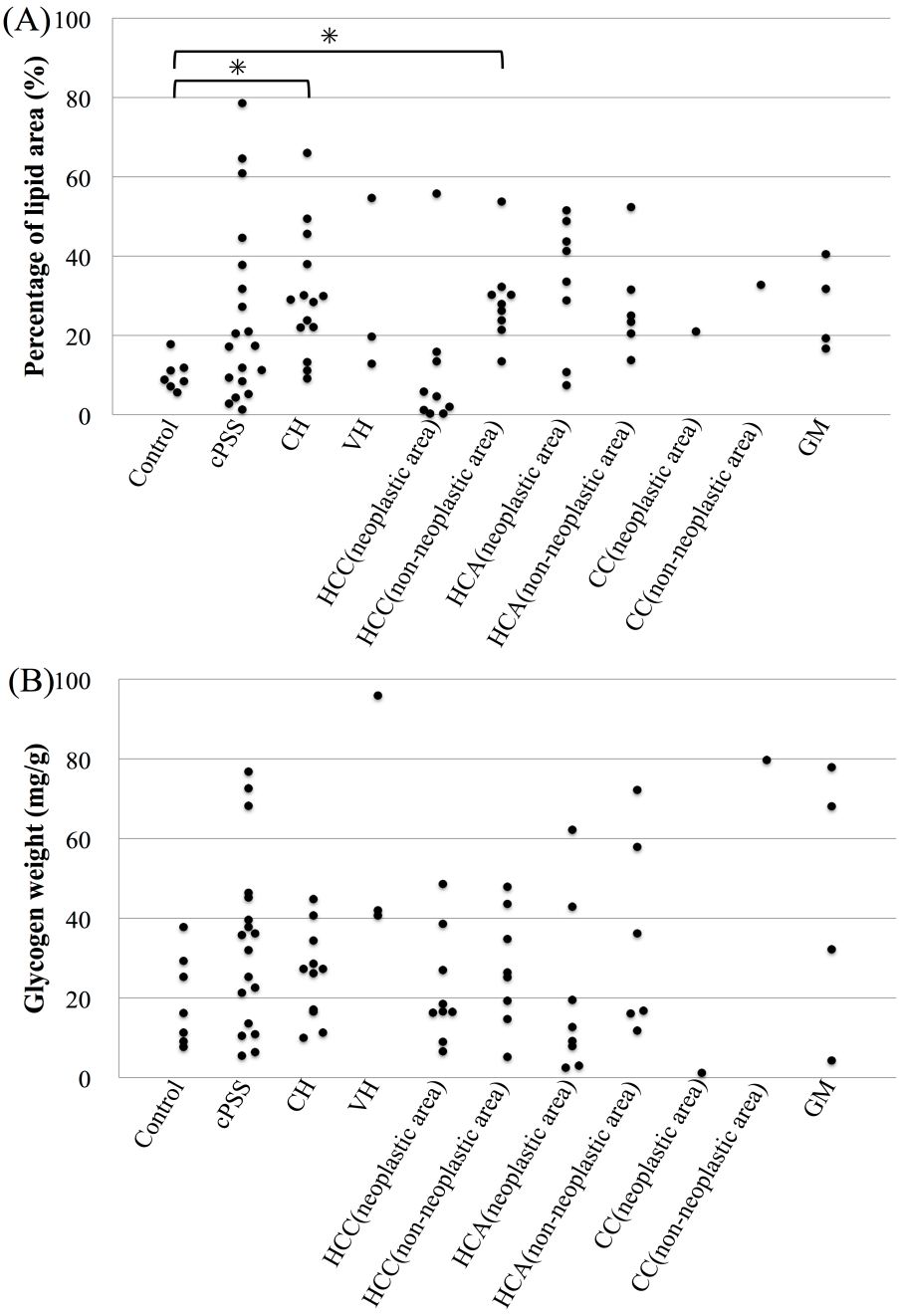
Histological feature of canine liver vacuolation in dogs with chronic hepatitis. HE staining. (A) Discrete clear hepatocyte vacuolation which is considered to contain lipid (arrows) and (B) cytoplasmic rarefaction which is considered to contain glycogen. bar = 50µm.

Figure 5



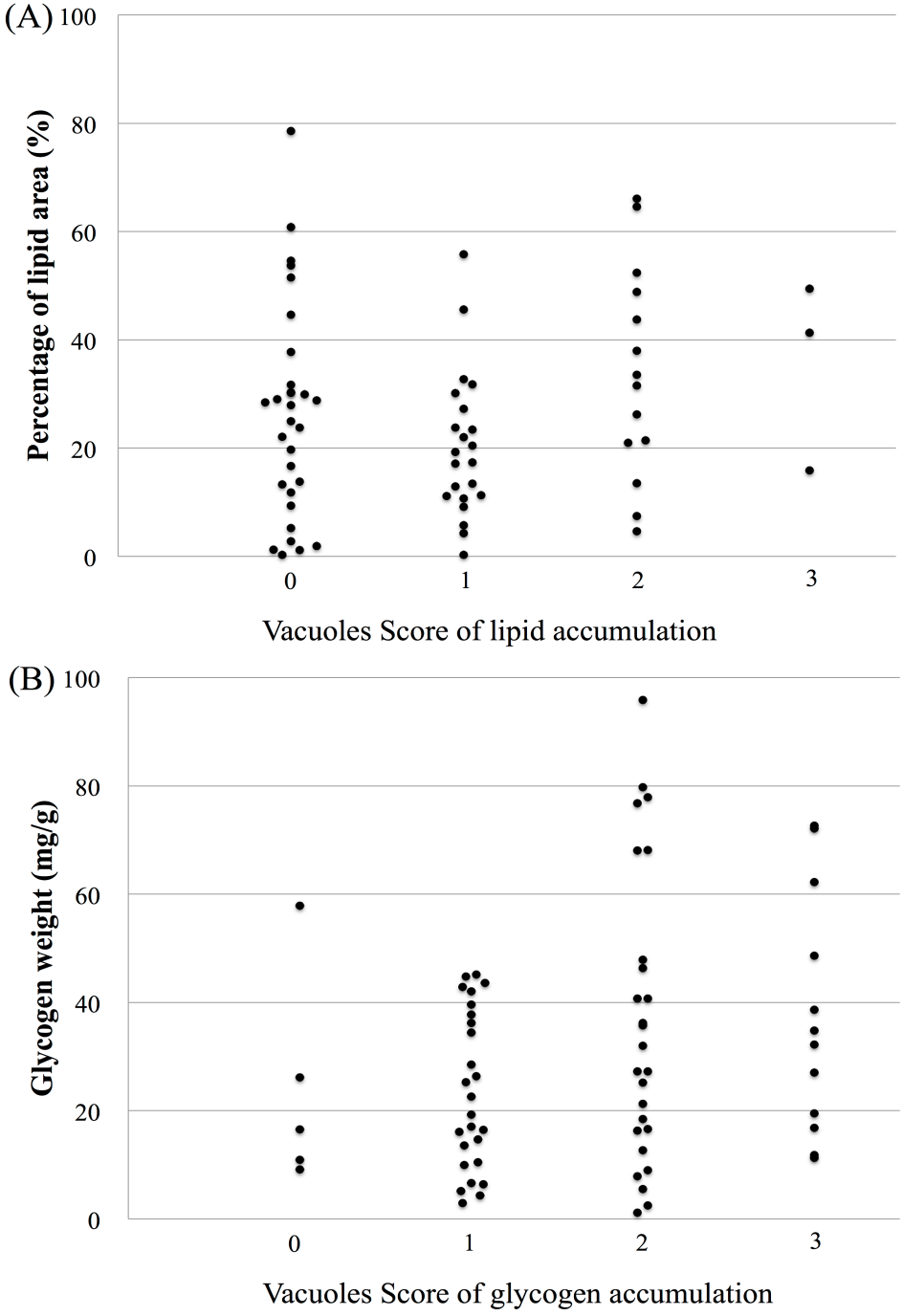
Frozen section of canine liver. Oil Red O staining of (A) a healthy control dog and dogs with cPSSS (B) mild and (C) moderate lipid accumulation. in the liver of. bar = 50µm.

Figure 6



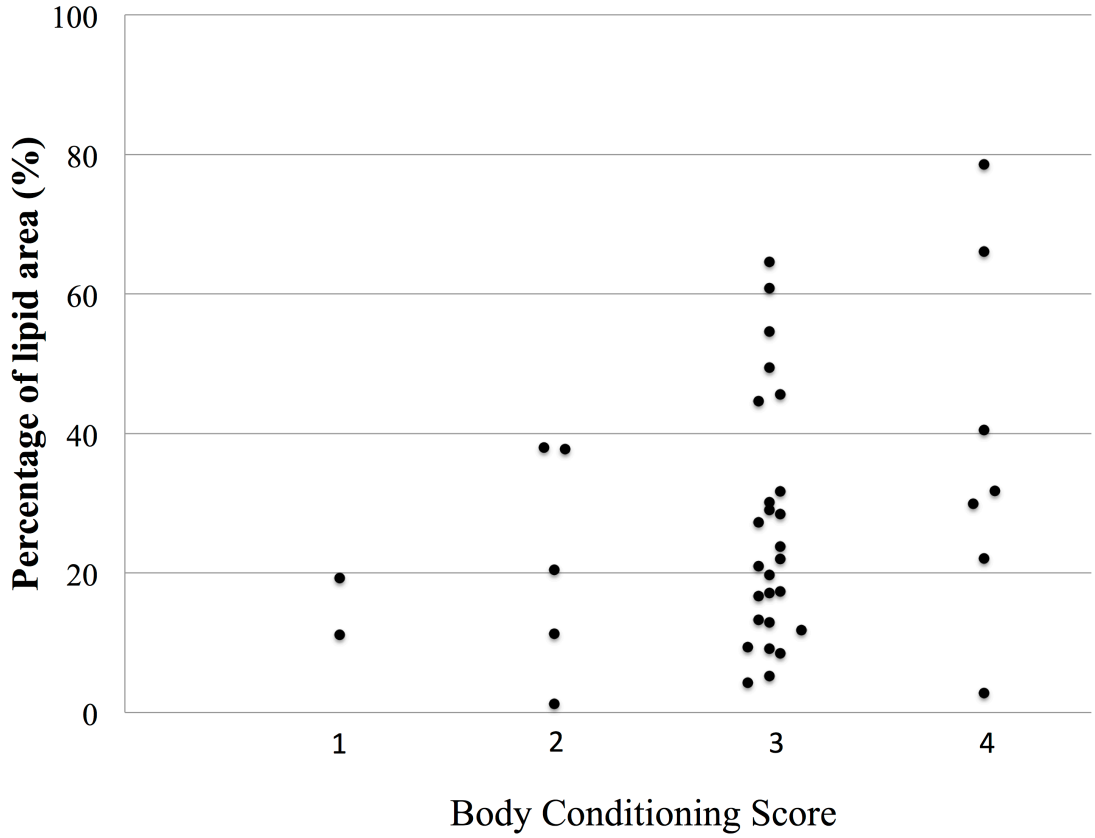
Dot plot of (A) lipid area percentage and (B) glycogen weight in each diagnosis group. cPSS: portosystemic shunt, CH: chronic hepatitis, VH: vacuolar hepatopathy, HCC: hepatocellular carcinoma, HCA: hepatocellular adenoma, CC: cholangiocarcinoma, GM: gallbladder mucocele. $^{*}P < 0.05$, Steel test

Figure 7



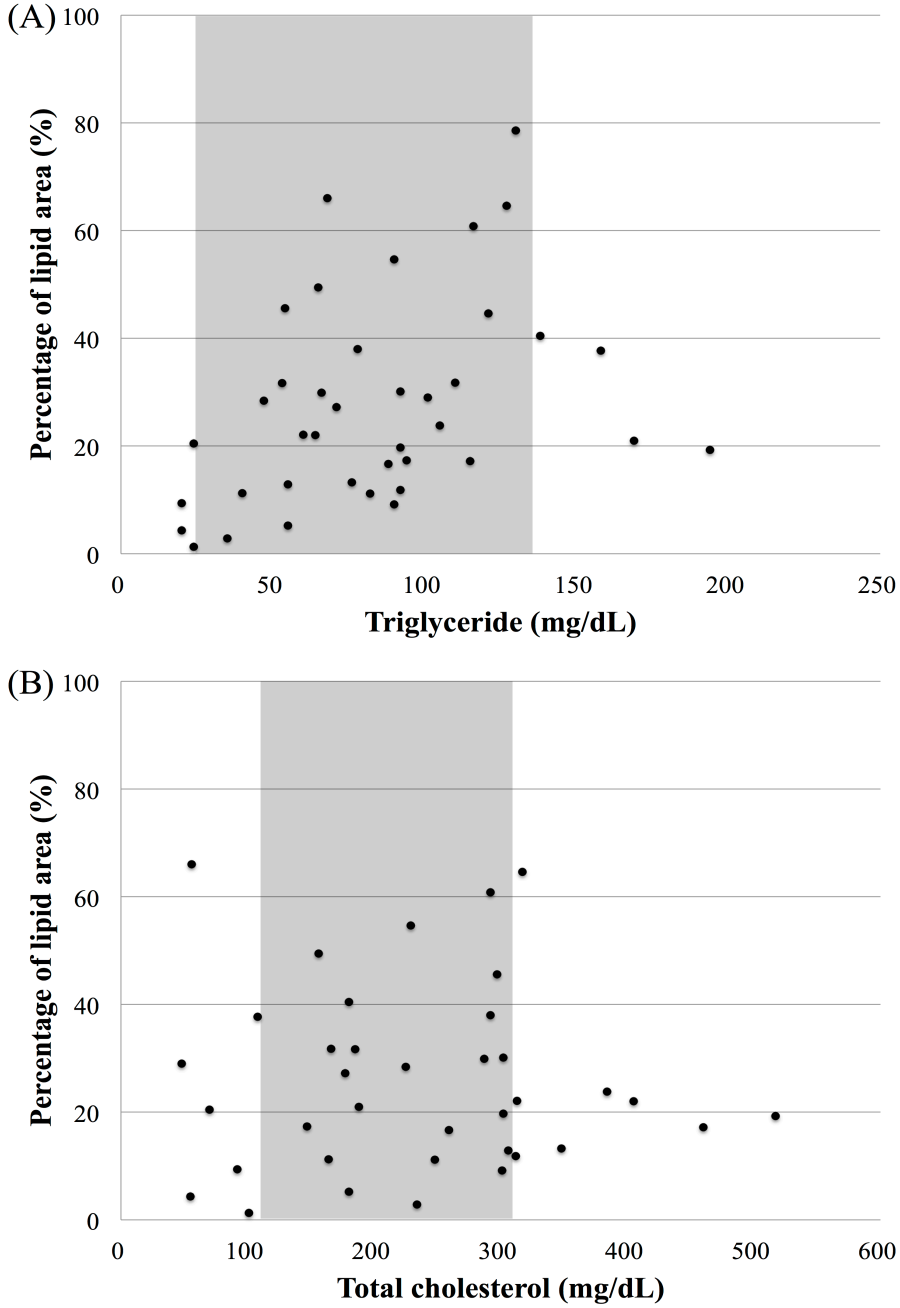
Dot plot of (A) lipid area percentage and (B) hepatic glycogen weight in each lipid vacuolation score group dogs with hepatobilially diseases.

Figure 8



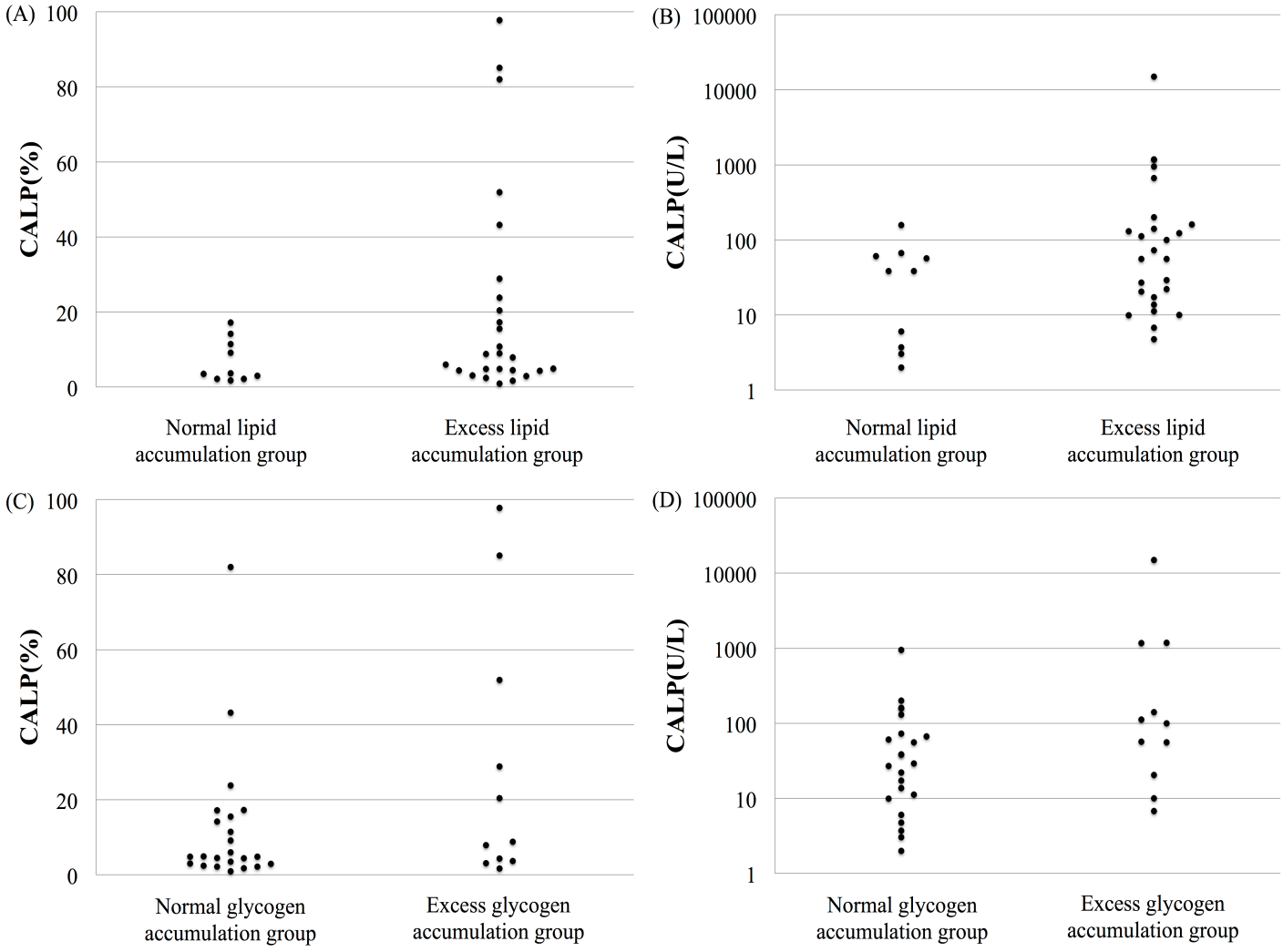
Dot plot of lipid area percentage in each body conditioning score group of dogs with non-tumor hepatobilially disease.

Figure 9



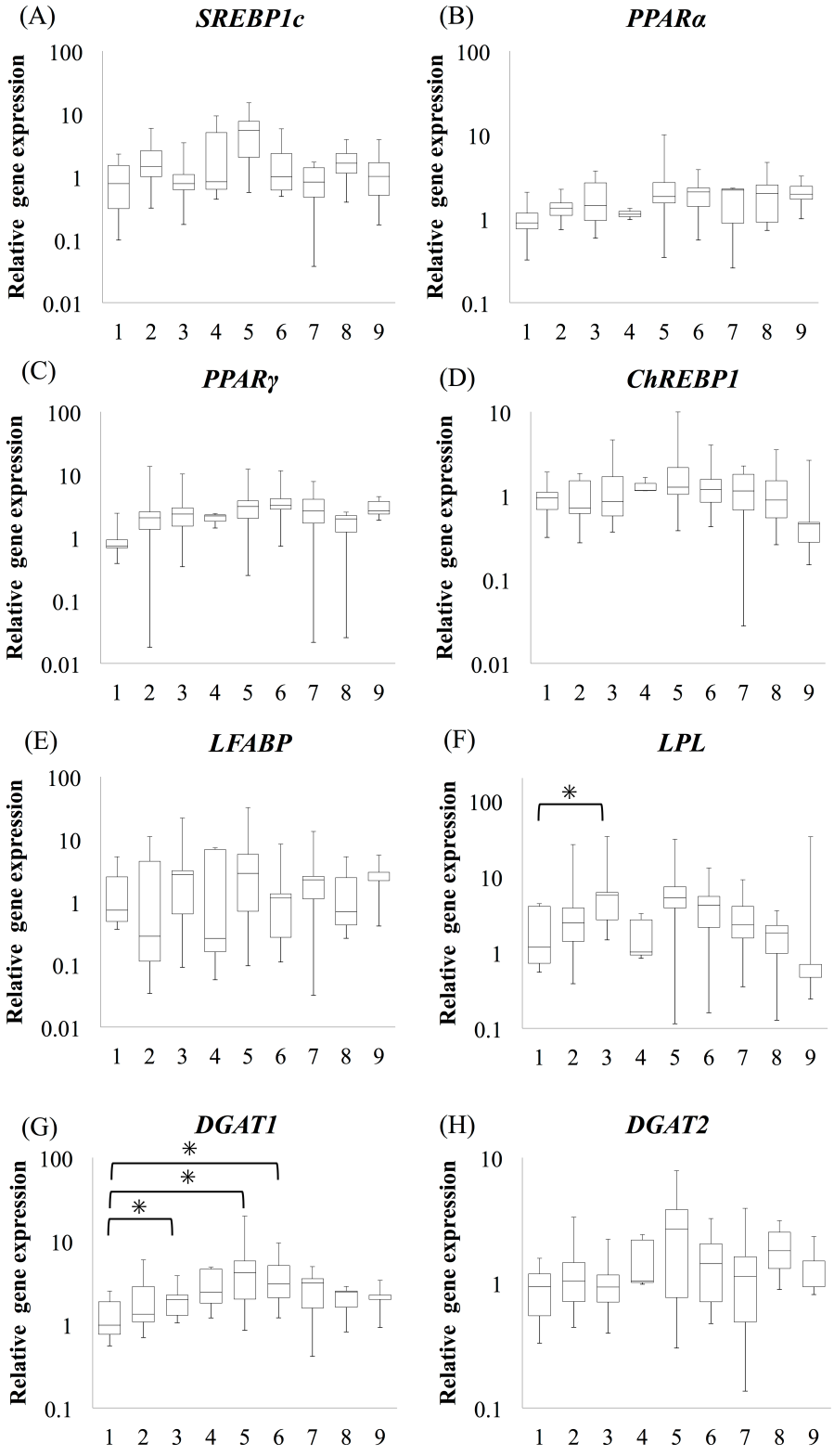
Scatter plot of percentage of lipid area and (A) plasma triglyceride concentration or (B) total cholesterol concentration in dogs with non-tumor hepatobiliary diseases. Grey area indicates the reference range of triglyceride or total cholesterol concentration in dogs.

Figure 10



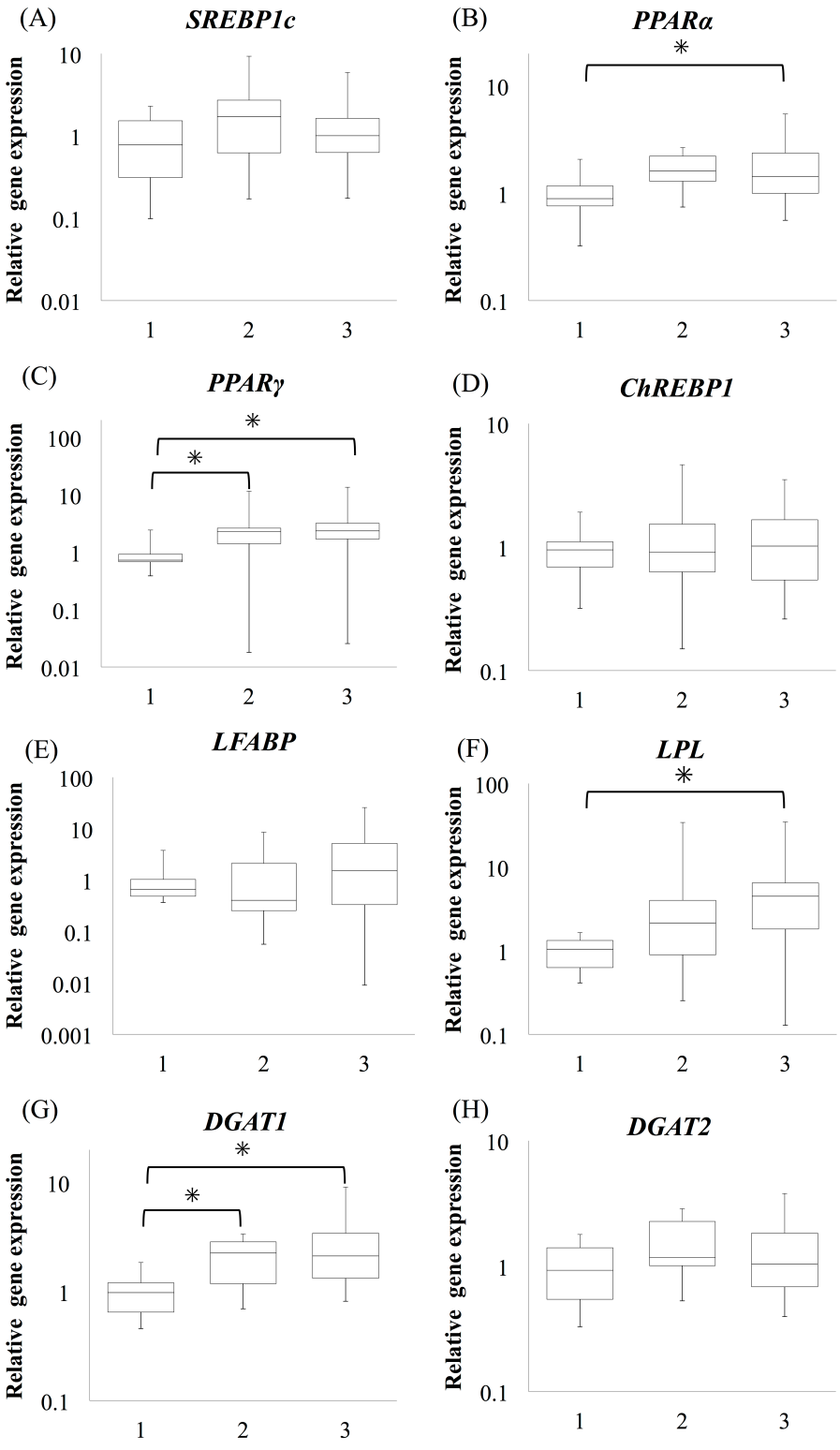
Dot plot of CALP (A) percentage and (B) activity in dogs with hepatobiliary diseases accumulated with normal level of lipid and high level of lipid in liver tissue. Dot plot of CALP (C) percentage and (D) activity in dogs with hepatobiliary diseases accumulated with normal level of glycogen and high level of glycogen in liver tissue.

Figure 11


(A)
SREBP1c
(B)
PPARα
(C)
PPARγ
(D)
ChREBP1
(E)
LFABP
(F)
LPL
(G)
DGAT1
(H)
DGAT2
Relative gene expression
100
10
1
0.1
0.01
1 2 3 4 5 6 7 8 9
*

Relative expression of (A) *SREBP1c*, (B) *PPARα*, (C) *PPARγ*, (D) *ChREBP1*, (E) *LPL*, (F) *LFABP*, (G) *DGAT1* and (H) *DGAT2* in each diagnosis group: (1) healthy control (2) cPSS, (3) CH, (4) VH, (5) HCC (neoplastic area), (6) HCC (non-neoplastic area), (7) HCA (neoplastic area), (8) HCA (non-neoplastic area) and (9) GM. $^{*}P < 0.05$, Steel test

Figure 12


(A)
SREBP1c
Relative gene expression
10
1
0.1
0.01
1
2
3
(B)
PPARα
*
Relative gene expression
10
1
0.1
1
2
3
(C)
PPARγ
*
*
Relative gene expression
100
10
1
0.1
0.01
1
2
3
(D)
ChREBP1
Relative gene expression
10
1
0.1
1
2
3
(E)
LFABP
Relative gene expression
100
10
1
0.1
0.01
0.001
1
2
3
(F)
LPL
*
Relative gene expression
100
10
1
0.1
1
2
3
(G)
DGAT1
*
*
Relative gene expression
10
1
0.1
1
2
3
(H)
DGAT2
Relative gene expression
10
1
0.1
1
2
3

Relative expression of (A) *SREBP1c*, (B) *PPARα*, (C) *PPARγ*, (D) *ChREBP1*, (E) *LPL*, (F) *LFABP*, (G) *DGAT1* and (H) *DGAT2* in each diagnosis group: (1) healthy control dogs or dogs with hepatobiliary diseases accumulated with (2) normal level of lipid and (3) high level of lipid in liver tissue. $^{*}P < 0.05$, Steel test

Chapter 3

Increase of serum alkalinephosphatase activity in dogs intravenously administrated with fat emulsion

ABSTRACT

In dogs, excess lipid accumulation causes hepatic vacuolation that is frequently observed with high serum ALP activity. In adult dogs, two ALP isozenzymes, liver ALP (LALP) and corticosteroid-induced ALP (CALP) can be detected in serum. In our previous study, dogs with hepatobiliary diseases accompanied with hepatic lipid accumulation showed higher serum ALP activity and increased expression of lipid synthesis associated genes. In Schnauzer, hyperlipidemia-associated vacuolar hepatopathy was reported with increased plasma triglyceride, cholesterol and ALP activity. However, since serum ALP activity is easily influenced by hepatocyte disorder and cholestasis of various reasons, direct relation between lipid metabolism and ALP activity should be examined. The objective of this study is to investigate the change of serum ALP activity, gene expression of ALP in liver, and alteration of lipid metabolism including hepatic lipid accumulation and gene expression when fat emulsion is experimentally induced to dogs by intravenous administration.

Four dogs were intravenously administrated fat emulsion corresponding to 50 % of daily energy requirement over 4.5 hours. Lipid accumulation and histological changes of the liver, plasma activities of liver enzymes (ALT, GGT, GOT, LALP and CALP), concentrations of triglyceride and total cholesterol and mRNA expressions of *LALP*,

CALP and 8 lipid metabolism-associated genes (*SREBP1c, PPARα, PPARγ, ChREBP1, LPL, LFABP, DGAT1* and *DGAT2*) were evaluated during fat emulsion administration experiment. After 4 days of lipid emulsion administration, 2 of 4 dogs showed increased hepatic lipid accumulation and all of 4 dogs had histologically enlarged hepatocyte. Activities of serum total ALP, LALP and CALP were increased gradually from day 0 to day 4 in all dogs, while other blood biochemical values did not change. Expression of *CALP* mRNA in the liver was increased in two dogs that showed increased lipid accumulation in liver. Expression of *PPARγ* mRNA of all dogs was increased on day 5 and 18 than day 0, however, expression of other 7 lipid metabolism-associated genes were not notably changed.

In the present study, intravenous fat emulsion administration induced increased serum ALP activity, expression of *LALP* and *PPARγ*. Moreover, 2 of 4 dogs showed increased lipid accumulation and CALP expression after fat emulsion administration. These results suggest that lipid overload would affect to activity and expression of ALP isoenzymes, especially CALP expression would relate to lipid metabolism disorder in hepatocyte.

Introduction

In dogs, there are some abnormalities of lipid metabolism, such as hypertriglyceridemia, hypercholesterolemia and excess lipid accumulation in liver. Excess lipid accumulation in the liver is recognized as hepatic vacuolation in HE stained section and can be observed in congenital portosystemic shunt (Hunt et al., 2014; Lee et al., 2011), nodular hyperplasia (Fabry et al., 1982) and other various hepatobiliary diseases as described in chapter Ⅱ in this thesis. Although the mechanism to induce lipid accumulation in canine liver is not clear in the light of lipid metabolism, hyperlipidemia-associated vacuolar hepatopathy has been reported in Schnauzer together with increased plasma triglyceride, cholesterol and ALP activity (Guilford W.G. Center, 1996).

Two ALP isozenzymes, liver ALP (LALP) and corticosteroid induced ALP (CALP) is produced in canine liver (Sanecki et al., 1987). CALP isoenzyme is unique to dogs, and induced by endogenous and exogenous corticoid hormone stimulation (Fittschen and Bellamy, 1984; Syakalima et al., 1997a; Wellman et al., 1982), or various hepatobiliary diseases as shown in chapter Ⅰ in this thesis. It has been reported that dogs with excess lipid accumulation showed higher serum CALP activity and percentage compared to dogs with normal lipid accumulation in the liver when studied with naturally

occurring hepatobiliary cases. Since hepatobiliary diseases would influence serum ALP activity, the direct relationship between high serum CALP activity and hepatic lipid accumulation is still unclear. No study evaluating the change of serum ALP isoenzymes after experimentally inducing fatty liver in dogs had been reported so far. The aim of this study is to experimentally inducing fat emulsion to dogs for evaluating the change in hepatocyte lipid metabolism including hepatic lipid accumulation condition and serum hepatic enzyme activities including ALP isoenzymes.

In terms of lipid metabolism associated gene expression in dogs, it has been reported that the mRNA expression of lipogensis associated genes, *SREBP1* and *LFABP,* was increased in liver of obese dogs (Kabir et al., 2005). To determine whether lipid metabolism related gene expression is altered in dogs with in liver of dogs with fat emulsion administration, I also measured 8 genes involved in lipid metabolism (Jump et al., 2013; Musso et al., 2009; Nguyen et al., 2008) in the liver tissue. The eight genes included 4 transcription factors which regulate hepatic lipid metabolism: sterol response element binding protein-1c (*SREBP-1c*), carbohydrate response element binding protein (*ChREBP1*), the peroxisome proliferator-activated receptor α (PPARα) and PPAR γ. I also measured expression of the gene coding lipid metabolism-associated enzymes, lipoprotein lipase (*LPL*) which hydrolyze lipid for uptake in hepatocyte, liver fatty acid

binding protein (*LFABP*) which delivery fatty acids in hepatocyte, diacylglycerol acyltransferase 1 (*DGAT1*) and *DGAT2*, which catalyzes the final step of triglyceride synthesis.

Materials and Methods

Animals

Clinically healthy 4 beagle dogs (castrated male: n=3, spayed female: n=1) were used in this study. The procedure was conducted in compliance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo (approval number; 924 approved, 2014). The mean body weight (BW) was 10.4 kg (range, 9.7-10.7) and the mean age was 5.8 years (range, 3.2 -7.8).

Fat emulsion administration, blood biochemical analysis and sample collection

During the study, all 4 dogs were fed with a standard food corresponding to daily energy requirement (DER). DER was calculated as follows (Pointer et al., 2013): $DER=1.8\times70\times BW^{0.75}$. Fat emulsion (Intralipos, Ohtsuka Pharmaceutical, Ltd, Tokushima, Japan) corresponding to 50% of DER was administrated intravenously over 4.5 hours each day. The day that the fat emulsion administration has been started was defined as day 1, and the administration was repeated from day 1 to day 4. Blood sample were drawn from jugular vein at 0, 4.5 and 9 hours after the beginning of fat emulsion administration. Body weight was measured everyday before lipid emulsion

administration. Plasma activity of ALT, GGT and GOT, serum total ALP activity and concentration of triglyceride and total cholesterol were measured by automatic blood chemistry measurement equipment (DRI-CHEM, Fujifilm Medical Co., Japan). Percentages of ALP isoenzymes (LALP, CALP and BALP) were measured by electrophoresis and colour densitometry analysis (LSI Medience, Tokyo, Japan). The activity of each isoenzyme was calculated from the percentage and total ALP activity (Itoh et al., 2002). Serum concentration of total bile acid was measured by the enzyme method (Monolis, Inc. Tokyo, Japan) on day 0 and 5. Ultrasound-guided biopsies were performed by using 14G Tru-cut type needles (Argon Medical Devices, TX, USA) on day 0, day 5 and 18.

Evaluation of liver tissue samples

All liver tissue samples were fixed in 10% formalin, processed and paraffin embedded, cut into sections, and stained with hematoxylin and eosin (HE) for histopathological evaluation. The severity of hepatocyte vacuolation was scored from G0 to G3 (G0 or absent = 0% of hepatocyte vacuolated, G1 or mild <33% of hepatocyte vacuolated, G2 or moderate 34-66% of hepatocyte vacuolated, G3 or severe >67% of

hepatocyte vacuolated) (Kleiner et al., 2005a). The number of hepatocytes was counted in 5 separate, randomly selected ×400 fields, and the mean number was calculated.

Another biopsy sample was placed into a Tissue-Tek container, which was then filled with Tissu-Tek OTC compound gel (Sakura Finetek, Torrance, CA). The sample was then frozen rapidly, being cut into 6μm slices, and then stained for lipid using a standard Oil Red O protocol (Hunt et al., 2014; Lee et al., 2011). The percentage of the area occupied by Oil Red O-stained lipid droplets in the liver tissue was calculated using Image J (http://rsbweb.nih.gov/ij/), in 5 separate, randomly selected ×200 fields(Liu et al., 2014).

RNA Extraction

Total RNA was extracted from the liver samples by using RNAspin Mini RNA Isolation Kit (GE Healthcare). The extracted RNA was then treated with DNase I (Invitrogen, Carlsbad, CA) and reverse-transcribed with a PrimeScript RT reagent kit (Takara Bioscience, Tokyo, Japan) according to the manufacturer's protocol.

Quantitative Polymerase Chain Reaction Analysis

Expression of *LALP*, *CALP* and 8 lipid metabolism-associated genes were quantified by real-time RT-PCR: The 8 lipid metabolism-associated gene were *SREBP1c, PPARα, PPARγ, ChREBP1, LPL, LFABP, DGAT1* and *DGAT2*. Three genes, *HMBS*, *RPL13A* and *RPL32*, were included as internal controls. Gene expression was quantified in triplicate samples using the Thermal Cycler Dice Real-Time System (Takara Bio Inc, Tokyo, Japan) with THUNDERBIRD SYBR qPCR Mix (Toyobo Co. Ltd, Osaka, Japan) according to the manufacturer's instructions. Primers were designed using Primer 3 (http://primer3.sourceforge.net/) or were selected by referring to previous publications (Peters et al., 2007). The sequences of the primer pairs used were shown in Table 4.

Initial incubation at 95℃ for 15 seconds was followed by 40 polymerase chain reaction (PCR) cycles consisting of denaturation at 95℃ for 5 seconds and annealing/elongation at 60℃ for 1 minute. The final denature was carried out for 15 seconds at 95℃, followed by the melting curve reaction consisting of a gradual temperature increase from 60℃ to 90℃.

The threshold cycle (Ct value) was determined using the second derivative maximum method. Before the samples were quantified, standard curves of the relative starting amount versus the Ct value were constructed using pooled liver cDNA. The amplification efficiency of each reaction was within 80% to 120%. The dissociation

curve was confirmed to have a single peak in each experiment and the PCR products were confirmed to be in agreement with their theoretic sizes by using agarose gel electrophoresis. The quantity of each transcript was determined by comparing the relative amount of the sample mRNA to that of RPL13A, RPL32 and HMBS mRNA using the ratio of mean Ct values (Peters et al., 2007). Relative gene expression was calculated as previously described.

Statistical analysis

Wilcoxon signed-rank test was used to test for significant differences in each biochemical parameters and mRNA expression between each time points. $P < 0.05$ was considered significant for the remaining statistical analysis in the present study.

Result

Lipid accumulation in liver tissue and histological evaluation

Plasma triglyceride concentration was changed rapidly by intravenous fat emulsion administration; markedly increased after administration and decreased to reference range 4.5 hour after the end administration. The maximum value of triglyceride concentration after fat emulsion administration was decreased day by day (Figure 13).

After 4 days of lipid emulsion administration, 2 of 4 dogs showed increased percentage of lipid area evaluated by ORO staining (Figure 14). In these 2 dogs (dog 1 and 2), the percentage of lipid area was kept high on day 18 (Figure 14E). By histological evaluation in HE sections (Figure 15), 2 of 4 dogs showed mild lipid vacuolation in day 0. The severity of one dog was decreased on day 5 and the severity of the other dog was kept on day 5. Glycogen vacuolation was observed in all of the 4 dogs on day 0, 5 and 18. The glycogen vacuolation severity score became more severe at day 5 compared to day 0 in 3 of the 4 dogs, and retained in 2 of 3 dogs after the fat emulsion administration period, on day 18 (Table 5). The mean number of hepatocyte per field in each time point was as follows, day 0 (median 70.4, range 67.4-75.8), day 5 (median 45.7, range 42.2-46.4) and day 18 (median 48.2, range 47.8-57.4). The mean number of hepatocyte per field was the

smallest on day 5, indicating that hepatocyte of these dogs were most severely swelled on day 5. However, there was no significant difference in the mean number of hepatocyte between day 0, 5 and 18.

Changes in blood biochemical value and gene expression of ALP isoenzyme in liver tissue

Total serum ALP activity was gradually increased in all of the 4 dogs exceeding the upper limit of reference range (47-254 U/L) on day 3 (Figure 16A). LALP and CALP activity was also increased (Figure 16B and C), but no significant difference in total ALP, LALP and CALP activity was observed between on day 0 and day 1, 2, 3 and 4 in this study. Plasma activity of GPT, GGT and GOT and concentrations of total cholesterol was not changed during this study (data not shown). Serum TBA concentration was within the reference range on day 0 and day 5 in 3 dogs, while beyond the normal range in 1 dog (21.8 nmol/mL, reference range: 0.1-10.0 nmol/mL) on day 0. Further, the gene expression of ALP isoenzyme genes was evaluated by RT-PCR. *LALP* expression was increased in all of 4 dogs on day 18 compared to day 0 (Figure 17A), and *CALP* expression was increased in 2 of 4 dogs (dog 1 and 2) on day 5 compared to day 0. The 2

dogs (dog 1 and 2) were the identical dogs that showed excess lipid accumulation in liver (Figure 17B).

Expression analysis of lipid metabolism-associated genes in liver tissue

In the relative expression of lipid metabolism-associated genes, *PPARγ* is increased on day 5 and 18 compared to day 0 (Figure 18C). The expression of the other lipogenesis-associated genes was not changed markedly from day 0 to 18 (Figure 18A, B and D-H).

Discussion

In the present study, intravenous fat emulsion administration induced increased serum ALP activity and increased gene expression of *LALP* and *PPARγ* in liver. Excess lipid accumulation and increased *CALP* expression in liver was also observed in 2 of 4 dogs at the end of fat emulsion administration for 4 days.

Two dogs showed increased hepatic lipid accumulation and the other 2 dogs did not show notable lipid accumulation in this study. Plasma lipid might also be absorbed to adipose tissue (Tilg and Moschen, 2010) as well as to hepatocytes, possibly at higher rates in the latter 2 cases. There might be an individual difference in lipid distribution. Although increased lipid accumulation was observed in only 2 of 4 dogs, hepatocyte swelling secondly to hepatocyte vacuolation was observed in all of 4 dogs on day 5 and day 18. The component of the hepatocyte vacuole in the 2 dogs without liver lipid accumulation would be expected as glycogen, intracellular edema or water (Guilford W.G. Center, 1996; Rothuizen, 2006). Since dogs were taken calorie more than DER by diet and fat emulsion in present study and intravenously induced fat emulsion would be spend firstly as energy, carbohydrate in diet, which corresponds to surplus calories, would be metabolized and stored as glycogen in hepatocyte. Although the mechanism of the accumulation of these contents after fat emulsion administration is unclear, other method for inducing hepatic lipid accumulation, such as high fat diet feeding (Inoue et al.,

2005) would be needed to study about canine hepatic lipid accumulation.

Serum ALP activity was increased gradually between day 1 to day 5, and decreased on day 18. Although serum TBA concentration was not increased on day 5, swelling of hepatocyte secondary to fat emulsion administration might have caused microenvironmental cholestasis resulting in increase of plasma LALP activity. Indeed, LALP expression is indicated to be up regulated by cholestasis in other species (Khan et al., 1998; Suzuki et al., 2006). After the hepatocyte swelling subsided, expression of *LALP* mRNA was increased on day 18. Nevertheless, serum LALP activity was decreased on day 18, indicating that the change of serum LALP activity is rather influenced by cholestasis than the change of production in liver.

Relative gene expression of *CALP* was increased on day 5 than day 0 in the two dogs that showed higher hepatic lipid accumulation in the liver on day 5. CALP is coded by intestinal ALP (IALP) gene in canine liver (Sanecki et al., 1987; Wellman et al., 1982) different from LALP, which is coded by the tissue nonspecific ALP gene together with BALP (Hoffmann and Dorner, 1975; Wellman et al., 1982). *IALP* gene is expressed in intestinal mucosa and plays a role in regulating fat absorption (Lalles, 2010). Since it has been reported that high fat feeding induced increased IALP expression in rat liver (Goseki-Sone et al., 1996), canine *CALP* expression might also be up-regulated in liver

associated with hepatic lipid metabolism.

In all of 4 dogs, relative gene expression of *PPARγ* was elevated after fat emulsion administration: the highest on day 5 and kept higher on day18 than day 0. Expression of *PPARγ* has also been up-regulated in mouse fed with high fat diet in the previous study. The elevation of canine *PPARγ* expression might be induced by triglyceride administration in this study. *PPARγ* has an important role in hepatic lipid metabolism, regulating the expression of LPL (Nakamura et al., 2014). However, *LPL* expression did not change in this study in spite of increased *PPARγ* expression. Increased expression of PPAR alone would not contribute to activation of lipogenesis. Compared to dogs with spontaneous hepatobiliary diseases, the degree of hepatic lipid accumulation was mild. Fat emulsion administration in this study might be too short and rapid to alter the expression of multiple lipid-metabolism associated genes.

In the present study, increased serum ALP activity, expression of *LALP* and *PPARγ* was observed by intravenous fat emulsion administration. Moreover intravenous fat emulsion administration for dogs partly induced increase in liver lipid area, CALP isoenzymes expression. The mechanisms of hepatic lipid accumulation to induce serum CALP activity are still unclear. Future study using improved fatty liver dog model would

be helpful to clarify the mechanism of CALP isoenzyme expression induced by hepatic lipid accumulation.

Table 4. Primers used for quantitative polymerase chain reaction analysis

Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size(bp)
LALP	TCAAACCGAGACACAAGCAC	GGGTCAGTCACGTTGTTCCT	172
CALP	CACACCTCATGGGCCTC	GCCTCCTTCCACAAAGAGGTA	224
SREBP1c	TGGATTGCACATTCGAAGAC	AGCTCATTGTGGCAGGAGAT	168
PPAR α	CACAGACACGCTCTCACCAG	AAGAAACCCTTGCAGCCTTC	174
PPAR γ	GAAGGATGCAAGGGCTTCTT	CCAAACCTGATGGCATTATGA	162
ChREBP1	GATGCCTATGTGGGCAATG	GGTAGCTGGTGAAGAAATCTGA	132
LFABP	TCAAGGCAGTGGTTCAGATG	TGGTATTGGTGATCACATCG	108
LPL	CTACCGGTGCAACTCAAAGG	GGAAGACTTTGTAAGGCATCTGA	161
DGAT1	TGGTGATGCTGATCTTGAGC	TGGTGATGCTGATCTTGAGC	150
DGAT2	CTTCCTTGTGCTTGGAGTGG	GACCTCCTGCCACCTTTCTT	135
HMBS	TCACCATCGGAGCCATCT	GTTCCCACCACGCTCTTCT	112
RPL13A	GCCGGAAGGTTGTAGTCGT	GGAGGAAGGCCAGGTAATTC	87
RPL32	TGGTTACAGGAGCAACAAGAAA	GCACATCAGCAGCACTTCA	100

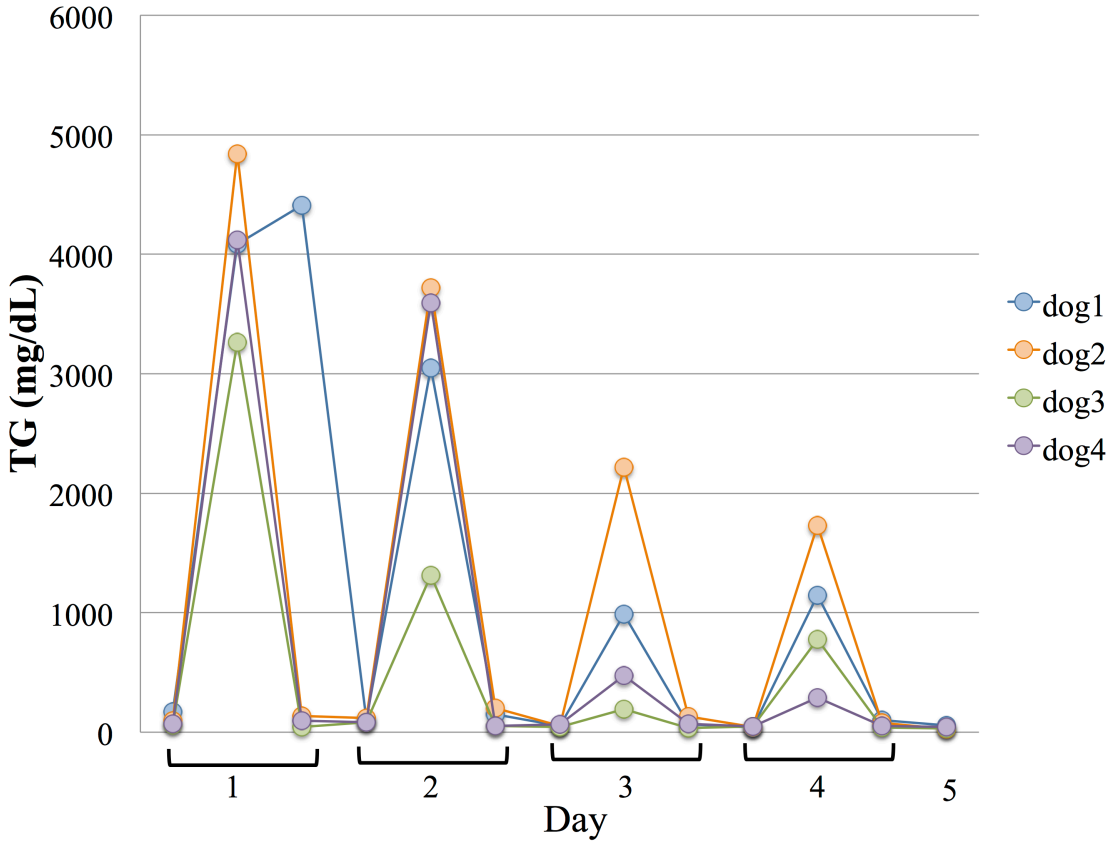
Table 5. Lipid and glycogen vacuolation severity score and mean number of hepatocyte

	Vacuolation severity score (Lipid)			Vacuolation severity score (Glycogen)		
	Day 0	Day 5	Day 18	Day 0	Day 5	Day 18
dog 1	1	0	0	1	1	2
dog 2	1	1	0	1	2	2
dog 3	0	0	0	1	2	2
dog 4	0	0	0	1	2	1

0:0%, 1:1~33%, 2:34~66%, 3:66%~ of hepatocyte vacuolated

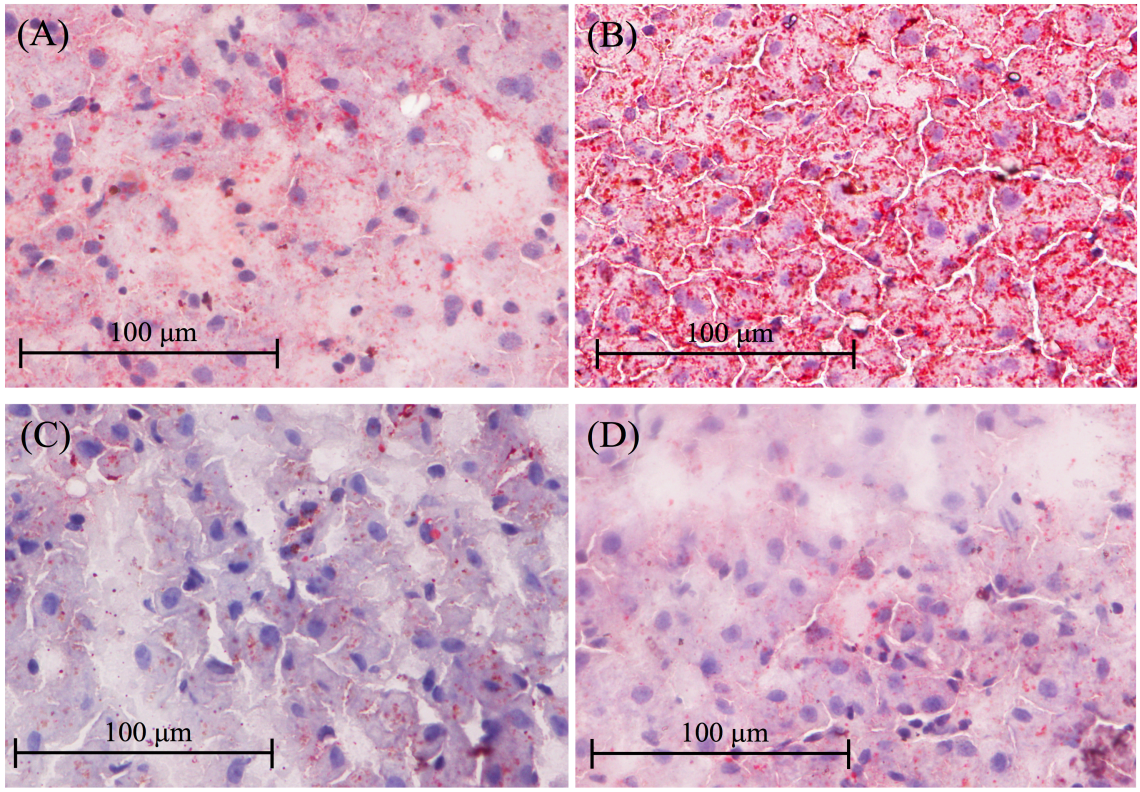
* The mean number of hepatocyte counted in 5 separate, randomly selected ×400 fields

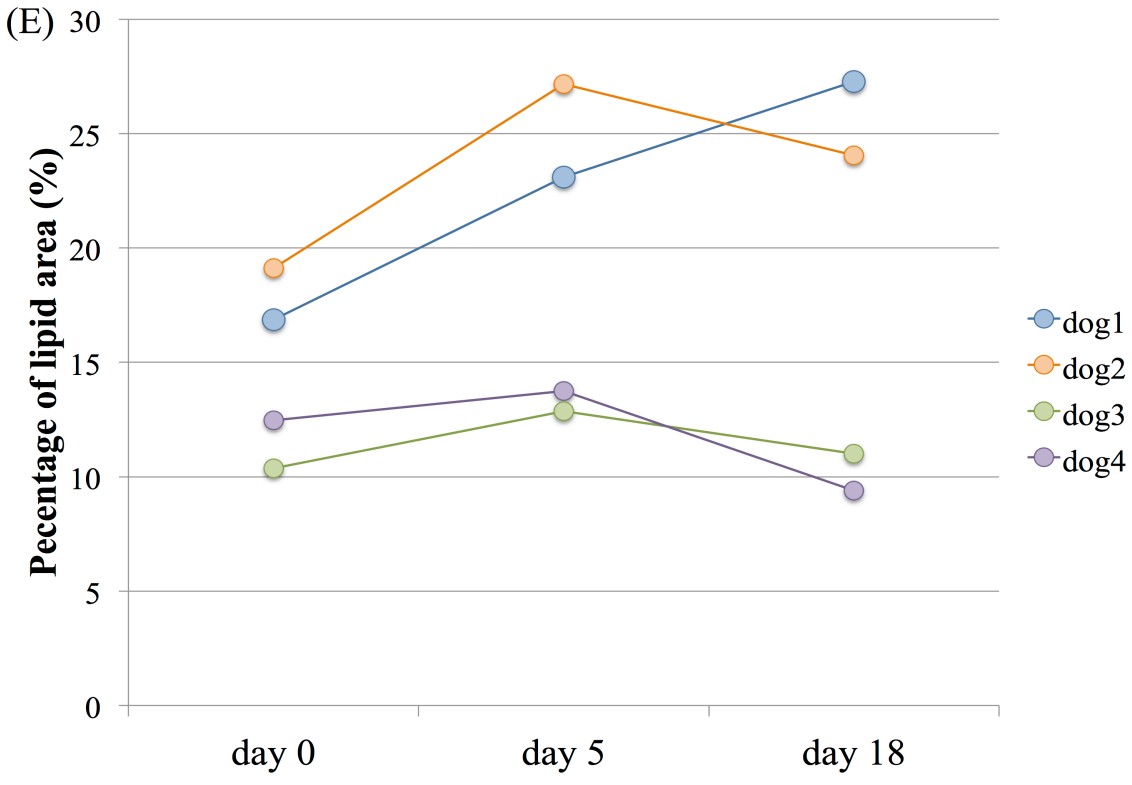
Figure 13



Change in plasma triglyceride concentration after fat emulsion administration. Plasma triglyceride concentration was measured before fat emulsion administration, immediately after 4.5 hour administration of fat emulsion and 4.5 hour after the end of fat emulsion administration each day from day 0 to day 4. Plasma triglyceride concentration was also measured on day 5.

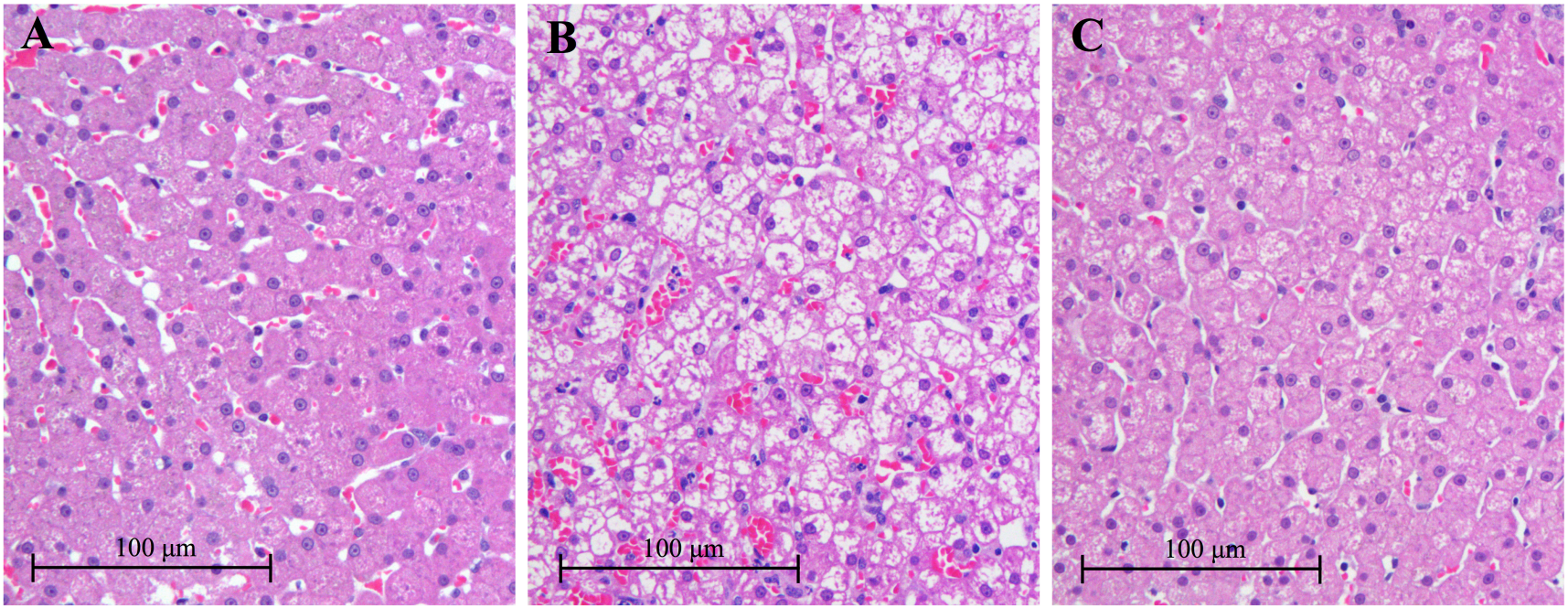
Figure 14


(A)
100 μm
(B)
100 μm
(C)
100 μm
(D)
100 μm


(E)
Pecentage of lipid area (%)
30
25
20
15
10
5
0
day 0
day 5
day 18
dog1
dog2
dog3
dog4

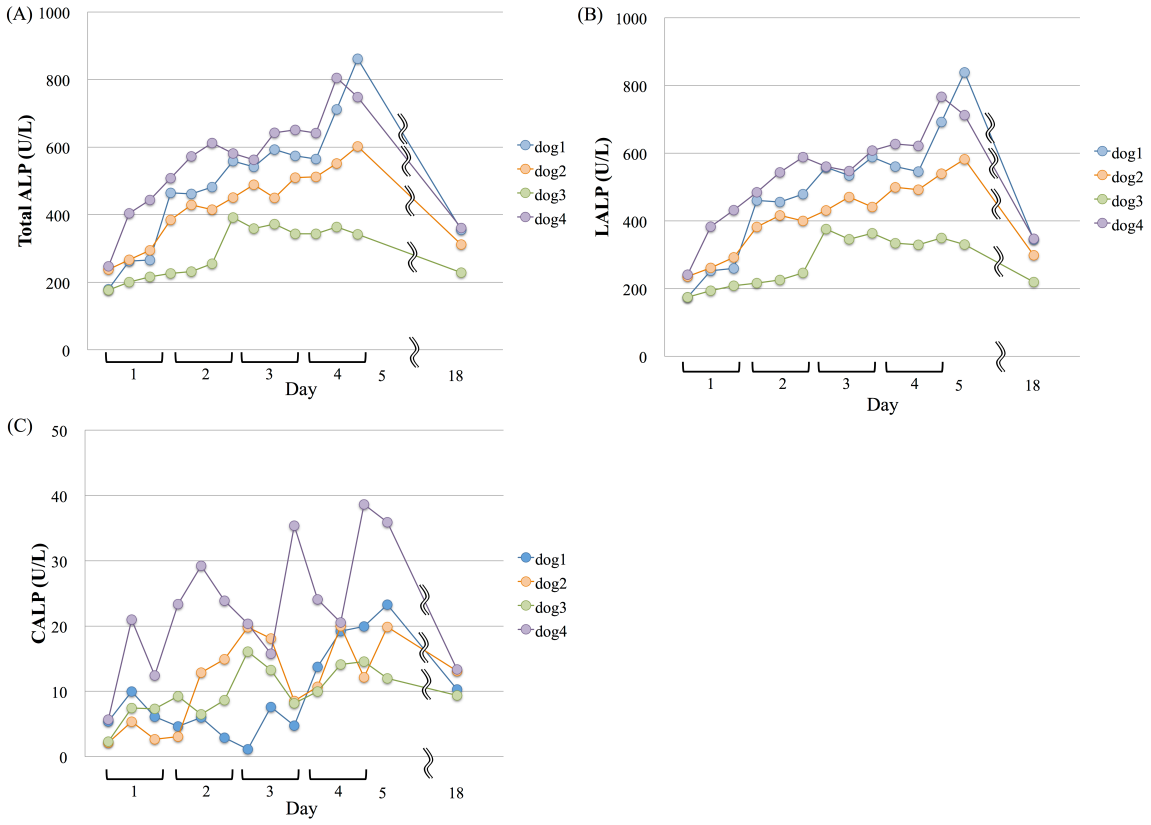
Frozen section of canine liver stained by Oil Red O staining. Liver section of dog 1 on (A) day 0 and (B) day 5. Liver section of dog 3 on (C) day 0 and (D) day 5. bar = 100μm. (E) Transition of lipid area percentages in 4 dogs after lipid emulsion administration.

Figure 15



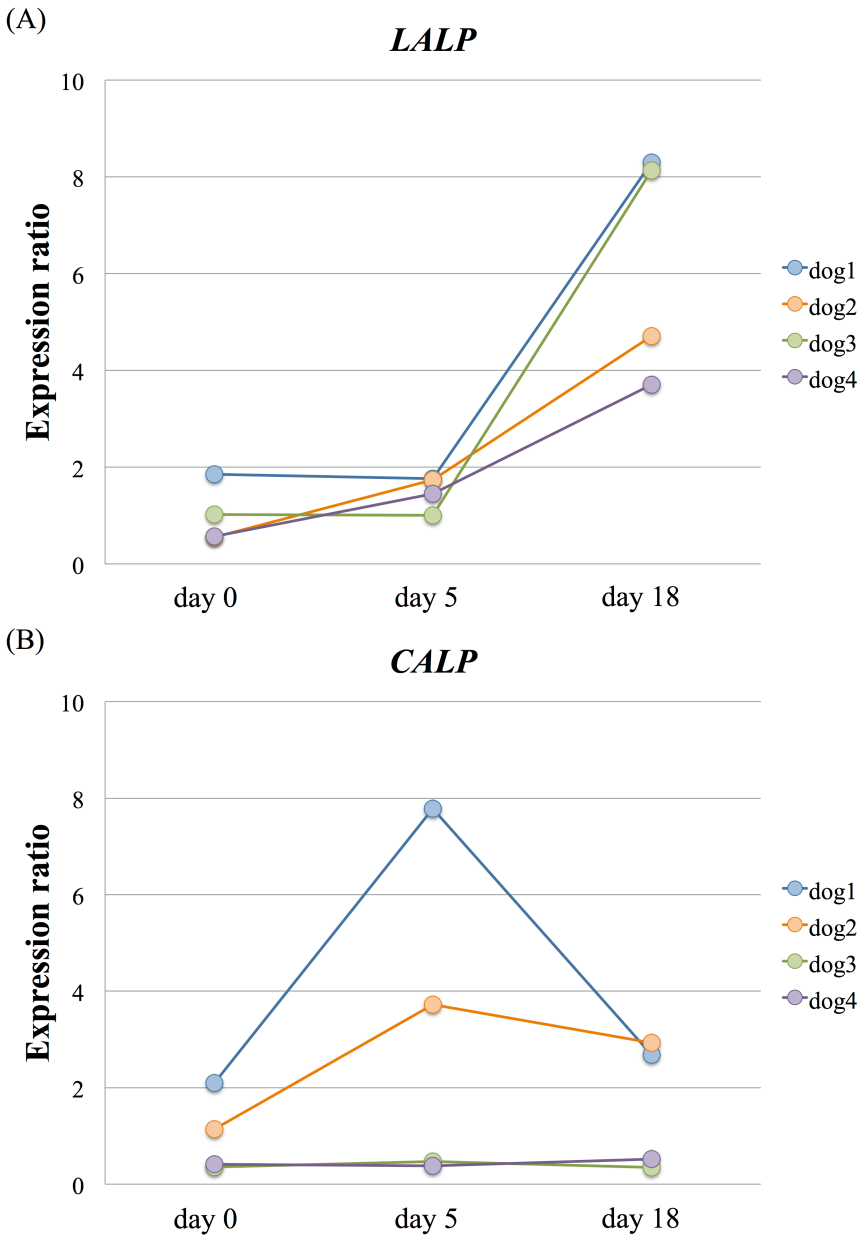
Liver section stained by HE staining. Liver section of dog 1 on (A) day 0, (B) day 5 and (B) day 18. bar = 100μm.

Figure 16



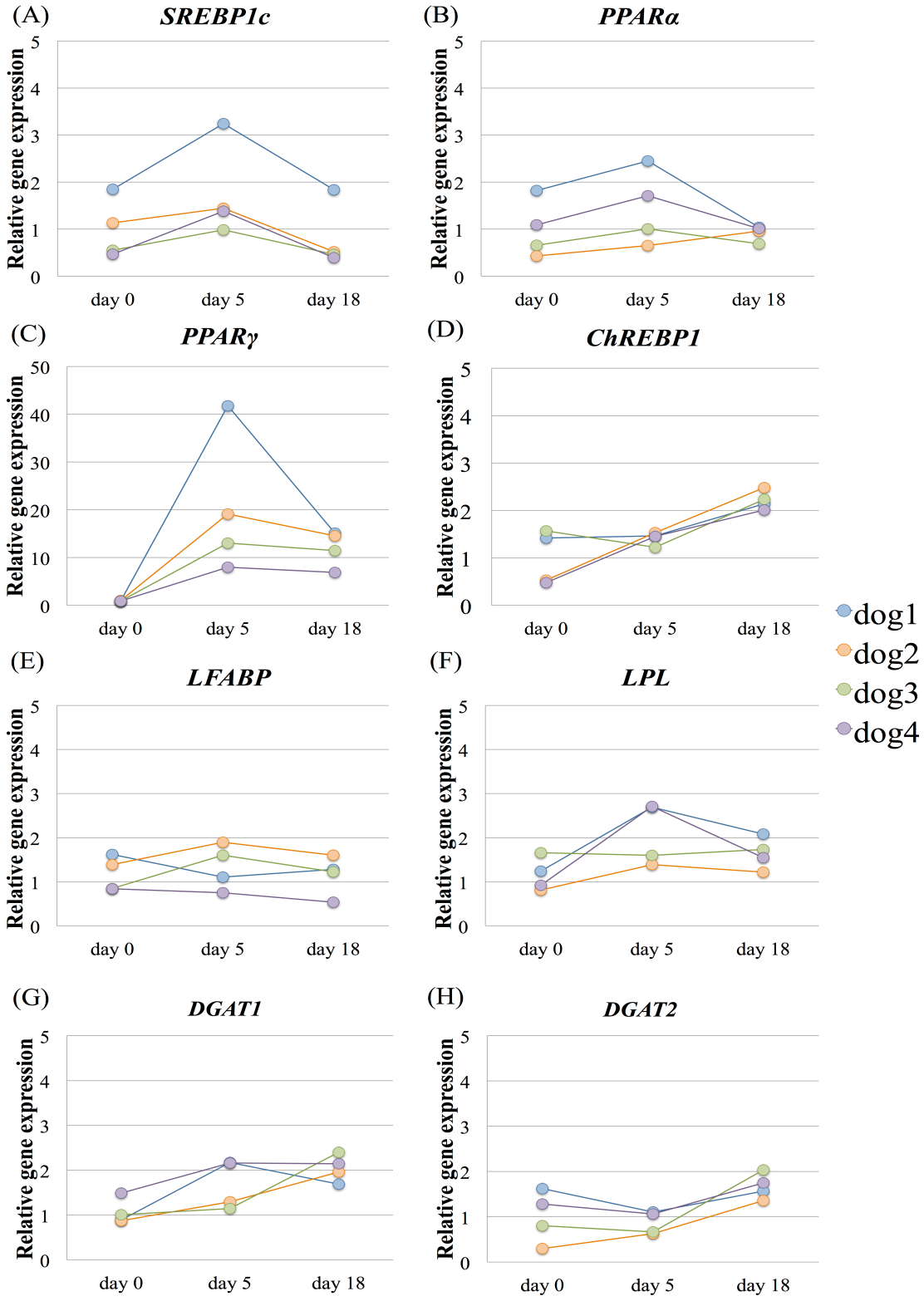
Changes in serum (A) total ALP activity, (B) LALP activity and (C) CALP activity in 4 dogs after fat emulsion administration. Analysis was performed before fat emulsion administration, immediately after 4.5 hour of fat emulsion administration and 4.5 hour after the end of fat emulsion administration each day from day 0 to day 4. Measurement was also performed on day 5 and day 18.

Figure 17



Relative gene expression of (A) *LALP* and (B) *CALP* in the liver of 4 dogs administrated with fat emulsion. Data of mRNAs were expressed relative to the geometric mean of three reference genes and were plotted as the result of calculation from the ratio of the mean relative expression on day 0

Figure 18


(A)
SREBP1c
(B)
PPARα
(C)
PPARγ
(D)
ChREBP1
(E)
LFABP
(F)
LPL
(G)
DGAT1
(H)
DGAT2
Relative gene expression
day 0
day 5
day 18
dog1
dog2
dog3
dog4

Relative gene expression of 8 lipid metabolism-associated genes in the liver of 4 dogs administrated with fat emulsion. Data of mRNAs were expressed relative to the geometric mean of three reference genes and were plotted as the result of calculation from the ratio of the mean relative expression on day 0.

Conclusion

The studies in this thesis were carried out to investigate the activity of serum ALP isoenzymes as well as the actual effects and mechanism of hepatic lipid accumulation in dogs with hepatobiliary diseases.

In chapter 1, serum ALP isoenzymes were measured in dogs with hepatobiliary disease without a history of glucocorticoid drug administration. Hyperadrenocorticism (Teske et al., 1989; Wellman et al., 1982) and glucocorticoid administration was considered to have increased the serum CALP level (Dorner et al., 1974; Syakalima et al., 1997a). However, the results reported in Chapter 1 showed that the canine serum CALP level was increased regardless of glucocorticoid administration or baseline of serum cortisol concentration in various hepatobiliary diseases. Although elevation of serum LALP activity is common in dogs with hepatobiliary diseases, the contribution of CALP to the total ALP activity needs to be considered even in conditions of apparent hepatobiliary diseases. Furthermore, dogs with vacuolar hepatopathy without the effects of endogenous/exogenous cortisol showed markedly high serum CALP. Glucocorticoid hormones were considered to have induced hepatocyte vacuolation secondary to glycogen accumulation and serum CALP elevation in previous studies (Dorner et al., 1974; Syakalima et al., 1997a). The result reported in chapter 1 suggests that the accumulation of substances other than glycogen could induce the vacuolar change and CALP production in dogs.

In chapter 2, the specific amount of hepatic lipids or glycogen accumulated in dogs with various hepatobiliary diseases to the activity of each serum ALP isoenzyme were assessed and compared. Dogs with hepatobiliary disease with a larger lipid accumulation than control dogs tended to show elevated serum CALP levels. Therefore, it was suggested that hepatic lipid accumulation was partly associated with serum CALP elevation. Because hepatobiliary diseases could influence serum ALP activity, a clinically healthy dog model of excess lipid accumulation is needed to clarify the relationship between canine serum CALP activity and hepatic lipid accumulation. This study also revealed that excess hepatic lipid accumulation occurs widely in dogs with hepatobiliary diseases. In humans, fatty liver was confirmed to develop into hepatitis, cirrhosis, and hepatocellular carcinoma (Dietrich and Hellerbrand, 2014; Musso et al., 2009). In this study, the liver of dogs with chronic hepatitis and non-neoplastic regions of hepatocellular carcinoma showed significantly higher lipid accumulation than that of control dogs did. It is likely that the clinical state of canine hepatic lipid accumulation is similar to that of humans, but the determination of the effect of lipid accumulation on canine hepatic diseases was beyond the scope of this thesis. Further studies on the progression of hepatobiliary diseases are required to reveal the impact of canine hepatic lipid accumulation. Moreover, gene expression analysis revealed that dogs in the excess lipid accumulation group showed increased expression of de novo lipid synthesis

associated genes (*PPAR γ*, *LPL*, and *DGAT1*). These results suggest that increased hepatic lipogenesis induced excess lipid accumulation in dogs. Increased expression of *PPAR γ* was also observed in the fatty liver of humans (Chao et al., 2000) and, therefore, canine hepatic lipid accumulation might have similar pathophysiology to human fatty liver disease. In future, studies focused on the mechanisms of canine hepatic lipid accumulation would show the usefulness and versatility of the dog in the fatty liver model animal, and contribute to developing new treatments for dogs with excess lipid accumulation.

In Chapter 3, induction of excess hepatic lipid accumulation was attempted by intravenously administering a fat emulsion, and the relationship between hepatic lipid accumulation and the activity as well as hepatic expression of serum ALP isoenzymes was verified. CALP expression and the amount of lipid accumulation were increased in 2 of the 4 dogs after fat emulsion administration. This result suggests that CALP expression was induced by lipid accumulation in the hepatocytes. All 4 dogs showed increased total serum ALP activity associated with hepatocyte swelling secondary to fat emulsion administration and, therefore, the relationship between the serum CALP activity and expression induced by lipid accumulation is still unclear. Moreover, the analysis of lipid metabolism-associated gene expression showed that only that of *PPAR γ* was increased by fat emulsion administration regardless of the difference in hepatic lipid accumulation.

These results indicate that the activation of lipogenesis was not induced by *PPAR γ* expression alone. Further studies using a physiological canine model of fatty liver (e.g., long-term feeding with a high-fat diet) would be useful for investigating the mechanisms of hepatic lipid accumulation and multiple factors assumed to be involved in lipid metabolism in dogs.

In conclusion, the series of studies reported in this thesis showed new findings of mechanisms associated with CALP increase. The results reported in Chapter 1 showed that serum CALP activity was increased in dogs with various hepatobiliary diseases regardless of glucocorticoid drug administration while results reported in Chapters 2 and 3 suggest that serum CALP increase was at least partly related to hepatic lipid accumulation in dogs. In future studies, it would be necessary to clarify the relationship between factors inducing CALP expression in hepatocyte and the genes associated with lipid accumulation. Furthermore, results described in Chapter 2 revealed that hepatic lipid accumulation occurred more extensively in dogs with hepatobiliary diseases, especially hepatitis and hepatocellular carcinoma than it did in healthy dogs. Increased expression of *PPARγ*, a lipogenesis-associated gene, was observed in the liver of dogs with excess lipid accumulation in the results described in chapters 2 and 3, and this would be an interesting gene to investigate further in an animal model of fatty liver. It was reported that lipid accumulation in hepatocyte develops into hepatitis and

hepatocellular carcinoma by inflammatory cytokine induction in human. In future, it would be expedient to investigate the effect of hepatic lipid accumulation on the inflammatory condition, immune state, and prognosis of dogs with hepatobiliary diseases. Further studies are also needed to examine the usefulness of diet and drug treatment in reducing lipid accumulation in the canine liver.

Acknowledgement

I would like to express my cordial gratitude to Dr. Koichi Ohno for great support and advice during this study. I also would like to express my profound gratitude to Drs. Hajime Tsujimoto, Yuko Goto-Koshino, Hideyuki Kanemoto, Kenjiro Fukushinma, Masaya Tsuboi, and Kazuyuki Uchida for their support of my work.

I wish to thank all the patients and their owners in my works and all the staffs in Veterinary Medical Center of the University of Tokyo and the Department of Veterinary Internal Medicine.

Finally, I am most grateful to my family, Mr. Akira Kojima, Mrs. Takako Kojima, Mr. Kei Kojima, and my dog Shichiemon, who encouraged me every of the way.

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