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Elucidating Hepatic Lipidosis in Stray Cats through Serum Biochemistry, Liver Histopathology and Liver RNA Expression of PPAR-δ and PPAR-γ

(Pengesanan Lipodesis Hepar pada Kucing Liar melalui Serum Biokimia, Histopatologi Hati dan Ekspresi RNA Hati PPAR- δ dan PPAR- γ)

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ABSTRACT

Early detection of feline hepatic lipidosis (FHL) with appropriate treatment can increase prognosis significantly. This study looks into the serum biochemistry and lipid composition of serum and liver samples in a group of stray cats (N=18) collected from pounds in Klang Valley, Malaysia. Alanine aminotransferase (ALT) in blood serum was used to detect for liver damage possibly due to FHL, confirmed through light microscopy, serum biochemistry (triglyceride, cholesterol, creatinine, and urea), liver triglyceride and cholesterol concentrations, and liver RNA expression of lipid droplet regulators peroxisome proliferator-activated receptors (PPARs). Differing severity of FHL in samples were divided and grouped using an adapted scoring method observing fatty change of liver (FCL) with trends between FCL groups investigated. Elevated serum ALT reflective of increasing FCL severity was observed with elevated concentrations of liver TAG and cholesterol levels. Serum TAG and cholesterol decreased with heightened FCL pointing to fatty acid oxidation and lipid restoration in the liver, supported by PPAR-γ expression which also propose macrophage activation for liver recovery alongside PPAR-δ for lipogenesis and inflammatory reactions. Elevated serum creatinine and urea levels with increasing FCL severity propose overall intact hepatic function in the stray cat samples.

Keywords: *Felis catus*; hepatic lipidosis; PPAR-δ; PPAR-γ; serum biochemistry

ABSTRAK

Pengesanan awal lipidosis hepatik felin (LHF) berserta rawatan bersesuaian dapat meningkatkan prognosis dengan ketara. Kajian ini meneliti serum biokimia dan komposisi serum lipid dan sampel hati sekumpulan kucing liar (N=18) yang telah dikumpul daripada pusat lindungan kucing terbiar di sekitar Lembah Klang, Malaysia. Alanina aminotransferase (ALT) di dalam serum darah digunakan bagi mengesan kerosakan hati yang mungkin disebabkan LHF, disahkan melalui mikroskopi cahaya, serum biokimia (trigliserida, kolesterol, kreatinina dan urea), kandungan trigliserida (TG) dan kolestrol hati serta pengekspresan asid ribonukleik hati daripada pengatur titik lipid reseptor-reseptor diaktifkan-pengproliferat peroksisom (RDPP) proliferator peroksisom -diaktifkan reseptor (PPARs). Keparahan yang berbeza antara sampel LHF dibahagi dan dikelompok menggunakan sebuah kaedah penilaian yang telah digubah untuk memerhati perubahan lemak hati (PLH), seterusnya mengkaji trend PLH antara kelompok. Kenaikan serum ALT seiring dengan keparahan PLH telah diperhati berserta kenaikan kandungan TG dan kolesterol hati. Penurunan serum TG dan kolesterol seiring

dengan keparahan PLH menunjukkan berlakunya pengoksidaan asid lemak dan pemulihan lipid di dalam hati, ini telah disokong oleh pengekspresan RDPP-γ yang juga mencadangkan pengaktifan makrofaj bagi memulihkan hati di samping RDPP-δ untuk lipogenesis dan reaksi-reaksi radang. Kenaikan kreatinina dan serum urea seiring dengan keparahan PLH mencadangkan kebolehan fungsi hepatik pada kucing-kucing terbiar yang dikaji.

Kata kunci: *Felis catus;* lipodesis hepar; PPAR-δ; PPAR-γ; serum biokimia

Introduction

Felis catus, the common cat, has been raised as companion animals for a myriad of reasons. However, their welfare comes into question when they become strays. Even when cared for domestically, cats are predisposed to FHL where triacylglycerol (TAG) is excessively deposited into the liver, increasing the organ's original weight by more than 50%, possibly causing secondary impairment of liver function and intrahepatic cholestasis (Valtolina & Favier 2017). Anorexia and distinct inappetence, when coupled with loss of weight, icterus and diarrhoea make up clinical signs leading to FHL (Kuzi et al. 2017).

Anorexia and malnutrition has been commonly linked to FHL. Healthy cats that develop lipidosis with underlying diseases and undergo extended periods of anorexia are characterised with 'secondary HL', whereas cats with 'primary HL' are healthy cats that have experienced accelerated weight loss from food deprivation due to stressors such as unfavoured changes in lifestyle, food, or even unintentional food deprivation by the owner (Armstrong & Blanchard 2009). Armstrong and Blanchard (2009) also posit that FHL development is hastened when relating the cat's original adiposity concentration against percent loss in calorie intake. Malnutrition is considered a factor in the disease in line with pathophysiological conditions including metabolic changes such as with obesity, and insulin resistance, besides deficiencies in essential fatty acids, antioxidants, proteins, B-vitamins and L-carnitine (Verbrugghe & Bakovic 2013). In cases of FHL without accompanying severe disease, enteral feeding can yield up to 88% recovery (Kuzi et al. 2017).

It was assumed that peripheral fat lipolysis would increase serum fat levels and into the liver, however, it was reported that FHL cats did not present significant differences in serum TAG or cholesterol concentrations when compared to healthy cats (Minamoto et al. 2018). Conversely, FHL cats present impaired carbohydrate metabolism and insulin uptake – increased concentrations of blood glucose, glucagon, lactate and non-esterified fatty acids (NEFAs) with low insulin levels – due to imbalances in the body's production of NEFAs, hepatic fatty acid (FA) rate of oxidation, FA *de novo* synthesis and hepatic TAG circulation associated with very low-density lipoproteins (VLDLs) (Valtolina & Favier 2017).

Nutritional needs high in protein is a consequence of the cat's dietary adaptations as obligate carnivores, rendering deficiencies in AAs and essential FAs detrimental to the cat's well-being, and may point towards FHL (Verbrugghe & Bakovic 2013). Urea cycle function, methylation reactions and substrate entrance metabolism are impaired with protein deficiencies as cats are incapable of reserving AA use even in conditions of malnutrition, consequently impairing detoxification reactions and subsequent hyperammonemia (Verbrugghe & Bakovic 2013).

Overconditioned cats present FHL to varying degrees as observed in their liver fat content – lean (2.5%), obese (5%), obese after undergoing rapid weight loss (10%) and FHL cats (34-49%) (Valtolina & Favier 2017). FHL-afflicted cats have an enlarged liver with yellowish discolouration, glistening, friable, and show rounded edges. Some FHL cats present lipid infiltration throughout all hepatocytes in a lobule while others concentrating at the periportal or centrilobular region. This excessive lipid deposition can cause inflammation or none at all, and though necrosis at the centrilobular region has been reported, it is uncommon (Zawie & Garvey 1984). Observations of affected hepatocytes can be done through light microscopy to detect macrovesicular and microvesicular steatosis. Macrovesicular steatosis demonstrate lipid droplets (LDs) in the hepatic cytoplasm with equivalent nucleus size or larger, which possibly displaces the nucleus to the periphery, while microvesicular steatosis present hepatocytes with LDs uniformly smaller than the cell's nucleus while filling up its cytoplasm (Cullen et al. 2006). FHL cats presenting both forms of lipid infiltration is possible (Cullen et al. 2006).

Besides lipid metabolism, the liver also plays a role in vital physiological functions such as urea production. Mitochondria in the liver catabolise urea from toxic ammonia, a highly-nitrogenous consequence

of protein catabolism, however, deficiencies in urea cycle enzymes can cause blood urea nitrogen (BUN) to decrease (Barmore et al. 2020). The drop in BUN levels could indicate severe liver complications like cirrhosis or failure of the organ (Higgins 2016) with the serum marker reducing even more when facing protein deprivation, anorexia, coupled with the advanced liver disease (Baum et al. 1975). Malnutrition also affects creatinine production as a product of muscle catabolism (Delanaye et al. 2017) presenting drastic plasma creatinine decline in patients suffering anorexia nervosa (Boag et al. 1985).

The depletion of muscle mass and protein in the diet reduces creatinine production, however, severe hepatic diseases can also produce the same outcome, possibly due to impaired production of creatinine in the liver (Takabatake et al. 1989). When protein deficient, the lack of essential amino acids leads to impairment of TAG excretion from the liver as apolipoprotein B100 becomes deficient, an important precursor to VLDL production which binds to TAG in the liver for its egress (Center et al. 1993). However, TAG and VLDL concentration in the blood of FHL cats have been reportedly higher than in control cats, where plasma TAG levels in FHL cats was at 62% against 25% in healthy, lean cats; total lipoprotein mass in serum showed VLDL in FHL cats was at 19% against 2% in healthy cats (Valtolina & Favier 2017).

Although the main cause of FHL remains vague, there is no doubt that the hepatopathy occurs in tandem with excessive deposition of lipids into the liver in the form of lipid droplets. Initially, these energy reserves were assumed to be inert, however, it was found that lipid droplets function as organelles associated with lipid metabolism and cellular energy homeostasis. Neutral lipids such as triacylglycerol (TAG) and sterol esters (SEs) generally make up LDs though concentrations of respective lipids vary according to cell type (Farese & Walther 2016).

Regulation of lipid droplets is carried out through the nuclear hormone receptor peroxisome proliferator-activated receptors (PPARs) by transcription of LD-associated proteins and LD remodelling through lipolytic and lipogenic enzymes (de la Rosa Rodriguez & Kersten 2017). PPARs have isomers localised to specific regions of the body and activate certain pathways such as PPAR-α in the liver for bodily adaptation towards food deprivation, PPAR-δ in skeletal muscle for coordination of muscle physiology adaptions to negative energy balance and endurance activities, and PPAR-γ in adipocytes for regulation of energy metabolism (Nakamura et al. 2014).

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

This research was regulated by the Institutional Animal Care and Use Committee (IACUC) of UPM under AUP-R050/2017. Cats (N=18) were collected from animal pounds located in the Klang Valley area. Only cats that were unhealthy - emaciated, dehydrated, lethargic, and were to be put down by the pound due to overpopulation - were included in this study. Under ideal body conditions (ranging from body conditions scores from 1 -3 (WSAVA 2013)) were observed; ribs could be observed with minimal subcutaneous fat. No signs of jaundice (yellowing of the sclera, gums and skin) were observed. Of the samples collected, only eighteen stray cats that were euthanised for their blood serum and liver tissue were used for this study. Serum ALT levels were analysed for all samples but only samples within the lowest and highest ranges of ALT obtained from the samples included in the study for liver histological evaluation and gene expression. The removal of outliers in the study (based on blood serum biochemistry values) reduced the final number of samples to be included. Liver samples extracted generally did not show significant signs of lipidosis; liver samples were brown in colour and generally did not show any yellowing and were firm with sharp borders. The cats were anaesthetised using Zoletil (0.3 mL/cat, Virbac, Hamilton, New Zealand) by intramuscular injection followed by extraction of blood sample from the jugular vein. Euthanasia was then conducted through intracardiac pentobarbital administration using Dolethal (10 mL/cat, Vetoquinol, England). Whole liver samples were extracted postmortem and stored in chilled PBS, followed by processing into respective downstream analyses: RNAlater (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania) for RNA gene extraction, liquid nitrogen (Alpha Gas Solutions, Selangor, Malaysia) for liver triglyceride and cholesterol analysis, and formalin (Sigma-Aldrich, Missouri, United States of America) for histopathology evaluation. Serum was analysed for alanine aminotransferase (ALT), triacylglycerol (TAG), cholesterol (Chol.) and creatinine (Creat). For liver TAG and cholesterol analysis, liver samples were homogenised and then lipid extraction was performed via Chloroform/Methanol method: chloroform, methanol (RCI Labscan, Bangkok, Thailand). Liver samples preserved in

formalin were histologically processed and stained using Hematoxylin and Eosin (HE) and scored for fatty change of liver (FCL). Gene expression was investigated using conventional PCR targeting genes responsible for energy regulation through lipid metabolism (PPAR-γ, PPAR-δ).

BLOOD SERUM EXTRACTION AND BIOCHEMICAL ANALYSIS

Blood samples were withdrawn from the jugular vein after anaesthesia. Blood was centrifuged at 2.5K RPM for 10 min at 4 °C (relative centrifugal force (RCF) = 511; Eppendorf Centrifuge 5702 R, rotor radius = 73 mm; Merck, Darmstadt, Germany). Sera obtained were then aliquoted into sterile Eppendorf tubes and stored at -20 °C before preservation at -80 °C. Blood sera were analysed at Veterinary Laboratory Services Unit (VLSU), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) using automated chemistry analysers TRX 7010 and Biolis 24i BioREX, Mannheim, Germany), and data were compared to the hepatic TAG and cholesterol concentrations and liver histological evaluation.

One-way ANOVA was used for statistical analysis (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp Statistics 26). Serum biochemistry and liver lipid concentrations were set at variables against FCL severity. Due to limited samples included in the study, a more accurate statistical analysis may have been hindered as no significant differences could be reported. The author acknowledges this may have also affected the high standard deviation in the reported data.

LIVER HOMOGENISATION FOR LIPID EXTRACTION

After euthanasia, whole liver samples were extracted post-mortem, washed with PBS, and stored in sterile Eppendorf tubes. The tubes were kept in a chilled container for transportation. Liver samples were sectioned into approximate 1 cm^3 square, stored in cryovials and into liquid nitrogen prior to lipid extraction. The standard Bligh and Dyer (Bligh & Dyer 1959) method was used for lipid extraction. Homogenisation was carried out for each cat sample using 0.22 g of liver and 2.2 mL of PBS. Protein quantification was carried out using 100 µL of the homogenate, diluted into five serial dilutions and quantified using Pierce BCA Protein Assay (Thermo Fisher, Illinois, United States of America). TAG and cholesterol analyses utilised 900 µL of the homogenate, respectively, quantified using Triglycerides liquicolor and Cholesterol liquicolor, respectively (Human, Wiesbaden, Germany).

HISTOLOGICAL EVALUATION

Histological processing was carried out on the cat liver samples to grade for FHL severity, as liver histological evaluation stands as the gold standard for fatty liver disease determination (Yerian 2011). Samples were histologically prepared and sectioned samples were stained using Harris Hematoxylin and Eosin (H&E) and examined under light microscopy.

HEPATIC SCORING

Severity of FHL was determined through the amount of steatosis present in the liver samples. A scoring method was used adapted from the Fatty Change of Liver (FCL) scoring method used by Kalaitzakis et al. (2017). Classification of liver fat content from Mertens (1992) point score scale was used, classifying six different degrees of liver fat content (Grades der Leberverfettung – GdL), which for this paper will then be referred to as FCL.

Determination of FCL severity was verified through light microscopy examination at 200× magnification between two veterinarians and the author conducting the study. Five different lobules for each liver histological sample was evaluated consisting of a portal triad and their respective central vein. The amount and size of lipid droplets were determined for each region between the portal triad and central vein, divided into three equal areas. Each divided area was scored for lipid droplet deposition as follows: no lesion (0 points), cloudy swelling (0.5 points), cloudy swelling with some small vacuoles (1.0 points), many small vacuoles (2.0 points), moderately sized vacuoles (3.0 points), large vacuoles (4.0) and many large and clumped vacuoles (5.0 points). Scores for each region were collected alongside all histological sections per cat sample with their median calculated against one of six degrees of FCL; FCL 0 (0.0 points), FCL 1 (0.5-1.0 points), FCL 2 (1.5-4.5 points), FCL 3 (5.0-7.0 points), FCL 4 (7.5-9.5 points), FCL 5 (>9.5 points).

QUALITATIVE POLYMERASE CHAIN REACTION (PCR)

RNA was extracted and purified from liver samples using Qiagen RNeasy Mini Kit (Qiagen, Maryland, United States of America) according to manufacturer instructions. For each sample, 20-30 mg was used with the addition of 2-mercaptoethanol (10 µL) into Buffer RLT. Primers used for genes of interest as listed in Table 1. References for genes used are as follows; PPAR-γ, from Zini et al. (2009); PPAR-δ, predicted sequence from NCBI, reference sequence: XM_011282251.3.

TABLE 1. Targeted genes and primer sequences for PCR of cat liver samples

Gene	Direction	Sequence (5'-3')
PPAR-γ	Forward	GCGATTCCTTCACTGATAC
	Reverse	TCTCTCCGTAATGGAAGACC
PPAR-δ	Forward	ATCAGCGTGCACGTCTTCTAC
	Reverse	CTCGGTCTCAGTCCCTCTTT

Conventional PCR was carried out using RNA extracted from the cat liver samples using Access RT-PCR System (Promega Corporation, Wisconsin, United States of America) and RNasin® Ribonuclease Inhibitors (Promega Corporation, Wisconsin, United States of America). With the exception of sample '69' due to limited RNA, each sample's RNA template was standardised to 100 ng/μL for reverse-transcription (RT) PCR using SensoQuest Labcycler® thermocycler (Sensoquest GmbH, Göttingen, Germany). Respective master mix and protocol for RT-PCR provided in Tables 2 and 3. Samples were then stored in -20 °C or observed through 2.5% agarose gel electrophoresis.

TABLE 2. Master-mix for one RT-PCR reaction of cat liver RNA

Item	Volume (μL)
Forward primer	1
Reverse primer	1
Nuclease free water	31
AMV/*Tfl* 5× reaction buffer	10
dNTP mix	1
$MgSO_4$ (25 mM)	2
AMV reverse transcriptase (5 u/μL)	1
Tfl DNA polymerase (5 u/μL)	1
RNAse inhibitor	0.5
RNA (100 ng/μL)	2

TABLE 3. RT-PCR protocol for cat liver RNA

Number of cycles	Temperature (°C)	Duration	Activity
1	45	45 min	Reverse transcription
1	94	2 min	AMV RT inactivation and RNA/cDNA/primer denaturation
40	94	30 sec	Denaturation
40	based on primer	1 min	Annealing
40	68	2 min	Extension
1	68	7 min	Final extension
1	4	∞	Storing

Electrophoresis was conducted using 80 V for 50 min and then viewed using Bio-Rad Gel Doc XR+ with Image Lab Software (Biorad, California, United States of America).

Results

The cats included in this study were mostly of poor health (body condition scores from 1-3) with ribs prominently observed with thin subcutaneous fat. Cats were lethargic, emaciated and dehydrated. However, jaundice was not observed in any of the included cats.

Serum biochemistry

Mean serum values of ALT, TAG, cholesterol, and creatinine of the stray cats Table 4 were within the 'normal' range based on University Veterinary Hospital's reference (UPM). Slight elevation of urea was observed at 10.08 mmol/L (normal range at 3-10 mmol/L), suggesting that the study population had elevated concentrations of serum urea. However, considering the exceeding value was not extremely beyond the reference range, any indication of severe liver damage due to impairment of liver urea cycle metabolism is not conclusive (Shangraw & Jahoor 1999). Dehydration in these stray cats may also have contributed to the elevated serum urea concentration (44% of the cats). About 28% of cats presented serum creatinine levels below the normal values. In conditions of chronic liver disease, the organ can reduce production of creatinine up to half the normal concentration, however, malnutrition, loss of muscle mass, in addition to sepsis and large volume ascites affecting satiety can also contribute to altered creatinine production (Slack et al. 2010). Over production of ALT was observed in 39% of the studied population, possibly indicating hepatic lipidosis (Center 2007), lesions similar to that of rats fed a high fat diet and undergoing steatohepatitis (Liu et al. 2016).

TABLE 4. Stray cat serum biochemistry

	Parameter	
Serum, unit	Mean (range minimum – maximum)	Reference
ALT, U/L	75.11 (17 – 229)	10-90[1]
TAG, mmol/L	0.79 (0.26 – 1.75)	0.28-1.80[2]
Chol., mmol/L	2.46 (1.3 – 4.1)	1.5-6[1]
Creat., μmol/L	65.17 (38 – 94)	60-193[1]
Urea, mmol/L	10.08 (5.0 – 29.6)	3-10[1]

ALT: alanine aminotransferase; TAG: triacylglycerol; Chol.: cholesterol; Creat.: creatinine (Veterinary Hospital UPM[1]; Klaassen 1999[2])

Histological evaluation for hepatic lipidosis of stray cats

The presence of and severity of FCL in the stray cats was determined through a scoring method adapted from Kalaitzakis et al. (2007). Lipid droplets can be observed as white, opaque circles deposited between the hepatocytes coloured with a purplish hue. No sample was categorised in FCL score 0 or 1.

When observing the histological sections (Figure 1(2 - 5)), FCL 2 samples presented a small number of lipid droplets, approximately 70-100 μm in diameter, indicating a lower severity of lipidosis. FCL 3 samples presented similar lipid droplet sizes at approximately 80-100 μm diameter, however, their numbers were more numerous in the histological sections and are indicative of a greater severity of lipidosis as compared to FCL 2 samples. Greater amounts of lipid deposition and lipidosis can be observed through the increased size and number of lipid droplets in FCL 4, taking more of a rounded shape, approximating 80-120 μm diameter. The highest severity of FCL, FCL 5, showcases liver samples with even larger, rounded lipid droplets, at around 160-170 μm diameter, and tend to clump together. FCL 5 in this study signifies the greatest severity of hepatic lipidosis. In FCL 4 and 5, lipid deposition can be observed as white inclusion bodies within the hepatocyte walls pushing cell nuclei to the periphery.

FCL severity and serum biochemistry

Data was grouped according to their FCL severity respective to their serum biochemistry and liver lipid concentrations; to compare for differences in concentration based on their grouping Table 5. ALT in serum increased with increasing severity of FCL. Serum TAG and cholesterol levels were seen to decrease in cats with increasing FCL. However, the opposite trend was observed in liver TAG and cholesterol concentrations increasing in concentration with higher FCL severity. Generally, serum creatinine and urea concentrations increased from the FCL 2 group to FCL 3-5 group.

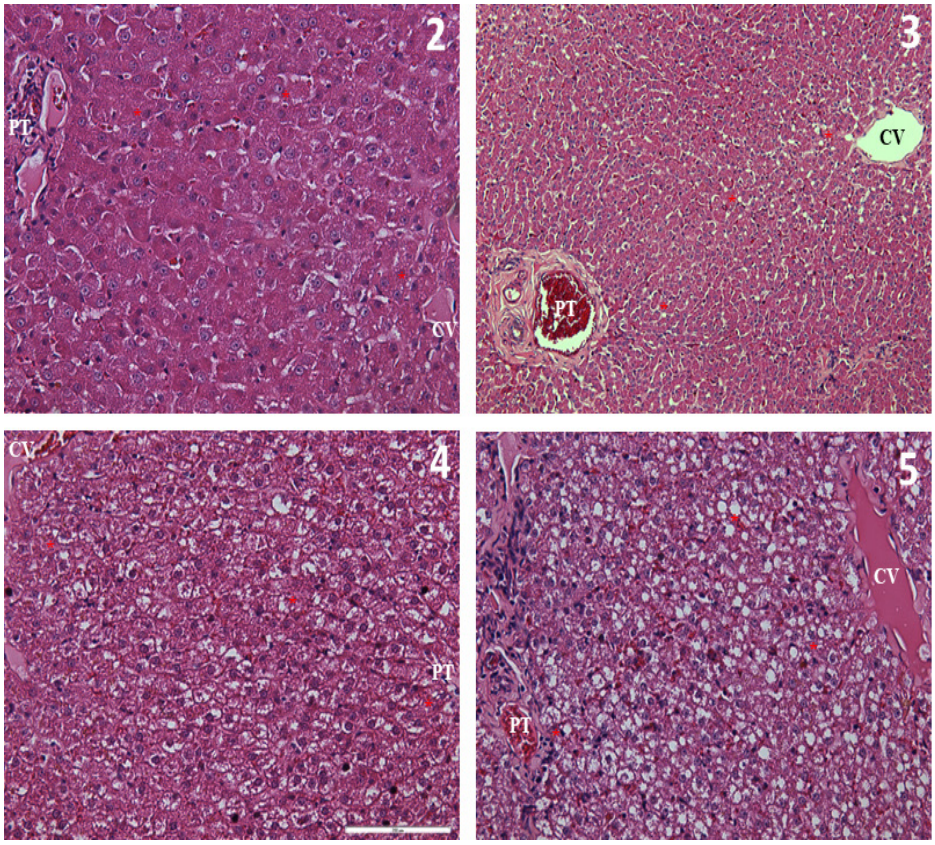


Figure X (a) – (d). Scoring lipidosis severity. Histological section from sample FC49 with GdL 4 severity (some vacuoles). Scale bar at 200µm.

FIGURE 1. Varying FCL severity in FHL observed by lipid droplet size and frequency in stray cat liver histological sections stained with H&E: (2) FCL 2: small number of lipid droplets observed, approximately 70-100 µm in diameter, (3) FCL 3: many lipid droplets observed, approximately 80-100 µm in diameter, (4) FCL 4: larger lipid droplets observed, approximately 80-120 µm in diameter, with deposition of lipid droplets into hepatocytes causing cell nuclei to be pushed to cell periphery, and (5) FCL 5: many larger lipid droplets observed, approximately 160-170 µm in diameter, with deposition of lipid droplets into hepatocytes causing cell nuclei to be pushed to cell periphery

FCL severity as numbered on sections (1-4). Lesions marked with red asterisks (*). Portal triad (PT), central vein (CV). Scale bar at 200µm

TABLE 5. FCL severity of stray cats compared to their serum biochemistry and liver lipid concentrations

Parameter	FCL severity		
	2 (Mean ± SD, n = 4)	3,4 (Mean ± SD, n = 3)	5 (Mean ± SD, n = 11)
Serum			
ALT, U/L	42.00 ± 34.51	61.33 ± 55.58	90.91 ± 65.51
TAG, mmol/L	1.24 ± 0.52	0.51 ± 0.22	0.71 ± 0.42
Chol., mmol/L	3.00 ± 0.54	2.37 ± 0.49	2.28 ± 0.77
Creat., µmol/L	56.25 ± 16.72	71.33 ± 6.11	66.73 ± 16.64
Urea, mmol/L	9.63 ± 1.28	8.87 ± 4.37	10.58 ± 6.97
Liver tissue			
TAG, pmol/µg	1.16 ± 1.17	1.09 ± 0.51	3.84 ± 3.54
Chol., pmol/µg	1.61 ± 0.60	2.14 ± 1.01	2.12 ± 1.25

SD: standard deviation; FCL: fatty change of liver; ALT: alanine aminotransferase; TAG: triglyceride; Chol.: cholesterol; Creat.: creatinine

ENERGY REGULATORY GENES AND FHL

Genes involved with energy regulation associated to lipid metabolism were investigated, specifically PPAR-δ and PPAR-γ. Average purity of extracted RNA samples was 2.11 based on 260/280 absorbance reading.

DISCUSSION

ELEVATED SERUM ALT PREDICTING FHL

From the 18 stray cats included in this study, four presented FCL 2, two presented FCL 3, one presented FCL 4 and 11 presented FCL 5. Notable observations were made regarding FCL severity. ALT concentrations increased concurrently with increasing severity of FCL (Table 5), signifying greater hepatic injury in more severe cases of FCL in the stray cats. The increased deposition of lipids into the liver of higher severity FCL cats (Figure 1) could trigger the production of ALT, widely accepted as a marker for hepatocellular damage. Detecting the expression of serum ALT is made more significant as it is a highly liver-specific enzyme found more abundantly in hepatocyte cytosol (Center 2007). Liver damage causes increased permeability of hepatocyte membranes leading to leakage of ALT into blood circulation (Washabau & Day 2013). ALT transfers ammonia to α-ketoglutaric acid (α-KG) generating pyruvate for gluconeogenesis (Center 2007), which may promote hepatic recovery while sustaining FHL. In line with hepatic recovery, ALT also functions in facilitating the movement of carbon and nitrogen, in the form of alanine, from body muscles to the liver. Protein synthesis and energy production can then occur, further promoting hepatic recovery, besides nitrogen elimination through urea discharge (Center 2007).

INVERSE LEVELS OF SERUM AND LIVER LIPIDS

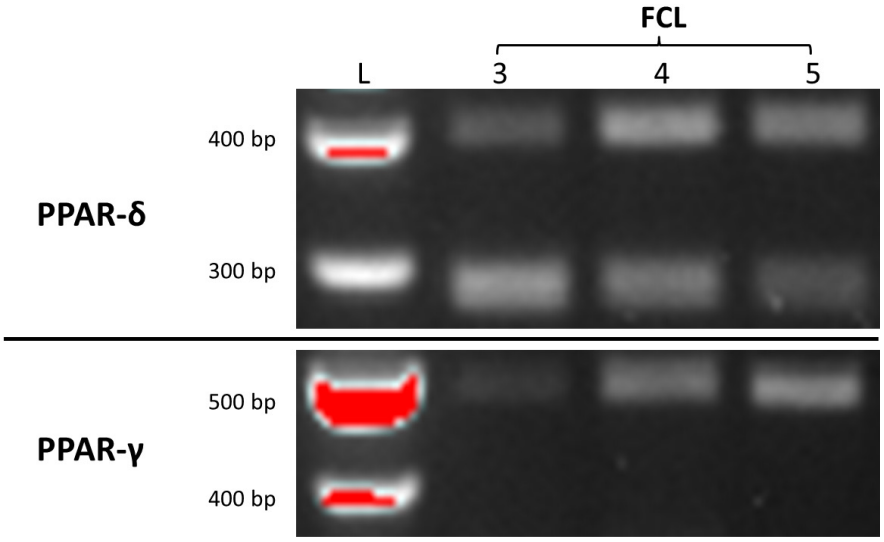
As FCL severity increased, serum TAG concentration decreased, however, the inverse trend was seen in the liver. Liver TAG concentration increased according to FCL severity. As the samples taken were of stray cats, they are mostly likely in a fasted or possibly malnourished state. In such conditions, adipose tissue of the cats would undergo lipolysis, circulating in the form of free fatty acids, reconstituted into the liver as TAG, and then exiting the liver in the form of very-low-density lipoproteins (VLDLs) (Softic et al. 2016; Verbrugghe & Bakovic 2013) choline, betaine, folate.

Serum and liver cholesterol concentrations were also seen to have an inverse concentration relationship against FCL severity. Serum cholesterol concentration seemed to decrease as FCL severity heightened, but its liver concentration was observed to increase with FCL severity. This observation contradicts a previous report by Center et al. (1993) where cholesterol levels were elevated in cats with severe FHL. However, Center reported jaundice in their study samples, a condition that was not observed in this study, which may explain the difference. This posits that the severity of FCL in this study was not as severe as the cases reported by Center. In a diet control study by Lawler et al. (2006), it was reported that total circulating cholesterol concentration was less influenced by environmental factors as compared to circulating TAG levels. The study by Lawler (2006), however, emphasised cholesterol heritability, looking into dietary restrictions and age, in contrast to this study which details liver physiology and its role in cholesterol metabolism.

The highest concentration of liver TAG and cholesterol in FCL 5 cats is similar to Hall et al. (1997) reporting 34% liver mass comprising of TAG in the liver of FHL cats as compared to 1% in the control group, whereas cholesterol esters was almost 17-times fold than in control.

SERUM CREATININE AND UREA PRESENT FUNCTIONING LIVER

In the Model for End Stage Liver Disease (MELD) serum creatinine is regarded as a significant factor. Serum creatinine is synthesised by the liver. With increased severity of FCL, comparing FCL 2 with FCL 3-5, Table 5 presented an increase of serum creatinine concentration. This contrasts values presented in impaired livers which would output low concentrations of creatinine, especially in cirrhosis (Cárdenas & Ginès 2009; Takabatake et al. 1989). As such, this may suggest severity of lipidosis in the stray cats has not reached chronic levels of hepatic dysfunction, even though the stray cats presented lipid deposition histologically and biochemically. Creatinine also functions as an analogue for body muscle mass (Thongprayoon et al. 2016) suggesting low concentrations of the enzyme to indicate malnutrition, muscle wasting and physical deconditioning (Ostermann et al. 2016). However, considering that the stray cats generally presented serum creatinine values within the normal range and even increased according to FCL severity, this may be a sign of inefficient glomerular filtration in the cats (Perrone et al. 1992). Renal pathologies have been studied relating elevated serum creatinine levels, chronic kidney disease (CKD), and NAFLD (Musso et al. 2014), with similar



L, ladder; bp, base pair

FIGURE 2. Agarose gel (2.5%) presenting gene expression of peroxisome proliferator activated receptor (PPAR)-δ and PPAR-γ, conventional PCR, from stray cat samples, with FCL 3-5, respectively

pathological markers investigating the two metabolic diseases (Kiapidou et al. 2020). This suggests a linkage between steatosis and renal pathologies.

High levels of urea were detected in the stray cats, among the contributing factors could be the unfavourable conditions they were kept in prior to sampling. As these cats were kept in animal pounds, they may have been stressed from the unfamiliar surroundings, individuals and other animals, and faced overpopulation, decreasing their water intake. Physiologically, FHL cats commonly present dehydration as a clinical sign (Armstrong & Blanchard 2009), similar to the elevated urea concentrations observed in this study (Table 5). However, diseased livers and cirrhosis patients have reported nearly 90% decrease in urea production (Mezey 1982). The liver metabolises urea and functions in the removal of hepatic nitrogen from the body, hence, damage to the liver impairs this ability (Glavind et al. 2016). Considering this, the stray cats present intact hepatic function as their urea production is slightly elevated from the normal range and may point towards hepatorenal complications.

PPAR-Γ AND PPAR-Δ IN FHL CATS

Expression of PPAR-γ was observed in the stray cats Figure 2, a gene associated with the repression of hepatic stellate cells (HSC) (Hinz et al. 2007). HSCs function in the storage of vitamin A and retinol, though, upon liver injury, HSCs are activated and differentiate into myofibroblasts. In this state, myofibroblasts contribute to fibrosis to recover the damaged liver. The expression of PPAR-γ in the stray cats then suggest that even at FCL 5 (considered the highest level of FHL severity in this study), damage through lipid deposition was not severe enough to induce myofibroblast differentiation. HSCs also function in lipid storage, containing retinol in their lipid droplets. As expression of the PPAR-γ gene was observed, it can be posited that the cats' HSCs are still functional in lipid storage.

Among other functions, PPAR-δ has been reported to increase glycolysis and pentose phosphate shunt activity, aimed to reduce hepatic glucose synthesis, however, its role in promoting hepatic lipogenesis (Tan et al. 2016) is of interest for this study. Liss and Finck (2017) reported elevated insulin signalling activity, reduced hepatic steatosis, and reduced gene expression for inflammatory responses upon PPAR-δ reaction with the of the GW0742 agonist. Kupffer and hepatic stellate cells, controllers of hepatic reactions to inflammation and fibrosis, express PPAR-δ (Tan et al. 2016). The link to steatohepatitis and PPAR-δ has also been observed in PPAR-δ -/- mice where the disease is made more severe due to failure of macrophages in the mice to transform M1 'killers' to M2 'healers' (Kang et al. 2009).

The nutritional needs of cats is also of concern in FHL as their main source of arginine stems from dietary intake and due to the cat's inability to downregulate urea cycle metabolism in conditions of malnutrition, have a higher requirement for arginine as compared to other species (Dor et al. 2018). Lacking of the amino acid affects immune response metabolism and in addition to ornithine deficiency, affects cellular proliferation, repair, and collagen biosynthesis (Ley 2017). In these stray cats undergoing malnutrition and FHL, lipidosis triggering inflammatory responses and macrophage action may induce detrimental consequences due to the uptake of insufficient arginine. Such consequences may include ammonia intoxication as the cat lacks the arginine required to mediate normal urea cycle metabolism (Valtolina & Favier 2017). Considering macrophage responses and their competing need for arginine, malnutrition faced by these stray cats may also hinder recovery of inflamed livers as macrophages become suppressed due to inadequate expression of inducible nitric oxide synthase (iNOS) (El-Gayar et al. 2003).

Liver recovery may further be supported by the presence of PPAR-γ expression (Figure 2). This gene induces the M1 to M2 macrophage monocyte shift (Orecchioni et al. 2019) where the M2 macrophages carry out elevated levels of metabolic glucose and fatty acid oxidation for energy production (Porta et al. 2015). Inducement of fatty acid oxidation may explain the elevated liver TAG concentration of the stray cats following the drop of serum TAG concentration with FCL severity (Table 5). TAG in cat serum was catabolised into fatty acids, reconstituted into the liver as TAG. The expression of PPAR-γ also posits ongoing tissue repair due to excessive hepatic lipid deposition where M2 macrophages utilise ornithine for collagen biosynthesis and cell proliferation (Ley 2017).

Conclusion

Supporting evidence with serum and liver biochemistry, as well as genetic factors that are linked to FHL have been presented in this study of stray cats. When grouped according to their FCL severity, trends can be observed reflecting increased FHL severity. In higher FCL groups, hepatic injury from excessive lipid deposition can be observed from elevated serum ALT levels. Serum TAG and cholesterol levels decreased with increasing FCL severity, exhibiting adipocyte lipolysis and concurrent fatty acid oxidation of the malnourished stray cats for energy, with the lipids reconstituting in the liver. PPAR-γ expression was reported, further supporting the occurrence of fatty acid oxidation and the decrease in serum TAG concentrations with increasing FCL. Expression of the gene also suggests induction of macrophage activity for hepatic recovery, supplemented by the expression of PPAR-δ, of which induces lipogenesis and inflammatory reactions. However, elevated serum creatinine and urea levels were observed with increasing FCL severity, a sign of intact hepatic function in its capacity to continue metabolising the enzymes and marks dehydration and probable catabolism of muscle tissue for energy supplementation.

Recommendations

Further studies investigating fatty acids can be conducted in relation to TAG and cholesterol concentrations. To further validate the presence of hepatic lipid deposition and inflammation, special stains can be used for histological analyses with oil red and trichrome staining respectively (Krishna 2013). Markers for FHL and related diseases can be further specified and optimised by conducting genetic sequencing of the feline liver profile.

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