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Review Article

Methionine Fermented Associated with Silybum Marianum is a Potential Hepatoprotective and Antioxidant in Cats with Inflammatory Liver Disease

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Abstract

The causes of almost all Feline inflammatory liver diseases have not been determined yet, but it is suspected that infectious agents or immune mechanisms may underlie the inflammatory response. In experimental liver disease models, methionine metabolites such as S-adenosyl-methionine have shown considerable hepatoprotective effects. Also Silybum marianum is considered to have hepatoprotective and antioxidant functions. The purpose of this study is to evaluate the hepatoprotective and antioxidant effects of fermented methionine associated with Silibinin in adult domestic cats affected by neutrophilic cholangitis. Twenty cats with neutrophilic cholangitis were enrolled in the study. Ten cats were daily orally administered with a formulation based on fermented methionine and Silybum marianum. Ten cats, served as control. Hematochemical, biochemical and oxidative stress parameters were evaluated at 0, 15, 30, 60 and 90 days. Leukocyte, Alkaline Phosphatase (ALP) and Alanine AminoTransferase (ALT) were lower ($p<0.05$) in T group than C group at day 90. Bilirubin, Gamma-Glutamyl Transferase (GGT) at days 30 and 90 were lower ($p<0.05$) in treated group than control group. In group T, ALT, AST and reactive oxygen metabolite derivatives values significantly decreased at day 90 ($P<0.05$) compared to T0. In the same group biological antioxidant potential levels significantly increase at the end of the treatment ($P<0.05$). These findings suggest that the formulation based on fermented methionine and Silibinin has two relevant effects on the defence of hepatocytes in cats with neutrophilic cholangitis, specifically, it acts against oxidative stress and inflammation. Further investigations are ongoing to confirm these preliminary results.

Introduction

Feline inflammatory liver disease includes a group of acquired inflammatory disorders such as cholangitis and less commonly hepatic parenchymal inflammation (hepatitis). The term cholangitis is preferred to cholangiohepatitis to describe an inflammatory liver disease. Cholangitis includes the histopathological abnormalities affecting the biliary tract with secondary, if any, involvement of the hepatic parenchyma. The World Small Animal Veterinary Association Standardization Committee (WSAVASC) recognizes three forms of feline cholangitis: Neutrophilic, lymphocytic, and chronic [1]. Liver biopsy and/or bile analysis (cytology and bacterial culture) are necessary for the final diagnosis of these pathologies [1].

Histopathological examination reveals lymphocytic hepatic infiltrates in the majority of cats with the lymphocytic form being mostly caused by an immune-mediated response [2]. On the other hand, the chronic form is associated with liver fluke [1].

Neutrophilic cholangitis is the most commonly reported form of cholangitis accounting for more than half (56.3%-90%) of inflammatory liver disease cases [1]. Most of the time, the development of this form is a consequence of a bacterial infection [3,4].

Hitherto, the use of antibiotics and supportive care is the elective treatment for the neutrophilic cholangitis in cats [1,3].

Several drugs, nutraceutical, and botanic extracts have cytoprotective properties. These products enhance natural defense mechanisms able to inhibit inflammation and fibrosis, to prevent apoptosis, or to protect against oxidant injuries maintaining an appropriate redox balance [5].

S-Adenosyl Methionine (SAMe), generated from L-methionine and ATP in a two-step reaction catalyzed by methionine adenosyltransferase, plays a central role in the trans-sulfuration process that generates glutathione [6,7]. This co-substrate is assumed to have anti-inflammatory and antioxidant effects [7]. Another agent is Silymarin, a flavonoid extracted from the milk thistle Silybum marianum [8]. It is composed of multiple flavonolignans including the most active

constituent the Silibinin (synonymous of silybin). This product has recently started to be considered as an effective treatment for a variety of diseases in human and veterinary medicine [9]. For example, it is used for the treatment of hepatobiliary disease having antioxidant, anti-inflammatory, and antifibrotic properties [5].

The purpose of this study is to evaluate the hepatoprotective and antioxidant effects of fermented methionine associated with Silibinin in adult domestic cats affected by neutrophilic cholangitis.

Materials and Methods

The study was performed at the Veterinary Clinic Napoli vet, in Naples (Italy) in the years 2015 and 2016. Owners' written consents were obtained before performing any procedure and all the samples were collected by the same clinician.

Experimental Animals

Twenty cats with neutrophilic cholangitis were enrolled in the study. The diagnosis of hepatic disease was based on clinical and radiographic signs, heapatic fine-needle aspirate cytology and hematochemical analysis.

All cats were treated with antibiotics (Amoxicillin-clavulanate SID 12.5-25 mg/kg) and intravenous fluid therapy, as a supportive care was used for rehydration and correction of electrolyte concentration.

The animals enrolled in this study, had a similar nutritional management consisting in high quality food with a highly digestible protein source as you can find in most of the commercially available gastrointestinal diets.

Ten cats (T group) were daily orally administered with a formulation based on fermented methionine and Silibinin (1 tab each 5kg b.w.). Ten cats, whose owners did not give consent for any supplemental therapy, were selected from the clinical database and used as control group (C group). The following criteria were used for case exclusion: concomitant metabolic disease or disorder potentially impacting liver functions (such as diabetes mellitus, gastritis, inflammatory bowel disease, chronic kidney disease, Cushing syndrome), and treatments with specific hepatoprotective products during the 30 days before the enrolment. Clinical condition, body weight, and body condition score were evaluated at 0 (T0), 15 (T15), 30 (T30), 60 (T60) and 90 (T90) days.

Sampling

Blood samples were collected from the jugular vein after an overnight fasting and divided into two tubes: a K3-EDTA anticoagulant tube for the whole blood, and a tube without anticoagulant for the serum. For a Complete Blood Count (CBC) a standard analytical device was used to evaluate: Hematocrit (HT), Hemoglobin (HG), Red Blood Cells (RBC), White Blood Cells (WBC), Neutrophils (N), Eosinophils (EO), Lymphocytes (LYM) at T0, T30 and T90 only. For the biochemical analysis an automated analyser was used to obtain creatinine (CREA), Blood Urea Nitrogen (BUN), Phosphorus (P), Total Proteins (TP), Albumins (ALB), albumin/globulin (A/G) ratio, Glucose (GLU), Alanine Transaminases (ALT), Alanine Aminotransferases (AST), Alkaline Phosphatases (ALP), Gamma-Glutamyl Transferase (GGT), Bilirubin (BIL) and Cholesterol (CHOL) values at T0, T15, T30 and T90.

The serum total oxidant levels and antioxidant capacity were assessed by d-ROMs (reactive oxygen metabolites derivatives) test and BAP (Biological Antioxidant Potential) test, respectively 10 at T0, T15, T30, T60 and T90 only for the T group.

Statistical Analysis

Data were analyzed using Kruskal-Wallis and Wilcoxon rank sum test using Graphpad Prism 7.02 (Graphpad Software©; San Diego, CA, USA). Test results were considered statistically significant when p-value < 0.05, whereas p-values < 0.10 represented a trend.

Results

Ten male and ten female cats with no differences in sex distribution between the T and C groups were included in the study.

Table 1: Hematochemical analyses: hematocrit (HT), hemoglobin (HG), red blood cells (RBC), white blood cells (WBC), neutrophils (N), eosinophils (EO), lymphocytes (LYM) data are expressed as median (minimum and *maximum)*; (a) indicate significant differences (P < 0.05); (b) indicate significant differences (P < 0.05) among experimental times within each group.

C group		HT	HG	RBC	WBC	N	EO	LYM
	Range	26-45%	8-15 g/dl	5-10 10^6mm^3	5.5-19 10^6mm^3	2.5-12 10^6mm^3	0- 1.5 10^6mm^3	1.5-7 10^6mm^3
T0		36,5	13,35	7,05	22,25	14,5	1,6	2,75
		(31,5-49,5)	(11,5-17,8)	(6,3-8,6)	(17,6-27,4)	(11,3-19,5)	(0,6-2,2)	(1,2-3,9)
T30		32,15	11,7	6,3	33,15	20,8	1,2	2,4
		(32,15-45,2)	(10,3-16)	(5,9-8,2)	(33,15-34,7)	(20,8-27,8)	(1,1-1,7)	(2,4-4,5)
T90		34,95	12,75	6,5	18,3	11,4	1,35	2,5
		(28,1-43,7)	(11-15,5)	(6-7,8)	(10,8-26,8)	(6,6-16,8)	(0,8-1,9)	(1,3-3,9)
C group		HT	HG	RBC	WBC	N	EO	LYM
	Range	26-45%	8-15 g/dl	5-10 10^6mm^3	5.5-19 10^6mm^3	2.5-12 10^6mm^3	0- 1.5 10^6mm^3	1.5-7 10^6mm^3
T0		35,35	11,5	6,7	23,6	17,5	0,95	1,65
		(27,5-54,4)	(10,3-16,9)	(5,9-10,5)	(12,9-32,9)	(7,7-27,9)	(0,7-1,6)	(0,7-3,1)
T30		34,3	11,8	6,45	19	12,8	1,3-5	2,3
		(31,2-48,4)	(9,5-16,5)	(6,1-8,9)	(14,9-29,9)	(8,6-19,7)	(0,6-1,7)	(1,4-3,7)
T90		38,85	13,5	7,4	13,25 [a, b]	9 [a, b]	1,4	1,9-5
		(32,2-44,2)	(10,3-16,1)	(5,9-8,6)	(9,3-15,3)	(6,4-9,8)	(0,9-2,1)	(1,1-2,6)

Table 2: Biochemical analyses: creatinine (CREA), blood urea nitrogen (BUN), phosphorus (P), total proteins (TP), albumins (ALB), albumin/globulin (A/G) ratio, glucose (GLU), alanine transaminases (ALT) alanine aminotransferases (AST), alkaline phosphatases (ALP), Gamma-glutamyl transferase *(GGT)*, bilirubin (BIL) and cholesterol (CHOL) data are expressed as median (minimum and *maximum);* (a) indicate significant differences (P < 0.05); (b) indicate significant differences (P < 0.05) among experimental times within each group.

C group		BUN	CREA	TP	ALB	A/G	CHOL	BIL	GLU	ALT	AST	ALP	GGT
	range	20-50 mg/dl	0,5-2mg/dl	6-8mg/dl	2,2-3,5mg/dl	0,8-1,3mg/dl	70-150mg/dl	0-0,5mg/dl	60-120mg/dl	7-40Ul/lt	7-40Ul/lt	7-50Ul/lt	0-10 Ul/lt
T0		31,5	1,2	7,3	3,25	0,95	66,5	3,45	132	213	130,5	220	18,5
		(21-54)	(0,9-1,5)	(5,6-8,6)	(2,7-4,2)	(0,7-1)	(43-100)	(1,9-4,9)	(56-210)	(118-339)	(66-262)	(181-421)	(12-35)
T15		27,5	1,25	6,9	2,95	0,9	56	4,4	107,5	349,5	185,5	362,5	28
		(18-46)	(0,90-1,99	(5,5-8,6)	(2,50-3,9)	(0,70-1)	(44-86)	(2,20-7,5)	(69-157)	(165-571)	(121-379)	(247-644)	(12-45)
T30		31,5	1,25	6,75	3	0,9	65,5	4	94	319,5	159,5	494	26,5
		(31-53)	(1,2-1,9)	(6,7-58,2)	(2,5-3,8)	(0,8-1)	(47-94)	(1,60-8,8)	(87-110)	(133-550)	(89-277)	(237-683)	(13-34)
T60		30	1,3	6,55	3	0,9	82,5	3,4	93	253,5	131,5	436	22,5
		(23-44)	(1,1-1,5)	(6-8,1)	(2,8-3,8)	(0,8-1)	(48-93)	(1,1-8)	(77-129)	(102-573)	(105-238)	(215-695)	(11-35)
T90		29,5	1,25	6,7	3,1	0,9	72,5	2,2	90	226	114,5	442,5	15,5
		(21-55)	(1,1-1,6)	(5,9-7,8)	(2,8-3,6)	(0,9-1)	(55-104)	(1-6,1)	(80-126)	(82-551)	(74-234)	(184-620)	(11-28)
T group		BUN	CREA	TP	ALB	A/G	CHOL	BIL	GLU	ALT	AST	ALP	GGT
	range	20-50 mg/dl	0,5-2mg/dl	6-8mg/dl	2,2-3,5mg/dl	0,8-1,3mg/dl	70-150mg/dl	0-0,5mg/dl	60-120mg/dl	7-40Ul/lt	7-40Ul/lt	7-50Ul/lt	0-10 Ul/lt
T0		34,5	1,25	7,45	3,4	0,95	51,5	3,6	82,5	218,5	161,5	211	15,5
		(12-88)	(0,8-1,8)	(6,5-9,2)	(3,2-4,4)	(0,7-1)	(32-109)	(2,3-8,4)	(66-229)	(94-439)	(87-451)	(155-553)	(9-25)
T15		35	1,4	7,35	3,1	0,9	55	5,1	91	334	198	291	14,5
		(15-67)	(0,91,9)	(6,28,8)	(2,84)	(0,7-1)	(33-93)	(1,87,3)	(69-167)	(177-488)	88-329)	(201-893)	(8-34)
T30		34	1,3	7,45	3,45	0,9	69	3,2 [a]	94,5	232	165,5	373,5	12,5 [a]
		(15-53)	(1,1-1,9)	(6,5-8,4)	(2,8-3,8)	(0,8-1)	(29-128)	(1-5,1)	(83-115)	(102-449)	84-429)	(119-480)	(8-24)
T60		37	1,3	7,4	3,45	0,9	76,5	1,6 [a]	99	166,5	117	273	12:00 AM
		(16-55)	(1,1-1,8)	(6,3-8,3)	(3-3,6)	(0,8-1)	(18-110)	(0,9-2,9)	(89-128)	(67-452)	45-381)	(98-373)	(7-19)
T90		32	1,25	7,4	3,7	1	99,5	0,7 [a, b]	92,5	82,5 [a, b]	74 [b]	188 [a]	9 [a, b]
		(13-43)	(0,9-1,7)	(6,9-7,9)	(3,1-3,7)	(0,9-1)	(43-122)	(0,3-2,1)	(81-105)	(32-231)	(34-238)	(78-290)	(6-13)

The majority of cats enrolled were domestic shorthair (13/20, 65%), other breeds were Persian (5/20, 25%), Chartreux Cat (1/20, 5%) and British Shorthair (1/20, 5%). The mean age for the cats belonging to the T group was 8 yr (range, 2-15 yr) and the body weight was 4.3±0.9 kg [Mean±Standard Deviation (SD)]. In the C group, the mean age was 9yr (range, 3-12 yr) and the body weight was 4.4±0.8 kg [Mean±Standard Deviation (SD)]. All animals completed the investigation. No adverse systemic effects were observed under the treatment based on clinical conditions and clinical pathology evaluation.

No significant differences were found between T and C groups at day 0 for hematochemical and biochemical analyses (Table 1-2). Comparing the CBC results between the two groups, a statistically significant reduction was found in the number of circulating neutrophils and leukocytes in the blood at T90 ($p<0.05$). Biochemical analysis revealed that ALP and ALT were lower in the T group than in the C group at T90 ($p<0.05$). Bilirubin and GGT at T30 and T90 were lower in the T group than in the C group ($p<0.05$).

In the T group, CBC results underlined significant differences between neutrophils and leukocytes at T90 compared to T0 ($P<0.05$). Bilirubin, ALT, AST, GGT and d-ROMs values significantly decreased at T90 compared to T0 ($P<0.05$). In the same group BAP levels significantly increased at the end of the treatment ($P<0.05$).

Discussion

The purpose of this study is to determine whether the administration of fermented methionine associated with Silibinin has a hepatoprotective activity in cats with neutrophilic cholangitis.

Our haematological and serum biochemical results can be considered typical for neutrophilic cholangitis disease cases. For example, common serum biochemistry findings in our samples were: increased activities of ALT, ALP, AST and increased concentration of total bilirubin and GGT [11]. Interestingly, these biochemical markers of hepatic injury showed a significant decrease following fermented methionine and Silibinin administration.

The d-ROMs test performed to obtain the ROS level in our cats has been already used for testing samples from several animal species, including mammalians [12, 13, 14, 10]. Although no data on cats has been published yet, some authors believe that reference values for dogs could be used for cats as well [15,10]. In our study, at the beginning of the treatment, animals in group T showed a high ROS level that significally decreased at the end of the treatment. The high ROS level

could be justified by an increased production of oxidant species and/or a decreased efficacy of the antioxidant system, while the decrease of ROS level could be due to the efficacy of our formulation.

Additionally, it has been demonstrated that the concentration of glutathione, an important endogenous antioxidant, is significantly lower in livers of cats with naturally occurring hepatic disease compared with healthy cats [16].

In our study, we used a formulation composed of two products with different effects on glutathione but unfortunately the level of glutathione did not detected in animals.

The first product, the fermented methionine, has cytoprotective activities including: augmentation of hepatocyteglutathione levels, improvement in membrane fluidity, modification of cytokine expression, alterations in DNA/histone methylation, and modulation of apoptosis [17]. The second one, the Silymarin, induces hepatic synthesis of glutathione by increasing cysteine availability [18]. This effect could be considered as a potential antioxidant mechanism in addition to the radical scavenging. Experiments in vitro showed how the combination of SAMe and Silymarin could be used for liver health support. These tests were also performed to study the characterization of the biological activity of this combination of products. Unfortunately, little is known about the combined effects of SAMe and Silymarin in vivo [19]. In literature, only a few studies investigated the actions of SAMe and Silymarin in cats but none of them reported their use in association [20, 21, 22]. The two products seem to protect hepatic cells from the typical effects of oxidative stress and damage that usually follow cholangitis. Furthermore, it is relevant to underline that the anti-inflammatory properties of our formulation were confirmed by the decrease in number of neutrophils and leukocytes in the T group at the end of the treatment.

Conclusions

These findings suggest that the formulation based on fermented methionine and Silibinin has two relevant effects on the defence systems of hepatocytes in cats with neutrophilic cholangitis, specifically, it acts against oxidative stress and inflammation. Further investigations are ongoing to confirm these preliminary results.

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