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Changes in Plasma Metabolites Concentrations in Obese Dogs Supplemented With Anti-oxidant Compound

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The aim of this study is to discuss the effect of anti-oxidant supplement (Rv-PEM01-99, Kibun Foods, Inc., Tokyo, Japan) on changes in energy metabolism in obese dogs. 200 mg/kg/day of Rv-PEM01-99 (equivalent to 5 mg kg/day of quercetin derivative) were applied for 6 weeks to the Beagle dogs fed high fat diet (HFD) or control diet (CD). In the present study, body weight (BW) decreasing effect of Rv-PEM 01-99 in obese dogs was not clear. However, plasma alkaline phosphatase (ALP) activities at the end of experiment were significantly decreased compared to those at the start of experiment in obese dogs supplemented with Rv-PEM 01-99 (paired-*t* test, $p < 0.05$). In control dogs supplemented with Rv-PEM 01-99, Plasma malondialdehyde (MDA), and triglycerides (TG) levels and lactate dehydrogenase (LDH) activities were significantly decreased compared to those at the start of experiment (paired-*t* test, $p < 0.05$). From these findings, Rv-PEM 01-99 seems to be not harmful for dogs. Anti-lipid peroxide effect and liver function improvement are expected in the dogs supplemented with Rv-PEM 01-99.

Keywords: anti-oxidant, dog, lipid metabolism, liver function, *Rhus verniciflua*

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INTRODUCTION

Recently, occurrence of obesity and its related metabolic disorders have increased in dogs and cats as in humans (1, 2). Obesity accompanied by visceral fat accumulation causes insulin resistance and is a risk factor for metabolic syndrome, diabetes mellitus, hypertension, dyslipidemia, cardiovascular diseases, musculoskeletal disorders, and some forms of cancer (3, 4). Obesity is defined as systemic low grade inflammation (5). Some of the inflammatory cytokines, such as tumor necrosis factor-α(TNF-α), IL-1β, and IL-6, and C-reactive protein (CRP) are released from adipose tissue in obese animals. TNF-α not only promotes inflammation, but also induces insulin resistance by inhibiting insulin receptor activation. IL-1β, and IL-6, C-reactive protein also causes insulin resistance (6). In obesity animals, oxidative stress, which results from an imbalance between the amount of pro-oxidants and antioxidants, also contributes to a cause of insulin resistance (6). Leptin (LP) and adiponectin (ADN) are secreted from adipose tissue in obese animals. LP increases serum TNF-α and IL-6 concentrations and induces insulin resistance while ADN has anti-inflammatory properties and decreases the release of proinflammatory mediators (7).

High concentrations of circulating non-esterified fatty acid (NEFA) from accumulated visceral fat is one of characteristics of lipotoxicity (8, 9). As such, large amount of circulating NEFA from excessive accumulated visceral fat induces oxidative stress and inflammation (10, 11). In obese animals, β-oxidation of fatty acids in mitochondria is markedly activated in various tissues and excess amount of reactive oxygen species (ROS) is produced. Overproduced ROS is attributed to the one of pathogens for obesity and its associated inflammatory diseases (12). Consequently, some antioxidants and anti-inflammatory supplements are thought to be effective for ameliorating obesity conditions in animals as well as human (13–15).

Obesity is caused by over calorie intake and physical inactivity. Diet therapy is the most effective to prevent obesity in dogs and cats. Recently some phytogenics are reported to prevent high-fat diet-induced lipotoxicity in animals (16, 17). Such phytogenics have anti-oxidant and anti-inflammation effects (18, 19).

In the present study, we supplied obese dogs fed high-fat diet with plant mixture having anti-oxidant effect, and measured changes in concentrations of metabolites and hormones, and activities of enzymes in plasma of the dogs. The aim of this study is to discuss the effect of anti-oxidant supplement on lipid metabolism in obese animals.

MATERIALS AND METHODS

Animals

Twenty healthy Beagles [average age: 2 years (1–3 years), average body weight (BW):10.4 kg (9.4–11.3 kg), average body condition score (BCS) of five scale: 2.4 (2–3)] were enrolled in this study. Prior to the study, 20 healthy beagle dogs were kept in controlled condition and were fed on control diet (CD) for 2 months. Then, they were randomly divided into four groups. Ten dogs of control group were provided CD. For the five out of 10 CD dogs, 200 mg/ kg/day of anti-oxidant compound (Rv-PEM01-99, Kibun Foods, Inc., Tokyo, Japan) were supplemented. Another group of 10 dogs were provided high fat diet (HFD). For the five out of 10 dogs in HFD group, 200 mg/ kg/day of anti-oxidant compound (Rv-PEM01-99, Kibun Foods, Inc., Tokyo, Japan) were supplemented.

All the dogs were kept individually in cages measured 54 cm(height) × 45 cm (wide) × 72 cm (depth) and provided the same condition for 6 weeks at Narita Animal Science Laboratory Co., Ltd. (Narita, Japan), with the environment maintained at 24.0 ± 2.0°C and 55.0 ± 10.0% relative humidity, and on a 12:12 h, light: dark cycle (light on 8:00 a.m. to 8:00 p.m.).

Abbreviations: ADN, adiponectin; AMPK, AMP-activated protein kinase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCS, body condition score; BUN, blood urea nitrogen; BW, body weight; CD, control diet; CRE, creatinine; GLU, glucose; HFD, high fat diet; INS, insulin; LDH, lactate dehydrogenase; LP, leptin; MDA, malondialdehyde; NEFA, non-esterified fatty acid; SE, standard error; TC, total cholesterol; TP, total protein; TG, triglyceride TNF-α; tumor necrosis factor alpha.

TABLE 1 | Comparison of compounds between control and high fat diet.

	Control diet	High fat diet
Metabolic energy (kcal/100g)	339	403
Water (%)	8.7	6.1
Protein (%)	21.7	20.3
Lipid (%)	10.1	21.4
Linoleic acid (%)	1.72	2.55
Arachidonic acid (%)	ND	0.04
Decanoic acid (%)	ND	ND
Lauric acid (%)	ND	0.02
Myristic acid (%)	ND	0.39
Myristoleic acid (%)	ND	0.11
Pentadecanoic acid (%)	ND	0.04
Palmitic acid (%)	ND	5.37
Palmitoleic acid (%)	ND	0.58
Heptadecanoic acid (%)	ND	0.13
heptadecenoic acid (%)	ND	0.11
Stearic acid (%)	ND	2.42
Oleic acid (%)	ND	9.37
Linolenic acid (%)	ND	0.13
Arachidic acid (%)	ND	0.04
Icosenoic acid (%)	ND	0.09
Eicosapentaenoic acid (%)	ND	ND
Behenic acid (%)	ND	ND
Docosahexaenoic acid (%)	ND	ND
Crude fiber (%)	2.7	3.9
Nitrogen free extracts (%)	50.5	42.9
Ash (%)	6.3	5.4

ND, not detected.

Induction of Obesity With High-Fat Diet in Dogs

HFD was designed by Nippon Pet Food, Co., Ltd. Differences in compounds between HFD and CD were as shown in **Table 1**. Calories in HFD were calculated as 403 kcal/100g food and those in CD food were calculated as 339 kcal/100 g food. HFD group dogs were given 38.8 kcal/kg /day of HFD, CD group dogs were given 30.8 kcal/kg/day of CD, respectively.

Supplementation With Plant Mixture

We used Rv-PEM01 prepared by Kibun Foods, Inc., Tokyo, Japan as antioxidant compound. Amount of each herb used for extraction (g) was as follows: *Rhus verniciflua* (90), *Ulmus hollandica* (60), *Polygonatum sibiricum* (50), *Lycium chinense* (10), *Ganoderma japonicum* (10), *Panax ginseng* (10) Total (230). Each of the six herbs was ground and added to the compound mixture (total volume is 230 g), which was then extracted with 10 vol of 70% ethanol in water at room temperature. The extracted solution was filtered, and the solvent was evaporated, filtered and lyophilized, yielding 60 g of lyophilized Rv-PEM01 from 230 g of herb powder (20). Urushiol, an allergenic substance found in *Rhus verniciflua* was completely removed. Two hundred

milligrams of Rv-PEM01 is equivalent to 5 mg of quercetin derivative.

Body Weight (BW) and Body Conditioning Score (BCS) Measurement

BW and BCS were measured at the time of each blood sampling. Each subject was evaluated by the same veterinarian on-site, and classified by a 5-point scale system (1) Very thin, (2) Underweight, (3) Ideal, (4) Overweight, and (5) Obese, commonly used in Japan (21).

Blood Sampling

Five milliliters of each blood was collected from the jugular vein of each animal into the heparinized tubes before the experiment (0 week) and at the end of experiment (6 week).Blood collection was performed before the morning feeding and collected sample were immediately centrifuged at 2,000 x g for 10 min, 4°C. These samples were stored at −80°C until use.

Metabolite, Hormone, and Enzyme Analysis

Glucose (GLU), total cholesterol (TC), triglyceride (TG), total protein (TP), blood urea nitrogen (BUN), creatinine (CRE) concentrations and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) activities were measured using an auto-analyzer (JCA-BM2250, JEOL Ltd., Tokyo, Japan) with the manufacture's reagents at FUJIFILM Monolith Co., Ltd (Tokyo, Japan). Plasma non-esterified fatty acid (NEFA) concentration was measured using a commercial kit, NEFA-C test (Wako Pure Chemical Industries, Inc., Tokyo Japan). Plasma insulin (INS), adiponectin (ADN), and tumor necrosis factor alpha (TNFα) were measured with Rat Insulin ELISA KIT(TMB) (AKRIN-010T, Shibayagi Co., Gumma, Japan), mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), TNF Alpha Dog Elisa kit (LS-F1347-1, Life Span Bioscience, Inc, Seattle, USA), respectively.

Leucocytic AMP-activated protein kinase (AMPK) activity was measured with a commercial ELISA kits, CycLex AMPK Kinase kit (CycLex Co., Ltd. Nagano, Japan).

The AMPK activity was measured at 30°C for 30min and was expressed as ng of phosphorylated substrates per minute per mg of protein (specific activity).

Protein concentration in white blood cell cytosol fraction was measured with the Bradford method using bovine serum albumin as the standard (22).

Statistics

All values are expressed as mean ± standard error (SE). Statistical significance was determined by paired-*t* test. The significance level was set at $p < 0.05$.

RESULTS

Changes in Body Weight (BW)

Changes in mean BW (kg) ±SE are shown in **Tables 2**, **3**. At the end of the experiment (6 weeks), animals of control diet (CD) group, high fat diet (HFD) group, CD+Rv-PEM01-99 group

TABLE 2 | Changes in biomarker levels in obese dogs fed with high fat diet.

	Without Rv-PEM01-99		With Rv-PEM01-99	
	0 W	6 W	0 W	6 W
BW (Kg)	10.4 ± 0.3	12.8 ± 0.4	10.5 ± 0.3	12.9 ± 0.4
INS (ng/mL)	0.3 ± 0.1	0.6 ± 0.2	0.1 ± 0.0	0.4 ± 0.1
Adipo (μg/mL)	27.9 ± 6.5	38.8 ± 7.8	21.3 ± 2.8	25.9 ± 3.9
AMPK (ng/mg protein min)	8.7 ± 0.8(3)	13.2 ± 0.9	12.7 ± 2.5(4)	13.7 ± 2.6
TNFα (pg/mL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
NEFA (mEq/L)	0.69 ± 0.05	0.61 ± 0.12	0.60 ± 0.07	0.60 ± 0.12
TG (mg/dL)	36.0 ± 1.2	78.8 ± 17.2	32.4 ± 3.7	59.2 ± 14.9
MDA (μmol/L)	1.14 ± 0.10	2.00 ± 0.40	1.39 ± 0.11	1.60 ± 0.29
TC (mg/dL)	120.4 ± 11.0	199.6 ± 29.5*b	139.2 ± 14.1	181.0 ± 10.6*a
AST (IU/L)	26.8 ± 0.4	32.4 ± 2.7	25.6 ± 0.4	32.8 ± 2.6
ALT (IU/L)	40.0 ± 2.4	33.0 ± 2.2	42.0 ± 2.7	41.4 ± 4.7
ALP (IU/L)	154.6 ± 16.6	141.2 ± 15.6	156.0 ± 13.7	85.2 ± 4.2*a
LDH (IU/L)	88.6 ± 12.8	82.4 ± 8.2	71.6 ± 10.5	70.6 ± 2.7
TP (g/dL)	6.7 ± 0.1	7.2 ± 0.2*b	6.4 ± 0.1	6.6 ± 0.1
BUN (mg/dL)	12.6 ± 0.7	17.4 ± 1.2*b	13.8 ± 0.6	12.2 ± 1.7
CRE (mg/dL)	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
GLU (mg/dL)	91.6 ± 2.9	93.6 ± 1.4	91.6 ± 3.1	94.8 ± 3.0

Data are presented as mean ± SE.

The numbers in parenthese indicate the number of animals examined.

*[*a] Significant ($p < 0.05$) when compared against 0 W of High Fat Diet +Rv-PEM01-99group (paired-T test).*

*[*b] Significant ($p < 0.05$) when compared against 0 W of High Fat Diet group (paired -T test).*

TABLE 3 | Changes in biomarker levels in control dogs fed with control diet.

	Without Rv-PEM01-99		With Rv-PEM01-99	
	0 W	6 W	0 W	6 W
BW (Kg)	10.5 ± 0.2	11.3 ± 0.3	10.3 ± 0.3	10.9 ± 0.5
INS (ng/mL)	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0
Adipo (μg/mL)	18.4 ± 4.4(4)	27.5 ± 3.7	28.3 ± 6.8	30.9 ± 4.2
AMPK (ng/mg protein min)	6.8 ± 1.3	11.1 ± 1.5	9.5 ± 4.8(4)	11.5 ± 1.2
TNFα (pg/mL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
NEFA (mEq/L)	0.73 ± 0.08	0.68 ± 0.12	0.83 ± 0.17	0.95 ± 0.18
TG (mg/dL)	30.6 ± 1.4	38.0 ± 7.4	40.2 ± 3.5	25.4 ± 3.5*a
MDA (μmol/L)	1.47 ± 0.19	1.46 ± 0.10	1.49 ± 0.14	0.98 ± 0.03*a
TC (mg/dL)	132.8 ± 3.7(4)	131.6 ± 10.0	118.8 ± 7.1	133.2 ± 6.8
AST (IU/L)	28.8 ± 0.4	35.2 ± 2.7	26.4 ± 0.4	35.8 ± 1.4
ALT (IU/L)	40.5 ± 2.9	30.2 ± 5.9	44.4 ± 4.4	42.0 ± 2.4
ALP (IU/L)	152.0 ± 4.3	166.6 ± 40.2	154.4 ± 4.4	160.8 ± 26.4
LDH (IU/L)	83.4 ± 6.3	113.6 ± 14.1	95.6 ± 7.6	60.8 ± 6.0*1
TP (g/dL)	7.2 ± 0.1	7.0 ± 0.2	6.4 ± 0.0	6.4 ± 0.0
BUN (mg/dL)	12.8 ± 0.7	12.0 ± 0.8	14.6 ± 0.2	13.8 ± 1.5
CRE (mg/dL)	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
GLU (mg/dL)	85.0 ± 1.4	79.8 ± 2.4	98.4 ± 2.7	95.6 ± 2.2

Data are presented as mean ± SE.

The numbers in parenthese indicate the number of animals examined.

**a Significant ($p < 0.05$) when compared against 0 W of Control Diet +Rv-PEM01-99group (paired-T test).*

(CD+Rv-PEM01-99) and HFD+Rv-PEM01-99 group (HDF+Rv-PEM01-99) showed 7.6, 23, 5.8, 22.9% of BW increase compared to the pre-experiment (0 week) BW, respectively. Compared to CD group, CD+Rv-PEM 01-99 group showed fewer increase in BW (5.8-percent gain) than CD group (7.6-percent gain) while HFD+Rv-PEM 01-99 group showed no changes in BW compared to HFD group over the 6 weeks of the experiment. Significant changes in BCS of all groups were not observed during the experiment.

Changes in Plasma Metabolites

Over the 6 weeks of the experiment, dogs in CD+Rv-PEM01-99 group showed significantly decreased TG concentrations (paired-*t*, $p < 0.05$) while dogs in HFD+Rv-PEM01-99 group showed increased TG concentrations. Plasma MDA levels in CD+Rv-PEM 01-99 group were significantly decreased (MDA: 0.98 ± 0.03 μmol/L). Although obese dogs in HFD group showed significantly increased BUN concentrations, obese dogs in HFD+Rv-PEM01-99 group showed decreased BUN concentrations at the end of the experiment. Plasma TNF-α activities in all group were not observed during the experiment. Plasma NEFA levels in all groups were increased within the healthy normal ranges.

Changes in Plasma Hormones

Although not significant, ADN concentrations in obese dogs fed HFD group were increased compared to those in control dogs at the end of the experiment. Plasma INS and ADN concentrations in all groups increased within the healthy normal ranges as AMPK activities in WBC cytosolic increased.

Changes in Plasma Enzymes

At the end of experiment, LDH activities in CD group were increased. However, LDH activities in control dogs supplemented with Rv-PEM 01-99 were significantly decreased (60.8 ± 6.0 IU/L). Significant changes in LDH activities in obese dogs were not observed.

DISCUSSION

In dogs and cats, the availability of functional food for weight loss, against behavioral disorders, and chronic inflammation, has been discussed by many researchers (23–28), and the role of supplements with anti-oxidant and anti-inflamatory effect has been reported (29, 30) There are many reports discussing anti-obesity effects of anti-oxidant polyphenols such as isoflavone (31–33) anthocyanin (34–36), catechin (37–39), and quercetin (40–42). Especially, quercetin glycoside is a well-known compound since its metabolic pathway route in human intestine is clarified (43). Yoshimura et al. demonstrated body fat reducing effect of quercetin glycosides on obese humans (44, 45). In our preliminary study, we observed BW decrease in mouse fed High fat +2%Rv-PEM 01-99 for 60 days. To investigate anti-obesity effect of Rv-PEM 01-99 in dogs, we developed obese dogs by feeding HFD for 6 weeks.

At the end of experiment, obese dogs fed HFD showed 23% BW increase and increased TC, TP, BUN concentrations. Although many researcher reported decreased serum/plasma ADN concentrations in obese animals (5, 46–48), plasma INS and ADN concentrations were increased in obese dogs fed HFD. Furthermore, obese dogs fed HFD seemed to show

no inflammation since changes in plasma TNF-αlevels were not observed. The activities of AMPK, an energy metabolic parameter, in WBC cytosol, were increased accompanied by plasma ADN increase. As we have previously proposed in cats (49, 50), the above mentioned conditions indicate no pathology, but a healthy state seen at an early stage of weight gain in dogs.

As shown in **Table 3**, since plasma TG and MDA levels, LDH activities were significantly decreased at the end of experiment in control dogs supplemented Rv-PEM 01-99, anti-oxidant effect and improvement effect of liver function and lipid metabolism are expected in healthy dogs by use of Rv-PEM 01-99.

In the present study, anti-obesity mechanism of Rv-PEM 01-99 in dogs was not clear.

As shown in **Table 1**, high fat food we used was specially designed to increase canine body weight by adding saturated fatty acid such as palmitic acid, stearic acid which contain in beef fallow, while control food we used was a commercial diet and was designed to meet the amount of lipid and linoleic acid for canine growth requirement. From our data as shown in **Tables 2**, **3**, we did not induce pathological obese condition by feeding HF food for 6 weeks. Therefore, it is thought that anti-obesity efffect of Rv-PEM 01-99 in dogs was not clear.

Yoshimura et al. (44, 45) administered 110–275 mg of enzymatically modified isoquercitrin for 12 weeks to human to investigate anti-obesity effect. *Rhus verniciflua* leaf contains physiologicall active substances such as fustin, fisetin, sulfuretin, qurercetin, and butein. (51). It was demonstrated that quercetin showed anti-oxidant (52), anti-inflammatory (53), anti-arteriosclerosis (54), anti-tumor (20, 55–57), anti-hypertension (58), and vascular atony effects (59). Simizu et al. have demonstrated the regulatory mechanism of apolipoprotein gene transcription by quercetin on the inhibition of cylomicron synthesis in human small intestine (60).

This study is a preliminary investigation. To discuss the effectiveness of Rv-PEM 01-99 for obese dogs, we have to design new experiment (1) enrolling many dogs, (2) using larger amounts of Rv-PEM 01-99, and (3) taking longer experimental period since Christmann et al. admitted effectiveness of weight management/loss food by use of 38–162 dogs for 6 months (24, 27).

ETHICS STATEMENT

Ethical approval for this study was from Narita Animal Science Laboratory Co., Ltd. Research Animal Ethical Committee (17-C042).

AUTHOR CONTRIBUTIONS

TA conceived and designed the study. KK collected data with TMu and TMi. YO and IY performed critical analysis and interpretation of data with KK. KS and KK designed Rv-PEM01-99. KK and YO wrote the manuscript.

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Conflict of Interest Statement: KS and KK were employed by company Kibun Foods Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.