

# Effects of supplementation with Shiitake powder, Lentinula edodes, on anti-oxidative activities and energy/ lipid metabolism in healthy dogs

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## Research Article

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# Abstract

Obesity is the number one health problem pets face. Effective food and supplement should be developed to improve anti-oxidative activities and energy /lipid metabolism in obese animals. In this study, we measured metabolites and hormone concentrations and enzymes activities related to anti-oxidant activities and energy /lipid metabolism in plasma of healthy dogs supplemented with shiitake powder to investigate effects of its supplementation on anti-oxidative function and energy /lipid metabolism in healthy dogs. Shiitake powder was supplemented with healthy dog at the high dose (800mg/kg body weights/day) and low dose (100mg/kg/day) for 4 weeks. In dogs supplemented with shiitake powder at the high dose, plasma SOD activities increased significantly and total cholesterol concentrations decreased and sirtuin1 mRNA expression increased in peripheral leukocytes. Plasma ALP and LDH activities tended to decrease in the high dose group. These reactions seemed to reflect of improvement of energy metabolism and hepatic function. Shiitake powder may ameliorate lipid metabolism in canine liver, and may improve anti-oxidative function and energy/lipid metabolisms in healthy dogs. To evaluate the usefulness of shiitake powder for obese dogs, further studies using overweight and obese dogs are needed. (186 words)

## Introduction

Obesity is one of the most widespread problems facing our society's health today. According to a report from World Health Organization (WHO), worldwide, the prevalence of obesity has nearly tripled since 1975, with 39% of the world's adults being overweight and 13% being obese in 2016 (World Health Organization 2019). Prevalence of obesity in dogs and cats has increased accompanying with aging in these years as in humans (Chandler et al. 2017; Okada et al. 2017). Obesity is a major risk factor for insulin resistance and type 2 diabetes caused by an imbalance between energy consumption and expenditure that leads to lipid accumulation (Stein and Colditz 2004). Ectopic accumulated visceral fat induces inflammation, mitochondrial dysfunction, hyperinsulinemia, and endoplasmic reticulum stress (Yazici and Sezer 2017; Longo et al. 2019). The liver plays a central role in the development of obesity-associated metabolic alterations. Indeed, hepatic mitochondrial dysfunction can cause the alteration of fat oxidation, reactive oxygen species (ROS) production, and oxidative stress (Nakamura et al. 2009). Amelioration of liver function is very important for prevention of obesity and its related metabolic disorders (Den Besten et al. 2015; Mollica et al. 2017). Effective food and supplement with antioxidative and anti-inflammatory activities should be developed for prevention of obesity and its associated metabolic disorders in animals.

Shiitake mushroom, *Lentinus edodes*, is a basidiomycete that has been consumed for more than 2000 years because of its nutritional value and health benefits (Gaitan-Hernandez et al. 2019). Shiitake has a low lipid content, high fiber content, and a considerable amount of protein, and extracts and pure compound of shiitake exhibit antibacterial, antifungal, cytostatic, antioxidant, anticancer, and immunomodulatory activities (Bisen et al. 2010; li et al. 2019). It has been reported that eritadenine, hypocholesterolemic factor isolated from shiitake, suppress biosynthesis of cholesterol in liver and

decrease plasma cholesterol concentrations in rodents (Shimada et al. 2003; Fukada et al. 2006). Shiitake shows various components except eritadenine for good health of animals (Li et al. 2019), and shiitake seems to be useful food to ameliorate hepatic functions of animals.

In this study, we measured metabolites and hormone concentrations and enzymes activities related to lipid metabolism in blood of healthy dogs supplemented with shiitake powder for 4 weeks. The objective of this study is to investigate effects of supplementation with shiitake powder on lipid metabolism in dogs and discuss potential application as supplement of shiitake powder to good health of dogs.

## **Materials And Methods**

### **Animals**

Twelve healthy Beagles (7 males, 5 females; average age, 2 years (1-3 years old); mean body weight:10.5kg (9.4-11.4kg; mean body condition score of 9-points scale, 5.0 (4.0-6.0)) were used. Body condition score (BCS) are evaluated with 9-points scale system (Sandoe et al. 2014). They were fed with commercial diet, DS-A (Oriental Yeast Co. Ltd., Tokyo, Japan) at 8 am daily, and were randomly divided into three groups, control and the low dose and the high dose groups.

### **Supplementation with shiitake powder**

Shiitake powder was prepared by Mori & Company, Limited (Kiryu, Japan). One hundred gram of dry powdered shiitake contains 78 mg of eritadenine as hypocholesterolemic factor. Shiitake powder was mixed with food and orally supplemented with dogs. Shiitake powder was supplemented at the dose of 800 mg/kg BW/day (the high dose group) and 100 mg/kg BW/day (the low dose group) with dogs for 4 weeks, respectively. The control group dogs were not supplemented with shiitake powder. The dose of shiitake powder was settled as 0.60~0.65 mg/kg body weight/day and 0.075~0.080 mg/kg/day as eritadenine, referring to effective dose of eritadenine in rats (Shimada et al. 2003; Fukada et al. 2006).

### **Blood sampling**

Five milliliter of each blood was collected from cephalic vein of each animal into the heparinized tubes before (0 week), 2 weeks and 4 weeks of the supplementation. Blood collection was performed before the morning feeding and collected samples were immediately centrifuged at 2,000 x g for 10 min at 4°C to obtain plasma. These plasma samples were stored at -80°C until use. For measurement of adenosine monophosphate activated protein kinase (AMPK) activities, peripheral leukocytes were collected from the buffy coat of collected blood (Takeguchi et al. 2005).

### **Metabolites, hormone, and enzyme analyses**

Plasma Glucose, total cholesterol, triglyceride, total protein, blood urea nitrogen (BUN) and creatinine concentrations, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were measured using an autoanalyzer (JCA-BM2250, JEOL Ltd., Tokyo,

Japan) with the manufacturer's reagents at FUJIFILM Vet Systems (Tokyo, Japan). Plasma free fatty acids (FFA) concentrations were measured using a commercial kit, NEFA-C test (Wako Pure Chemical Industries, Inc., Tokyo, Japan). Plasma insulin and adiponectin concentrations were measured with Rat Insulin ELISA Kit (AKRIN-010T, Shibayagi Co., Gunma, Japan) and Mouse/Rat adiponectin ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), respectively [Kawasumi et al 2018]. AMP-activated protein kinase (AMPK) activities were measured with a commercial ELISA kit, CycLex AMPK Kinase kit (CycLex Co., Ltd., Nagano, Japan). The AMPK activities in leukocytes were measured at 30°C for 30 min and were expressed as  $\mu\text{g}$  of phosphorylated substrate per min per mg of protein. Protein concentrations were measured with the Bradford method using bovine serum albumin as the standard (Bradford 1976). Malondialdehyde (MDA) concentrations were measured using a commercial kit (Northwest Life Science Specialties, LLC, Vancouver, WA). Superoxide dismutase (SOD) activities were measured using a commercial kit ((Northwest Life Science Specialties, LLC, Vancouver, WA). Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities were measured by the previously reported methods by Kaloustian et al (1969) and Bergmeyer and Bernt (1974), respectively. The M/L ratio was calculated as MDH activities by LDH activities and used as sensitive marker to reflect elevated energy metabolism including more ATP production in tissues (Li et al. 2012).

### **Quantitative real-time PCR (q-PCR) analysis of SIRT1**

Total leukocyte RNA was extracted from the blood samples using TRIzol (Invitrogen), according to the manufacturer's protocol. Total RNA (1 $\mu\text{g}$ ) was reverse transcribed by QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Genomic DNA was removed by DNase treatment, and cDNA was synthesized. After inactivating the reverse transcription reaction by heating at 95°C for 3 min, the cDNA product was used for q-PCR. Reactions were carried out with Perfect Real Time TYBR Premix Ex Taq II (Takara, Tokyo, Japan) using an ABI 7300 Real Time PCR Sequence Detection System (Applied Biosystems, Foster City, CA) and the following shuttle PCR protocol: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 35 s, in 20  $\mu\text{l}$  reaction volumes containing 2  $\mu\text{l}$  template cDNA, 0.8 $\mu\text{l}$  primers (0.4 $\mu\text{l}$  ROX Reference Dye, and 6.0 $\mu\text{l}$  distilled water. Primers shown in Table 1 were used for SIRT1 and beta-actin mRNA (21). Following the real-time PCR, the fragment was subjected to dissociation-curve analysis to avoid nonspecific PCR amplification. Each SIRT1 value was normalized to that of beta-actin mRNA.

### **Statistical analysis**

All values were expressed as means  $\pm$  standard error (SE). Statistical significance was determined by Paired-*t* test. The significance level was set at  $p < 0.05$ .

## **Results**

### **Body weight and body condition scores of dogs**

As shown in Table 2, body weights (BW) and body condition scores (BCS) of dogs were maintained within ideal range (13) through experimental periods with shiitake powder supplementation. All dogs did

not show any symptoms of diseases.

### **Plasma metabolite and hormone levels in dogs (Table 3)**

Glucose, triglyceride, total protein, blood urea nitrogen (BUN), creatinine concentrations were not changed in plasma of dogs in three groups after shiitake powder supplementation for 4 weeks. Plasma free fatty acids (FFA) concentrations increased in both low dose and high dose groups. Plasma total cholesterol concentrations decreased significantly in the high dose group. Plasma insulin concentrations were not changed in all three groups. Plasma adiponectin concentrations trended to increase in the high dose group.

### **Enzymes and M/L ratio in dogs (Table 4)**

AST and ALT activities were not changed. ALP and LDH activities tended to decrease in the high dose group. M/L ratio increased in the high dose group after shiitake powder supplementation. Leukocytes AMPK activities were not changed in the three groups.

### **Molecules related to oxidative stress in dogs (Table 5)**

Expression of sirtuin1 mRNA were increased significantly, and plasma SOD activities increased in the high dose group. Sirtuin1 expression and SOD activities in the control and the low dose group were not changed. Plasma malondialdehyde (MDA) concentrations increased gradually in all groups, however the differences among groups were not observed.

## **Discussion**

In dogs supplemented with shiitake powder at the high dose, decreasing in plasma total cholesterol concentrations and increasing in sirtuin1 mRNA expression in peripheral leukocytes were significant. Plasma ALP and LDH activities decreased in the high dose group, and these changes seem to reflect of amelioration of hepatic function. A higher plasma M/L ratio reflects elevated energy metabolism, including more ATP production via glycolysis, in liver and skeletal muscle (Li et al. 2102). Increased plasma M/L ratio might indicate increased ATP production in liver of dogs supplemented with shiitake powder.

Eritadenine, a kind of alkaloid and a hypocholesterolemic compound found in shiitake mushroom, has a wide range of effects on lipid metabolism such as an increase in the liver microsomal phosphatidylethanolamine concentrations, a decrease in the liver microsomal  $\Delta 6$ -desaturase activity, and an alteration of the fatty acid and molecular species profile of liver and plasma lipid (Shimada et al. 2003). Hypocholesterolemic action of eritadenine might be associated with a modification of hepatic phospholipid metabolism, and secretion of lipoprotein as transporter of cholesterol to blood might be decreased (Sugiyama et al. 1995).

On the other hand, sirtuin is an NAD<sup>+</sup>-dependent protein deacetylase and is a master metabolic regulator in different metabolic tissues (Li 2013), and depresses lipid synthesis and activates fatty acid oxidation resulting in ameliorating reactive oxygen species (ROS) stress (Jiang et al. 2012).

Hypocholesterolemia observed in dogs with supplemented with shiitake powder seems to be induced by eritadenine, and increasing of plasma FFA and adiponectin concentrations and SOD activities might be induced by some components in shiitake powder, which activates sirtuin1. Polyphenols such as resveratrol show the effect to increase sirtuin1 activities (Shi et al. 2018; Deng et al. 2019). One species of mushroom shows activating effect of sirtuin1 and is applied to improvement of neurodegeneration (Scuto et al. 2020). Shiitake must also contain useful components to activate sirtuin1.

Activated AMPK and SIRT1 regulate the activity of the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 $\alpha$ ), further up-regulate its expression (Canto et al. 2009). AMPK/SIRT1/PGC-1 $\alpha$  signaling pathway acts as an energy-sensing network and plays crucial regulatory role in mitochondrial biosynthesis, lipid metabolism, and oxidative stress (Canto et al. 2009). Promotion of AMPK/SIRT1/PGC-1 $\alpha$  signaling pathway improves oxidative stress in mice with myocardial ischemia (Tian et al. 2019), liver function in obesity and non-alcoholic fatty liver disease in mice with high-fat diet (Liou et al. 2019), neuroinflammation and neurodegeneration in Meniere's disease (Scuto et al. 2020), insulin resistance in type 2 diabetes mellitus (Yan et al. 2018; Jung et al. 2018), and other metabolic disorders of human. Flavonoids such as resveratrol have the effect to activate AMPK/SIRT1/PGC-1 $\alpha$  signaling pathway, however no flavonoids or lignans are found in a kind of mushrooms including shiitake (Mattila et al. 2001). Further studies are needed to clarify what components activate sirtuin 1 in shiitake powder.

It has been reported that overweight and obesity, aberrant lipid metabolism in liver, are usually associated with shorter lifespan (Cirulli et al. 2018; Sakaue et al. 2020). Similarly, obesity has detrimental effects on the health and longevity of dogs and cats (German 2006). Obesity is the number one health problem pets face. Shiitake powder can ameliorate lipid metabolism in tissues of dogs, and may be useful as anti-obesity food for dogs. Limitations of our study include small number of samples and biological and environmental variables (age or sex). To evaluate the usefulness of shiitake powder as supplement for improvement lipid metabolism, we should further study using overweight and obese dogs.

## Conclusion

In this study, we measured metabolites and hormone concentrations and enzymes activities related to lipid metabolism in plasma of healthy dogs supplemented with shiitake powder to investigate effects of its supplementation on lipid metabolism in dogs. Shiitake powder was supplemented with dog at the high dose (800mg/kg body weights/day) and low dose (100mg/kg/day) for 4 weeks. In dogs supplemented with shiitake powder at the high dose, plasma total cholesterol concentrations decreased and sirtuin1 mRNA expression in peripheral leukocytes increased significantly. Plasma ALP and LDH activities tended to decrease in the high dose group, and these reactions seemed to reflect of improvement of hepatic

function. Shiitake powder can ameliorate lipid metabolism in tissues of dogs, and may be useful as anti-obesity food for dogs.

## Declarations

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### Author contributions

AK, YO and HU contributed to the conception and design of the work and drafted the work. IY and TA contributed to the analysis and interpretation of data. YM and NT prepared shiitake powder. TA and KK contributed to the design of the work and final approval of the version to be publication. All authors read and approved the final work.

### Data availability

The data in this study are available from the corresponding author on reasonable request.

### Compliance with ethical standards

Ethical approval for this study was from Narita Animal Science Laboratory Co., Ltd. Research Animal Ethical Committee (21-C034).

**Conflicts of interest** The authors declare that they have no conflict of interest.

**Consent to participate** Not applicable.

**Consent of publication** All authors give consent for publication.

### Statements □ Declaration

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