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## Examination of the antidiabetic effects of mulberry leaves on a rat model of spontaneous type 2 diabetes mellitus (OLETF rats) and the contents of trace elements in the living body

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**Abstract** Spontaneous type 2 diabetes mellitus model rats (OLETF rats) and control rats were each divided into two groups: one treated with feed containing 2.5% mulberry leaves and the other given a standard diet. After 3 weeks, the contents of 7 trace elements were determined in each organ, namely zinc, magnesium, calcium, phosphorus, iron, manganese and copper. I then conducted a comparative study of the blood glucose levels and the effects of the trace elements in vivo on the kinetics associated with the intake of mulberry leaves. As a result, I discovered a slight inhibitory action of mulberry leaves on hyperglycemia. In comparison with the control group, the OLETF-standard diet rats showed a lower content of zinc in the liver but higher contents in the spleen, pancreas, fat and urine, whereas the mulberry leaves-supplemented OLETF rats exhibited no significant differences from the control group in the zinc contents in the liver, spleen or urine. In comparison with the control group, the OLETF-standard diet rats showed less magnesium in the lungs, liver, and pancreas but larger amounts in the urine and spleen, whereas the mulberry leaves-supplemented OLETF rats exhibited no significant differences from the control group in the magnesium contents of the lungs and urine. I also detected changes in each of the other trace elements in some of the organs, resulting from the administration of mulberry leaves. This study suggests that mulberry leaves may effectively replenish trace elements that the body lacks in individuals with impaired glucose tolerance, and lower blood glucose possibly by maintaining insulin secretory functions and/or improving insulin action.

### Introduction

Essential trace elements are not simply components of the body, since they are deeply associated with metabolism and play coenzyme roles that enhance the expression of enzyme activity or stabilize their structures. Studies using blood and urine samples from patients have reported variations in the contents of trace elements in various diseases, and that these variations vary among dis-

eases.<sup>1),2)</sup> In addition, deficiencies in trace elements, including zinc (Zn) and magnesium (Mg), have been reported to have close correlations with conditions such as arteriosclerosis and heart diseases, suggesting close links between the dynamics of trace elements and diseases and their complications.<sup>3),4)</sup> Even studies on diabetes mellitus using a rat model of experimental diabetes (STZ rats) caused by streptozotocin administration have reported marked changes in the trace element

contents of each organ for conditions such as uncontrolled diabetes alone or accompanied by diabetic nephropathy.<sup>5),6)</sup>

Meanwhile, revention and treatment of diabetes mellitus have long been associated with studies on, and the use of, plants and animals found in the immediate environment. Among these, mulberry leaves are a natural product widely used in Chinese or herbal medicine for the treatment of diabetes mellitus, since they have been reported to have preventive effects on diabetes as well as suppressive effects on hyperglycemia.<sup>7)</sup> In addition, although people have been brewing these leaves for consumption as tea, mulberry leaves have been gaining more and more popularity as a health supplement due to the increasing health consciousness of the population.

Previous studies have reported improvement of insulin resistance in skeletal muscles as well as improvement in insulin secretion as the mechanisms of action for the blood glucose lowering effects of mulberry leaves.<sup>7),8)</sup> It has also been reported that the constituents of mulberry leaves exhibit an inhibitory action towards alpha glucosidase, and that they suppress glucose absorption from the intestinal tract, thereby minimizing postprandial hyperglycemia.<sup>9)</sup> Furthermore, the action of dietary fibers found in the leaves and the formation of good enterobacterial flora may also be correlated with improvement in sugar metabolism.

Otsuka Long Evans Tokushima Fatty (OLETF) rats are an inbred strain derived from Long Evans rats, that represent a model for diabetes mellitus.<sup>10),11)</sup> OLETF rats were first reported as rats with naturally occurring type 2 diabetes mellitus accompanied by obesity. These rats exhibit a significant increase in blood glucose at approximately 10 weeks of age and develop diabetes mellitus. Their characteristics consist of insulin resistance leading to hypersecretion of insulin at early stages of the disease, and a gradual decrease in insulin secretion functions as the disease progresses.

Changes in the balance of trace elements in the body may have an influence on the onset of diabetes mellitus. In this study, I measured the contents of trace elements

found in each organ of OLETF rats during the first stage following the onset of diabetes, and examined the antidiabetic effects of the administration of mulberry leaves, as well as their effects on the balance of the trace elements in each organ, in order to investigate the possibilities and usefulness of mulberry leaves as an alternative medicine.

### *Materials and Methods*

#### 1. Animals and experimental design

This study involved 4-week-old male OLETF rats, and Long Evans Tokushima Otsuka (LETO) rats as non-diabetic controls, all of which were supplied by Tokushima Research Institute, Otsuka Pharmaceutical Company. For 3 weeks prior to the experiments, all rats were fed the standard diet. Subsequently, I divided them into 4 groups of 5 and each group was fed either the standard diet or feed containing 2.5% mulberry leaves for a period of 3 weeks. The amounts of food and water were unrestricted. I used Oriental MF from Oriental Yeast for the standard diet, and for the feed mixed with 2.5% mulberry leaves, I simply added powdered mulberry leaves to the standard diet. The rats were kept in a room maintained at  $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , with  $45 \pm 5\%$  humidity and lighting between 8 a.m. and 8 p.m. Food consumption and body weight were measured periodically.

#### 2. Measurement of blood glucose level

The casual blood glucose level (taken at any time of day) was measured by the glucose oxidase method at the start of the experiment and after each feeding session for a period of 3 weeks.

#### 3. Measurement of specimens and contents of trace elements

The rats were sacrificed using carbon dioxide after approximately 6 hours of fasting, after which I immediately removed the cerebrum, cerebellum, lungs, heart, liver, spleen, pancreas, kidneys, prostate glands, testes, muscles (femoris muscles), and subcutaneous fat (abdomen). I also collected urine and blood samples. Subsequently, I performed wet-ashing using nitric and perchloric acids, dissolved them in 6N HCL and set them at a constant volume with ultrapure water. I

measured the zinc (Zn), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn) and copper (Cu) contents by a flame atomic absorption spectrometric method, and used a colorimetric method with molybdic acid to determine the phosphorus content. I used a creatinine correction procedure to determine the contents of trace elements in the urine. For the analyses, I used reagents for measuring poisonous metals, and the laboratory glassware was soaked overnight or longer in 1:1 nitric acid to remove any metal ion contaminants.

#### 4. Statistical Analysis

All the results obtained from measurements of the composition of trace elements in the feed, the blood glucose level, and the contents of trace elements found in the organs, blood and urine of each group were expressed as the mean  $\pm$  SD, and all tests were performed using Student t-tests. The level of significance was set at  $P < 0.05$ .

### Results

#### 1. Composition of trace elements in the feed, and changes in food consumption and body weight

Table 1 shows the contents of trace elements found in the feed containing 2.5% mulberry leaves and the standard diet. I found significantly higher amounts of Ca, Fe and Zn in the feed containing 2.5% mulberry leaves than in the standard diet.

The mean food consumptions for the groups

were  $29 \pm 3$ g/day,  $31 \pm 5$ g/day,  $27 \pm 4$ g/day, and  $29 \pm 5$ g/day for the OLETF-standard diet group, mulberry leaves-supplemented OLETF group, control group, and mulberry leaves-supplemented control group, respectively, showing no significant differences among the groups.

Regarding the body weight, the OLETF-standard diet group and the mulberry leaves-supplemented OLETF group were  $249 \pm 10$  g and  $272 \pm 16$ g at the start of the experiment, and  $419 \pm 16$ g and  $404 \pm 16$ g after the 3-week feeding session, respectively, showing no significant difference in the weight increase.

#### 2. Blood glucose level

Although the blood glucose levels of the OLETF rats exhibited no significant difference from the control group at the start of the experiment (the levels for the OLETF-standard diet group, mulberry leaves-supplemented OLETF group and control group were  $133 \pm 12$ mg/dl,  $132 \pm 7$ mg/dl and  $128 \pm 9$ mg/dl, respectively), the levels in the OLETF-standard diet group increased significantly ( $244 \pm 26$  mg/dl;  $n=5$ ,  $p < 0.01$ ) after the 3-week feeding session compared with the control group ( $183 \pm 16$ mg/dl). Although the mulberry leaves-supplemented OLETF group ( $209 \pm 12$ mg/dl;  $n=5$ ,  $p < 0.05$ ) showed a higher level than the control group, it was significantly suppressed compared with the OLETF-standard diet group ( $p < 0.05$ ). Meanwhile, the mulberry leaves-supplemented control group ( $185 \pm 15$ mg/dl) showed no significant change in the blood glucose level compared with the control group.

#### 3. Contents of trace elements of each organ in vivo

Figure 1 shows the contents of Zn and Mg in each specimen. In comparison with the control group, the OLETF-standard diet group exhibited a lower content of Zn in the liver ( $18.17 \pm 0.75$  vs.  $20.20 \pm 1.48$   $\mu$ g/g), but higher contents in the spleen ( $16.28 \pm 0.19$  vs.  $15.53 \pm 0.57$   $\mu$ g/g), pancreas ( $18.36 \pm 2.22$  vs.  $15.01 \pm 0.98$   $\mu$ g/g), fat ( $5.69 \pm 0.16$  vs.  $4.94 \pm 0.5$   $\mu$ g/g) and urine ( $2.84 \pm 0.9$  vs.  $1.26 \pm 0.25$   $\mu$ g/g Cr). In comparison with the control group, the OLETF-standard diet group exhibited lower contents of Mg in the lungs ( $154.34 \pm$

Table 1. Mineral concentrations in the diets ( $\mu$ g/g)

	Mulberry leaves-supplemented diet	Standard diet
Ca	$7780.9 \pm 84.4^*$	$7104.4 \pm 78.0$
Mg	$3770.0 \pm 77.8$	$3775.2 \pm 199.9$
P	$744.0 \pm 241.9$	$920.4 \pm 120.2$
Fe	$120.8 \pm 12.0^*$	$94.1 \pm 12.8$
Mn	$56.7 \pm 3.1$	$56.3 \pm 3.4$
Zn	$42.0 \pm 1.5^*$	$37.0 \pm 1.0$
Cu	$5.7 \pm 1.5$	$4.0 \pm 0.6$

\*  $p < 0.05$  compared with the standard diet.

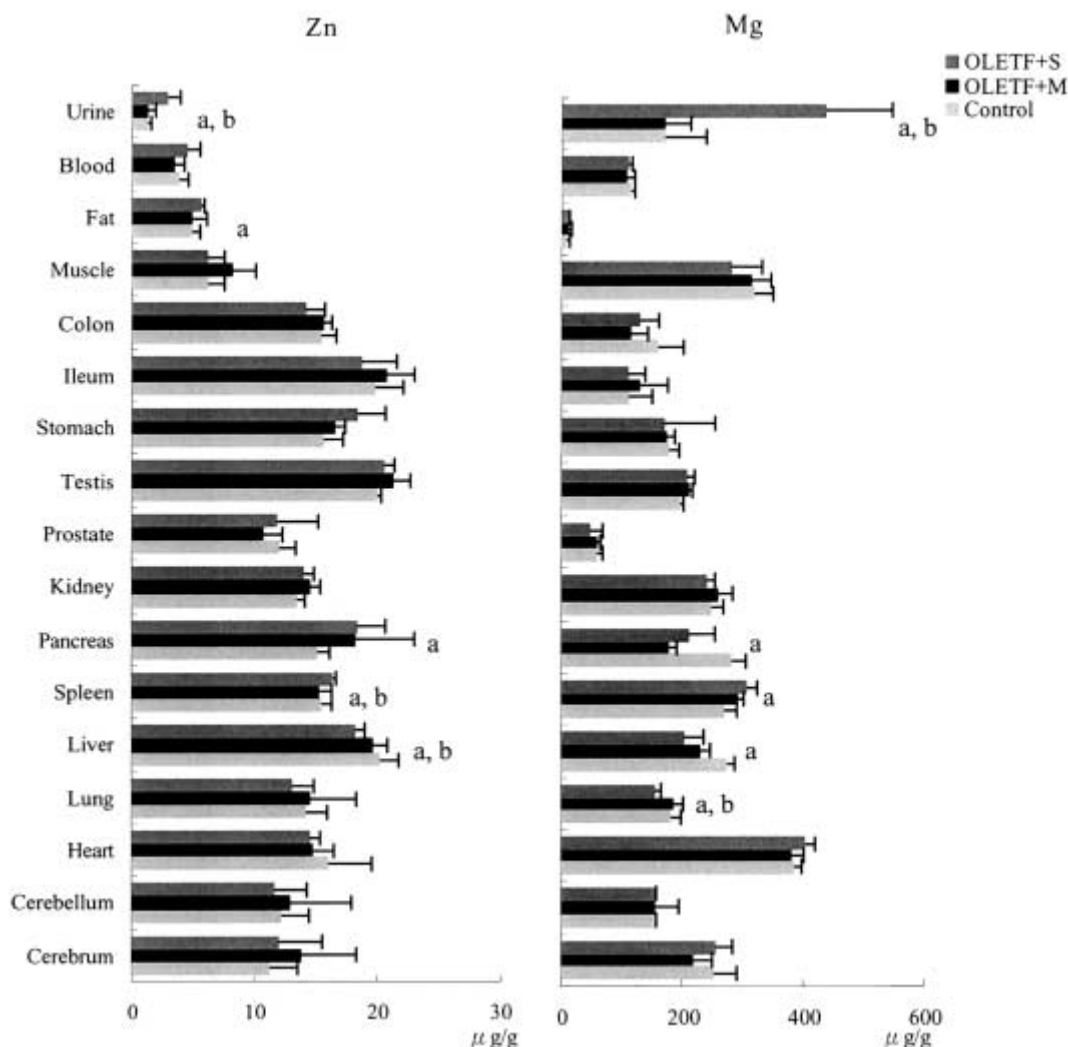


Fig. 1 Zn and Mg levels in the rat organs.

OLETF+S : OLETF-standard diet group

OLETF+M : Mulberry leaves-supplemented OLETF group.

Values are means  $\pm$  SD (n=5).

Significant differences (p < 0.05)

a : between OLETF+S and Control

b : between OLETF+M and OLETF+S.

9.09 vs.  $181.93 \pm 15.43 \mu\text{g/g}$ ), liver ( $202.63 \pm 30.35$  vs.  $274.88 \pm 11.47 \mu\text{g/g}$ ) and pancreas ( $212.22 \pm 41.24$  vs.  $281.35 \pm 21.40 \mu\text{g/g}$ ), but higher contents in the spleen ( $306.51 \pm 16.22$  vs.  $268.67 \pm 20.86 \mu\text{g/g}$ ) and urine ( $436.53 \pm 108.05$  vs.  $170.68 \pm 66.57 \mu\text{g/g}$  Cr).

Figure 2 shows the contents of Ca and P in each specimen. In comparison with the control group, the OLETF-standard diet group exhibited lower contents of Ca in the heart ( $16.50 \pm 1.08$  vs.  $23.45 \pm 5.98 \mu\text{g/g}$ ) and kidneys ( $30.50 \pm 1.64$  vs.  $35.25 \pm 3.02 \mu\text{g/g}$ ), but a higher content in the pancreas ( $85.64 \pm$

$9.53$  vs.  $67.09 \pm 10.99 \mu\text{g/g}$ ). Phosphorus was found in a smaller amount in the kidneys ( $2623.3 \pm 86.2$  vs.  $2895.2 \pm 62.5 \mu\text{g/g}$ ), but in a larger amount in the prostate glands ( $2434.2 \pm 148.9$  vs.  $2201.5 \pm 62.5 \mu\text{g/g}$ ).

Figure 3 shows the contents of Fe, Mn and Cu in each specimen. In comparison with the control group, the OLETF-standard diet group exhibited lower contents of Fe in the cerebrum ( $14.86 \pm 2.05$  vs.  $18.82 \pm 2.66 \mu\text{g/g}$ ) and cerebellum ( $14.91 \pm 0.57$  vs.  $20.35 \pm 2.99 \mu\text{g/g}$ ), but higher contents in the lungs ( $89.88 \pm 11.63$  vs.  $97.97 \pm 14.42 \mu\text{g/g}$ ), spleen ( $254.56 \pm 15.03$  vs.

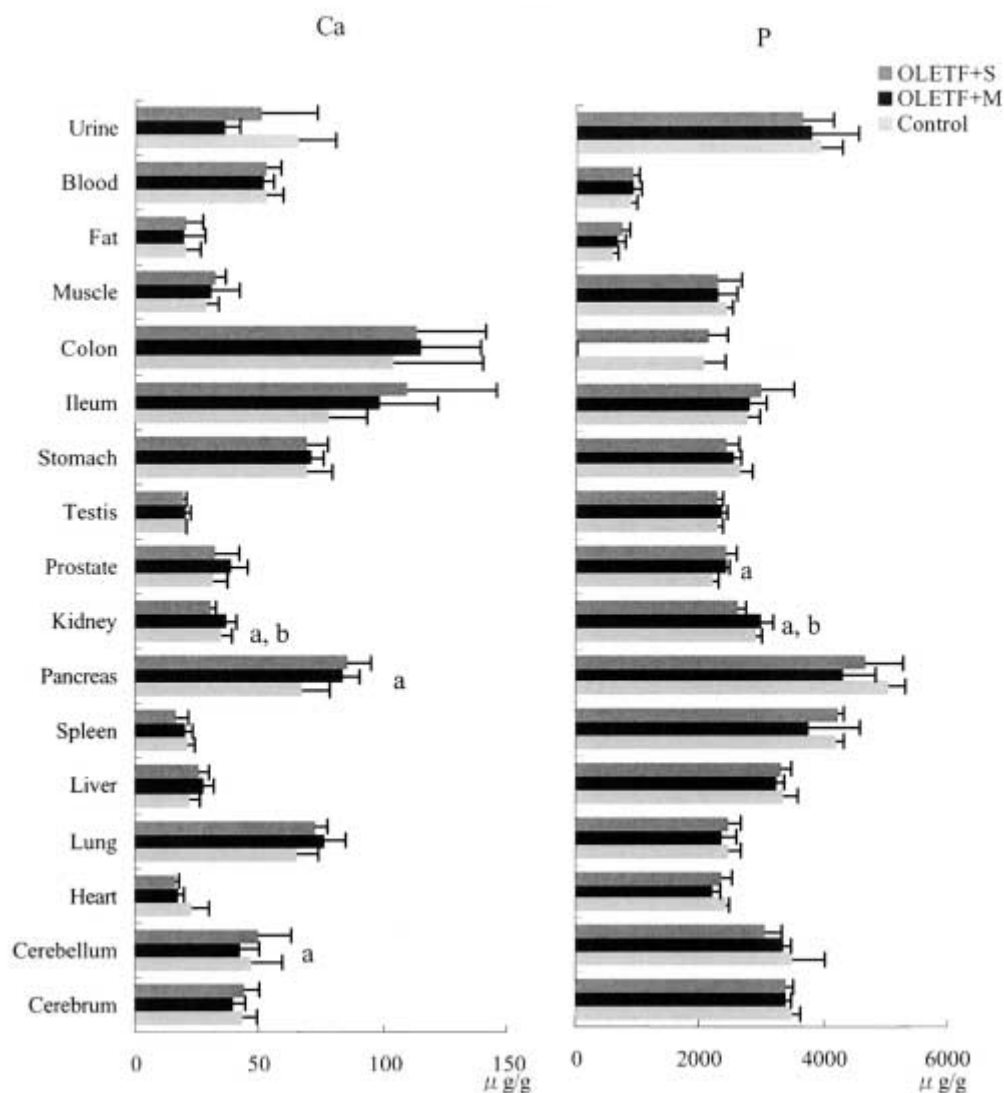


Fig. 2 Ca and P levels in the rat organs.

OLETF+S : OLETF-standard diet group.

OLETF+M : Mulberry leaves-supplemented OLETF group.

Values are means  $\pm$  SD (n=5).

Significant differences (p < 0.05)

a : between OLETF+S and Control

b : between OLETF+M and OLETF+S.

$201.78 \pm 29.42 \mu\text{g/g}$ ) and testes ( $18.87 \pm 1.38$  vs.  $16.03 \pm 0.74 \mu\text{g/g}$ ). Mn was found in a smaller amount in the heart ( $0.35 \pm 0.04$  vs.  $0.46 \pm 0.04 \mu\text{g/g}$ ), but in a larger amount in the testes ( $0.34 \pm 0.02$  vs.  $0.30 \pm 0.02 \mu\text{g/g}$ ). Cu was found in smaller amounts in the cerebrum ( $1.52 \pm 0.22$  vs.  $1.95 \pm 0.12 \mu\text{g/g}$ ), cerebellum ( $1.77 \pm 0.07$  vs.  $2.12 \pm 0.31 \mu\text{g/g}$ ) and testes ( $1.62 \pm 0.07$  vs.  $1.77 \pm 0.03 \mu\text{g/g}$ ).

#### 4. Effects of feed containing 2.5% mulberry leaves

I examined the effects of mulberry leaves by comparing the contents of the trace elements in the organs of the mulberry leaves-supplemented OLETF group with those in the organs of the OLETF-standard diet group and control group that exhibited a significant difference in the contents. I calculated the variations in the ratio of each trace element in each specimen between the OLETF-standard diet group and the mulberry leaves-supplemented OLETF group, and studied the changes between the 2 groups, as

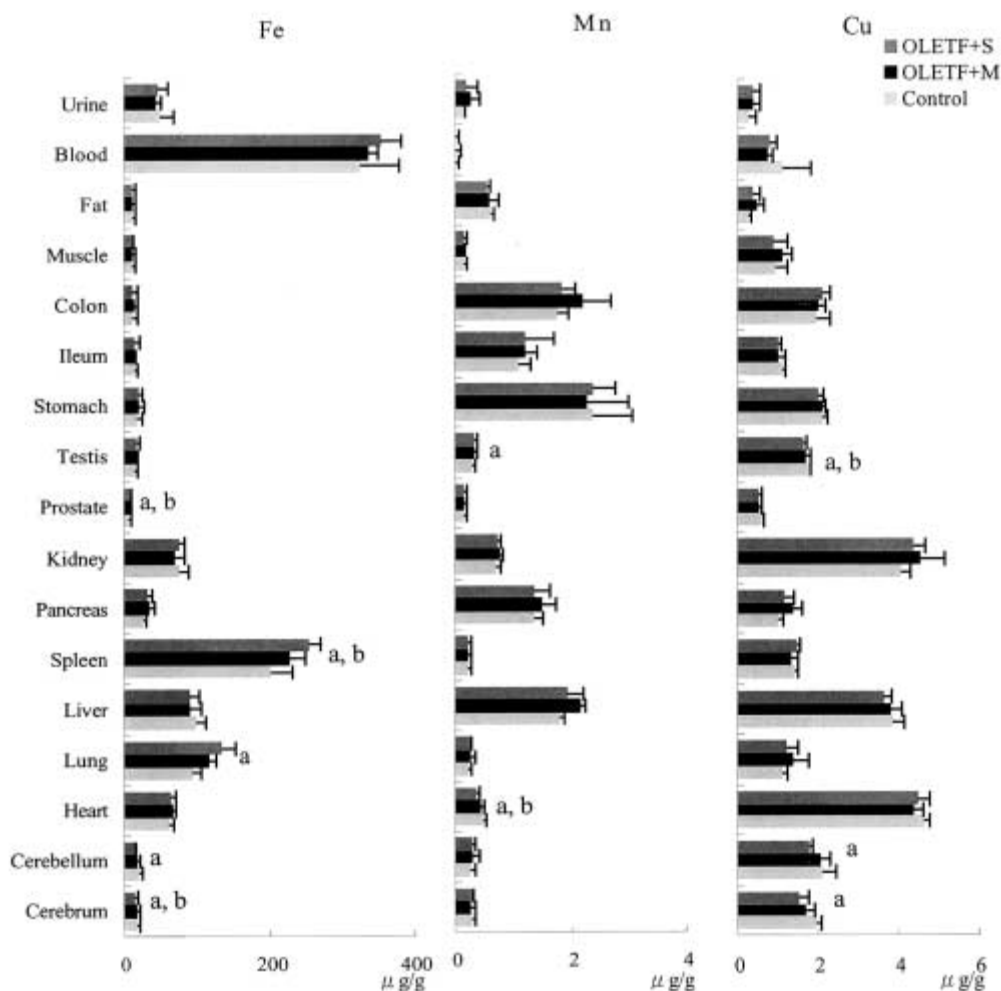


Fig. 3 Fe, Mn and Cu levels in the rat.  
 OLETF+S : OLETF-standard diet group.  
 OLETF+M : Mulberry leaves-supplemented OLETF group.  
 Values are means  $\pm$  SD (n=5).  
 Significant differences ( $p < 0.05$ )  
 a : between OLETF+S and Control  
 b : between OLETF+M and OLETF+S.

shown in Figure 4.

The Zn content decreased in the urine and spleen, but increased in the liver, showing no significant differences from the control group. I observed no changes in the pancreas and fat. The Mg content decreased in the urine, but increased in the lungs, showing no significant differences from the control group. I observed no changes in the liver, spleen and pancreas.

The Ca content increased in the kidneys and showed no significant difference from the control group. There were no changes in the heart and pancreas. The P content increased in the kidneys and showed no significant difference from the control group. There were no

changes in the prostate glands.

The Fe content increased in the cerebrum and decreased in the spleen and testes, showing no significant differences from the control group. There were no changes in the cerebellum and lungs. The Mn content increased in the heart and showed no significant difference from the control group. There were no changes in the testes. The Cu content increased in the cerebellum and showed no significant difference from the control group. There were no changes in the cerebrum and testes.

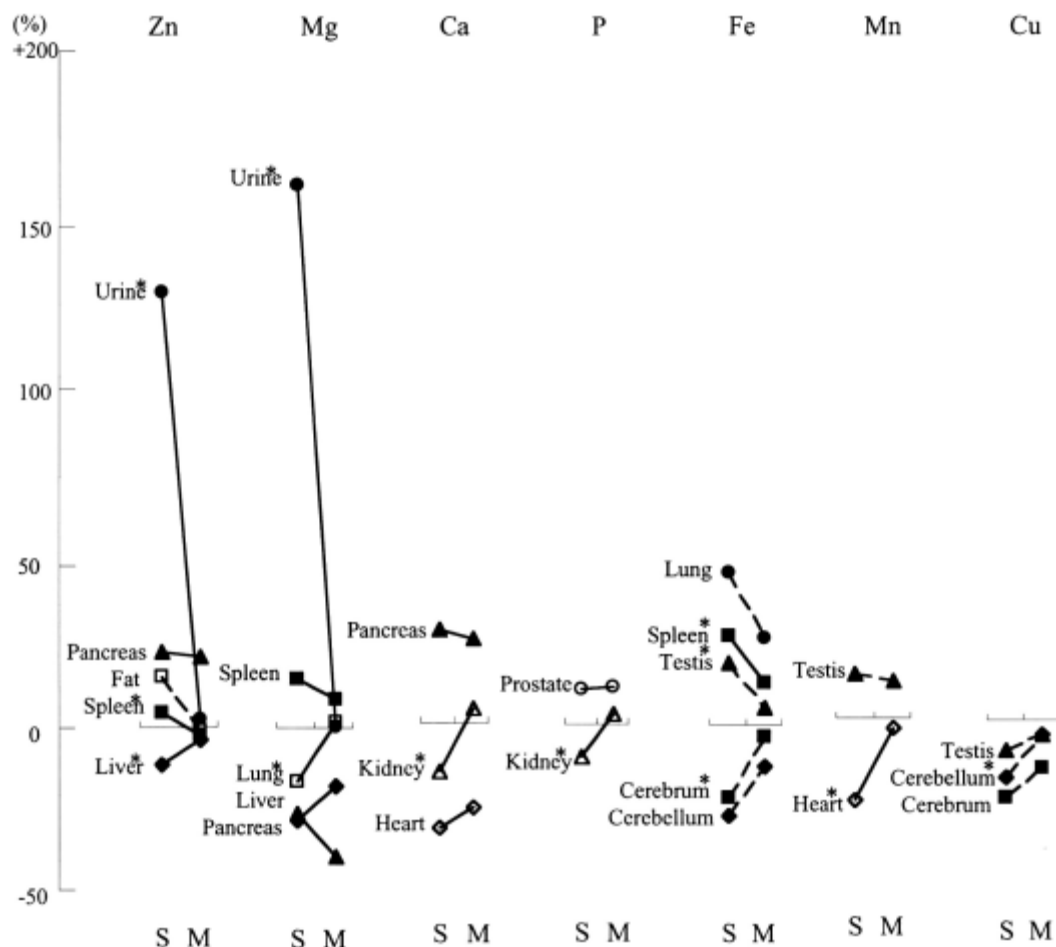


Fig. 4 Comparison of the relative rates of trace element contents in the organs of the OLETF-standard diet and mulberry leaves-supplemented OLETF groups to those in the control group.

S : Relative rates of the contents in the OLETF-standard diet group to those in the control group.

M : Relative rates of the contents in the mulberry leaves-supplemented OLETF group to those in the control group.

\* :  $p < 0.05$ , OLETF-standard diet group vs. mulberry leaves-supplemented OLETF group.

## Discussion

Although I observed a higher incidence of hyperglycemia in the mulberry leaves-supplemented OLETF group than in the control group, the blood glucose level was significantly lower compared with that in the OLETF-standard diet group, revealing a slight inhibitory action of mulberry leaves on hyperglycemia. The fact that no differences were found for the rate of increase in body weight and food consumption strongly indicates that the inhibitory action on hyperglycemia is due to the antidiabetic effects of

mulberry leaves. Meanwhile, no drop in blood glucose was observed in the mulberry leaves-supplemented control group, suggesting that the leaves act via mechanisms different from those involving the stimulation of insulin secretion, such as the effects of sulfonylureas.

With respect to the antidiabetic components of mulberry leaves, Asano et al. reported various alkaloids with an inhibitory action on alpha glucosidase, including 1-deoxynojirimycin.<sup>9)</sup> However, Iizuka et al. reported that a hot-water extract from mulberry leaves improved insulin resistance and

normalized insulin-stimulated glucose uptake.<sup>8)</sup> In addition, Chen et al. reported that, similar to the hot-water extract from mulberry leaves, an ethanol-insoluble fraction also had components with a blood glucose lowering effect that did not affect the control group.<sup>7)</sup> This strongly suggests that the antidiabetic action of mulberry leaves derives from various constituents.

The onset of diabetes mellitus has been reported to be associated with abnormalities in trace elements. With regard to Zn, Quarterman et al. reported in 1972 that Zn-deficient rats exhibited impaired glucose tolerance with a decrease in insulin secretion or sensitivity, and claimed that Zn deficiency was the culprit for diabetes mellitus and one of the precipitating factors.<sup>12)</sup> Consequently, this also suggests that the administration of Zn itself has antidiabetic effects. In fact, in a study conducted by Tobia et al. where rats with spontaneous diabetes mellitus (BB Wistar rats) were fed 3 types of diets containing Zn (1000ppm, 50ppm, and 1ppm) for a period of 60 days, the group treated with 1000ppm of Zn showed a delayed onset of diabetes.<sup>13)</sup> The feed containing 2.5% mulberry leaves contained significantly larger amounts of Zn compared with the standard diet. However, the study conducted by Tobia et al. did not report any antidiabetic effects for the diet containing 50ppm of Zn, which is similar to the level in the mulberry leaves-supplemented diet used in this study. Therefore, the antidiabetic actions of mulberry leaves may be attributable to the effects on the Zn balance in the body. Lau et al. reported that Zn excretion in the urine increased 3.4-fold in STZ rats.<sup>6)</sup> Some reports have suggested that individuals with abnormal glucose tolerance might be Zn-deficient due to increased Zn excretion.<sup>14)</sup> Zn is necessary for the stabilization of proinsulin, and is also vital for antioxidant enzymes such as catalase, peroxidase and Zn-SOD acting against free radicals generated by stress. In this study, I observed an increased Zn content in the pancreas of OLETF-standard diet rats. I speculate that this was caused by the obesity-induced increase in the insulin demand, and that the pancreas actively sequestered the Zn. Meanwhile, the Zn excretion rate in the urine

increased, and as the condition progressed, the amount of Zn sequestered in the pancreas became deficient due to exhaustion and damage to the pancreas beta cells leading to decreased insulin secretion, from which I infer the possibility of deterioration in the homeostatic control. Some reports have indicated a positive correlation between the urinary excretions of glucose and Zn in patients with type 2 diabetes mellitus.<sup>15),16)</sup> Such an increase in the excretions rate of Zn in the urine may be attributed to an increase in the excretion rate in the renal glomerulus or deterioration of the tubular reabsorptive functions.

By comparing healthy subjects and patients with non-insulin-dependent diabetes mellitus (NIDDM), Minami et al. reported a significant increase in the urinary Zn excretion in the NIDDM patients, and that the urinary Zn content did not decrease to the level observed in the control group even after treatment of the diabetes.<sup>17)</sup> In this study, Zn excretion in the urine was 2.2-fold higher in the OLETF-standard diet group than in the control group, whereas the mulberry leaves-supplemented diet decreased the urinary Zn excretion in OLETF rats to a level that was not significantly different from the control group despite the presence of hyperglycemia. This result strongly suggests that mulberry leaves have properties that help retain Zn in the body. With regard to the effect of Zn, an experiment conducted by Sharon et al. where mice with spontaneous diabetes mellitus (db/db mice) were given a large amount of Zn revealed increased tyrosine kinase activity due to insulin stimulation without any changes in the insulin receptors in the muscle, which therefore improved insulin resistance.<sup>14)</sup>

With regard to Mg, since Mather et al. reported that the Mg level in serum was significantly lower in patients with diabetes mellitus than in healthy subjects, there have been increased reports of hypomagnesemia.<sup>18)</sup> In addition, most of the reports have indicated increased Mg excretion in the urine.<sup>19),20)</sup> Fort et al. reported that the increased Mg excretion in the urine of patients with diabetes mellitus decreased the concentration in the serum, and that Mg tended to exhibit a



negative balance in the body of diabetic patients.<sup>21)</sup> Although the Mg content in the blood exhibited no significant difference from the control group after the 3-week feeding session, decreases in the Mg contents in the pancreas, liver and lungs had already been observed at that point, suggesting that the Mg content in the body was already leaning toward a negative balance. The amount of Mg excreted in the urine was 2.6-fold greater in the OLETF-standard diet group than in the control group, but in the mulberry leaves-supplemented OLETF group, the excretion in urine was suppressed to a level similar to that in the control group, suggesting Mg retention in the body. According to Baydas et al., although administration of Mg to rats with experimental diabetes mellitus had no influence on the blood glucose level, the total cholesterol and triglyceride levels decreased, revealing improved metabolism.<sup>22)</sup> From these observations, I infer that Mg has no antidiabetic effects and is not a preventive factor of the onset of the disease, but rather it is mainly correlated with the onset of the disease complications. The mulberry leaves-supplemented OLETF group showed the same level of Mg content in the lungs as the control group.

In a study conducted by Schneider et al. using model rats with alloxan-induced diabetes mellitus, decreased Ca absorptivity in the duodenum was observed, suggesting that Ca tended to be deficient in diabetes mellitus.<sup>23),24),25)</sup> Minami et al. detected Ca deposits in the kidneys of STZ rats with nephropathy, and reported that the Ca content of the kidneys increased in patients with diabetes mellitus, but that this may vary depending on the stage of the disease.<sup>26)</sup> I found no significant differences in the Ca and P contents of the kidneys between the mulberry leaves-supplemented OLETF group and the controls. Even for Fe, Mn and Cu, the levels found in the mulberry leaves-supplemented group tended to be similar to those of the control group.

The results of this study suggest that mulberry leaves may effectively replenish trace elements that the body lacks in individuals with impaired glucose tolerance. Through further examination of the correla-

tions between diseases and the biologically active components in foods in relation to the kinetics and metabolism of trace elements found in the body, I anticipate the discovery of preventive measures against the onset of diabetes mellitus and easily applicable nutritional measures that would be accompanied by changes in living habits through effective incorporation of accessible health supplements into the diet such as mulberry leaves.

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