Improve the intestinal bacterial flora environment on renal function recovery in chronic kidney disease by efficacy of administration of plant enzymes

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ABSTRACT

In renal failure, it is believed that decreased intestinal barrier function is caused by deterioration of the intestinal microflora environment and by intestinal mucosal disorder. This deterioration further promotes chronic kidney disease and unavoidably causes cardiovascular disorders and kidney death. Accumulation of uremic toxins also occurs. In this study, we investigated the effect of improving the intestinal microflora environment by administration of plant enzymes on chronic kidney damage, one of the sequelae of type 2 diabetes.

We examined the effect of administering plant enzymes in renal disorder model mice. The concentration of plant enzymes was 500 mg/kg/day, and the same amount of distilled water was administered to the control group. We administered these enzymes to mice by oral administration for over 2 weeks.

The control group received (Please provide the same type of information as above). Intestinal function was assessed by examining intestinal immunity and gastrointestinal action. Kidney function was assessed by histopathological analysis of glomerular tissue of the kidney and by measuring mouse blood and urinary protein levels.

Gut-associated lymphoid tissue function, including intestinal lymphocyte count and secretory immunoglobulin A level, was decreased, which led to restoration of kidney function. Histological findings of the glomerular tissue of the kidney showed restoration of kidney function. The mouse blood and urinary protein test results showed decreased urinary protein levels at 5–7 weeks relative to the levels in the control group.

Administration of plant enzymes improved the intestinal microflora environment, which led to improved intestinal function and reduction in the molecular weights of proteins at the nutrient absorption stage, which reduced the burden on the kidneys. Consequently, renal function recovered. The mice were treated to induce renal injury and examined how the gut microbiota changed to ensure that certain gut bacteria changed. In this study, it was found that some protective factors for the kidney are produced from intestinal bacteria.

Keywords: Sequelae of type 2 diabetes, Kidney function, Urinary proteins, *plant enzyme,* Blood urea nitrogen, Creatinine

INTRODUCTION

Recently, it has been shown that there is a close relationship between constipation and reduced kidney function. Increased severity of constipation is known to be associated with increased severity of renal dysfunction. Interestingly, intestinal bacteria have been shown to have a role in these problems [1]. Renal failure is a disease in which the function of the kidney's declines. Renal insufficiency includes acute renal failure and chronic renal failure. Acute renal failure can be completely cured in patients exhibiting temporary declining kidney function [2]. In chronic renal failure, the function of the kidneys gradually decreases. It is difficult to sufficiently suppress progression, and the number of patients on dialysis has been increasing, with >300,000 patients in Japan.

The main cause of renal failure and constipation in dialysis patients is a shortage of dietary fiber. Patients with a bad kidney should not ingest too much potassium. Therefore, vegetables rich in dietary fiber, dairy products, beans, and marine algae cannot be ingested sufficiently, which causes kidney failure and leads to constipation. Dialysis patients cannot take up much moisture. Because the amount of water that is incorporated into feces is also limited, the stool becomes hard, which makes defecation difficult [3].

Comprehensive measurement of metabolite concentrations, such as uremic toxins accumulating in the blood at the time of renal failure by metabolomic analysis, has shown that mice administered lubiprostone had decreased blood concentrations of indoxyl sulfate and hippuric acid, which are considered to be uremic toxins derived from enterobacteria. These results suggest that lubiprostone reduces the accumulation of uremic toxins through changes in the intestinal environment and intestinal microflora and inhibits the progression of chronic kidney disease. On the basis of the results of this study, the intestinal tract is considered to be the third uremic substance excretion pathway that functions similarly to urinary excretion and hemodialysis, and suggests that lubiprostone used as a remedy for constipation could also be used as a new therapeutic agent for chronic kidney disease [4].

In the present study, we investigated the effect of improving the intestinal microflora environment by administration of plant enzymes on chronic kidney damage, one of the sequelae of type 2 diabetes [5].

METHODS

Animals and breeding conditions

Five-week-old male type 2 diabetic nephropathy db/db mice were used in this study. Preliminary breeding was carried out for 1 week to acclimate the mice to the rearing conditions: a constant room temperature of 22°C ± 3°C, a relative humidity of 60%, and water and feed given ad libitum.

Experimental groups

The control group and the plant enzyme-administration group consisted of the db/db mice described above.

Method of administration

After 1 week of preliminary breeding, gavage by oral gastrectomy was performed every day until the end of the experiment. The concentration of plant enzymes was 500 mg/kg/day, and the same amount of distilled water was administered to the control group. We administered these enzymes to mice by oral administration for over 2 weeks.

Measurement item

Histological findings of the glomerular tissue of the kidney: Histological tissue samples were treated with 4% paraformaldehyde for 16 h and embedded in paraffin. A 6-μm thin section was stained with hematoxylin and eosin (HE). Renal cell tissue and kidney glomerular cell tissue were examined under a microscope. The renal glomerular cell tissue number was measured in five randomly selected visual fields.

Staining method of renal sections (HE method): The HE staining procedure is shown in Figure 1A, and the method of alcohol dehydration and penetration is shown in Figure 1B. Mouse kidney section specimens from each group were prepared, stained with Mayer's hematoxylin solution for 5 min, and rinsed with running water for 20 min. The samples were then dyed with 1% eosin solution for 2–4 min and lightly washed with water for 10 s, and the extra eosin liquid was washed. This procedure was repeated twice. The samples were dehydrated at 70%, 80%, 90%, 95%, and 99% (I, II) ethanol for 10 s each simultaneously with the valve color, dehydrated at the same time, penetrated with xylene I and II, and then sealed with oikit.

Figure 1: Protocol of HE stain (A) and alcohol dehydration and penetration (B).
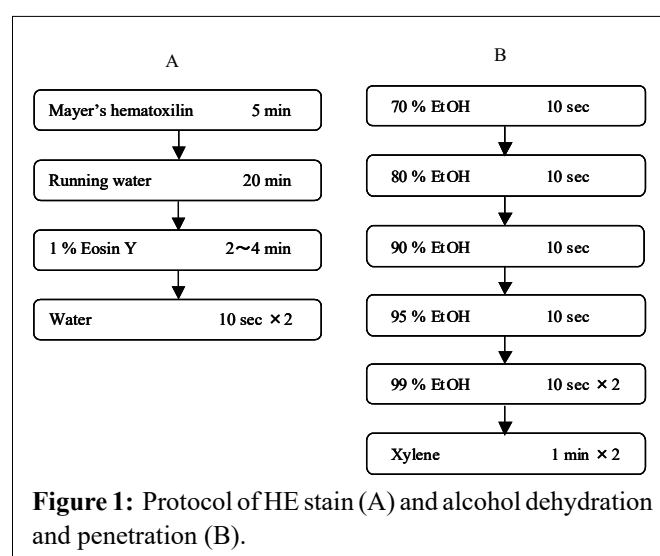
Intestinal immunity [humoral immunity: immunoglobulin A (IgA)] and intestinal action: IgA was measured by performing an enzyme-linked immunosorbent assay (ELISA) using a mouse IgA ELISA Quantitation Kit (BETHYL) and serum. A kit for the colorimetric determination of mouse IgA by sandwich ELISA was used. The ELISA kit consisted of a coat antibody, a horseradish peroxidase-labeled antibody, and a standard. The measurement range was 15.625–1000 ng/mL, and serum and plasma were used as measurement samples. Detection was performed using the coloration and assay methods. The number of sandwich ELISA (construction) assays was 1000.

The following procedure was used [6, 7]:

1. Affinity purified antibody was diluted 100-fold with immobilized antibody diluent.

2. A 100-µL aliquot of the above antibody solution was added to each well of the microplate and allowed to stand (solid phase) for 60 min at room temperature.

3. Washed twice with Tris-buffered saline (TBS) supplemented with TBS.

4. A 200-µL aliquot of 1% bovine serum albumin (BSA)–TBS was added to each well and allowed to stand at room temperature for 30 min.

5. Washed twice with TBS supplemented with TBS.

6. Standard serum and serum samples were diluted in diluent (1% BSA–TBS with 0.05% Tween 20) as follows: standard serum at a concentration of 0.24 mg/mL was diluted 240–15,360 times to give working standard solutions of 1000, 500, 250, 125, 62.5, 31.25, and 15.6 ng/mL.

Serum samples: Mice serum samples were diluted in the concentration range of the working standards.

1. For each plate, 100 µL of the serially diluted standard serum, as described above, was added in duplicate to each well, and appropriately diluted sample serum was similarly added in duplicate to each well.

2. The wells were allowed to stand at room temperature for 60 min.

3. The wells were washed twice with TBS.

4. Enzyme-labeled antibody IgA was diluted 80,000 times, and 100 µL of each was added to each well and allowed to stand at room temperature for 60 min.

5. The wells were washed three times with TBS.

6. Enzyme substrate solutions were prepared by adding 100 µL of each to each well and allowed to stand at room temperature for 5–30 min. To quench the reaction, 10 µL of 2M H_2SO_4 was added to each well.

7. The absorbance at 450 nm was measured using a microplate reader.

8. In the concentration calculation method, a standard curve was created with the X axis as the density and the Y axis as the absorbance (logarithmic scale). An approximate curve was obtained in a concentration range with good linearity, and the concentration of each sample was calculated from the equation [8, 9].

Mouse blood test for kidney disease

Urinary proteins: Proteins such as albumin and globulin remain in the blood even though it is filtered by renal glomeruli. However, when the glomeruli are injured, the proteins appear in the urine; hence, proteinuria is one of the indices of kidney damage. Urinary proteins were measured using a Urinary Protein Measurement Kit (New Urise BT) from TERUMO Co., Ltd. (Japan).

Blood urea nitrogen (BUN): BUN represents the nitrogen content of urea in the blood and is physiologically synonymous with urea. Urea, together with other compounds such as creatinine and uric acid, is a terminal metabolite of nitrogen-containing substances. Urea is synthesized from ammonia and CO_2 produced by deamination of amino acids primarily in the liver by the urea cycle. BUN is filtered through renal glomeruli, partially absorbed in tubules, and then excreted in the urine; hence, blood and urine measurements are indicative of kidney function. In vivo, the ingested protein becomes an amino acid, and the nitrogen component of the degradation product eventually becomes urea and is excreted in the urine. Because of this, urea in the blood cannot be excreted outside the body if renal function decreases; hence, its concentration increases, and this is one of the indicators of kidney function. Compared with creatinine, the urea blood concentration tends to change because of nutritional conditions such as dehydration; therefore, it is inferior to creatinine as an indicator of kidney function. A DetectX® Urea Nitrogen (BUN) Colorimetric Detection Kit (Funakoshi Co., Ltd., Japan) was used to calorimetrically measure BUN.

Creatinine: Creatinine is the metabolic end product of creatine and is an anhydrate obtained by nonenzymatically removing H_2O from creatine. Creatine is synthesized in the liver and kidney from three amino acids (glycine, arginine, and methionine), and most of it is held in skeletal muscle as creatine or creatine phosphate. Creatinine, which is one of the nonproteinaceous nitrogen compounds in blood, is filtered through the renal glomeruli and excreted in the urine almost without resorption. Therefore, measurement of blood creatinine is an index of filtration function in the kidney, and its clearance is a useful parameter to evaluate kidney function. A creatinine measurement kit was used (Creatinine Assay Kit) from Funakoshi Co., Ltd. (Japan).

Statistical analysis

The experimental results are expressed as mean ± standard deviation (SD). As the first test, each test sample in the administration group and the control group was evaluated by performing Student's t-test was performed to compare one group (control) with each test sample in the administration group. Measurement data were statistically processed using Stat View software version 5.0 (HULINKS, Japan) and presented as mean ± standard error. Variance analysis was used for intergroup comparisons of continuous variables. Differences were determined to be statistically significant for P values <0.05. Reproducibility was assessed by performing each experiment more than once. This study was approved by the Suzuka University of Medical Science Animal Research Ethics Committee (Ref: 423/16).

RESULTS

Histological findings of the glomerular tissue of the kidney

Histological changes in the kidney: The renal function recovery by plant enzyme administration in a diabetes model mouse (db/db mice) was examined. Microscopic
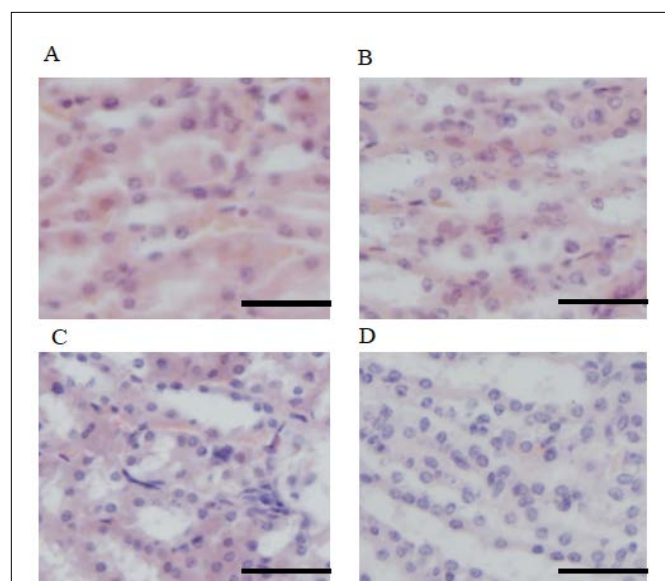
Figure 2: Study of renal function recovery by administration of plant enzymes using diabetes model mouse (db/db mice). Histopathological alterations of the kidney sell and glomeruli of the kidney regions in db/db mice. Microphotographs of coronal sections of the kidney cell in control (A) and plant enzyme (B) were stained with hematoxylin and eosin. Microphotographs of coronal sections of the glomeruli of the kidney in control (C; kidney failure) and plant enzyme (D; Improvement of renal failure) were stained with hematoxylin and eosin. Bars = 40 mm (A-D)

photographs of kidney histopathological changes in db/db mice and coronal sections of the kidney cells in the kidney region control group (Figure 2A) and the plant enzyme-administration group (Figure 2B) were stained with HE. Microscopic histological findings from glomerular corpuscles in the control group (Figure 2C) and the plant enzyme-treated group (Figure 2D) kidney microscopic histological findings showed recovery in the plant enzyme-treated group in all the kidney tissues.

Intestinal immunity (humoral immunity: IgA) and intestinal action

The intestinal (IgA) concentration was examined to examine the effect of reducing intestinal immunity (humoral immunity: IgA) and the burden of the kidney by intestinal action. As can be seen from Figure 3, intestinal (IgA) concentration was increased from the 5th week to the 7th week of the plant enzyme administered group as compared with the control group ($P < 0.05$)..

### Mouse blood and urinary protein test of kidney disease

Urine protein: Kidney function was measured by mouse urinary protein (Urine protein) measurement of kidney disease. As shown in Figure 4, the decrease of the numerical value of urine protein ($p < 0.05$) was observed from the 5th week to the 7th week of the plant enzyme administered group as compared with the control group.

BUN analysis: BUN represents the nitrogen content contained in urea in the blood and is physiologically synonymous with urea. Kidney function was assessed by measuring BUN in a mouse blood test of kidney disease. As shown in Figure 5, a decrease in BUN concentration was observed from the fifth to the seventh week of the plant
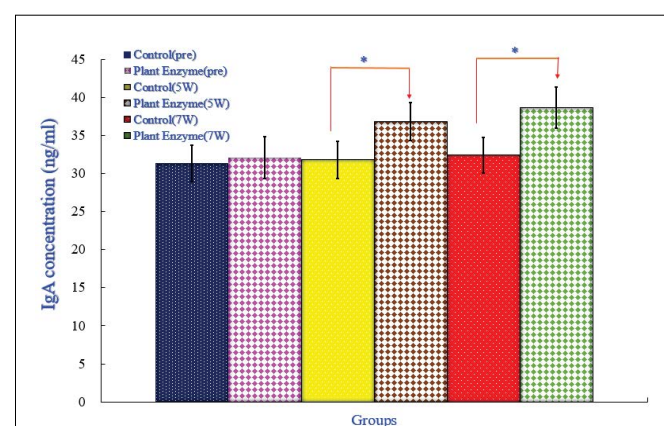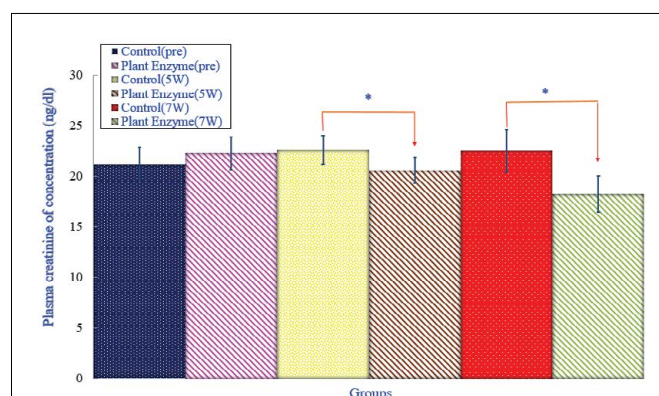

Figure 3: The effects of the plant enzymes of repeated administration on the levels of IgA in mice. Changes in the levels were obtained. The results measured represent ± S.D. IgA concentration (ng/ml) were also measured (*$P < 0.05$), *significantly different from the control group ($p < 0.05$).
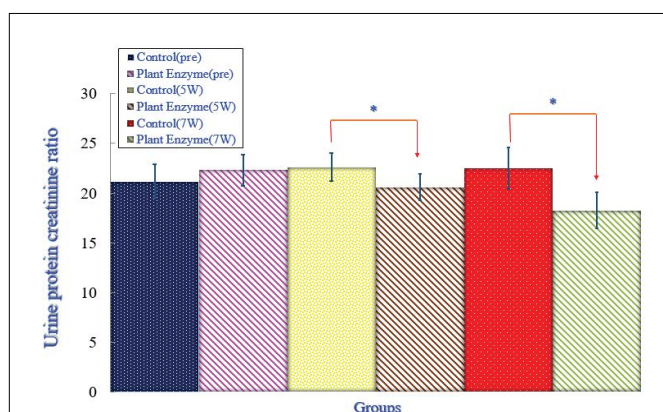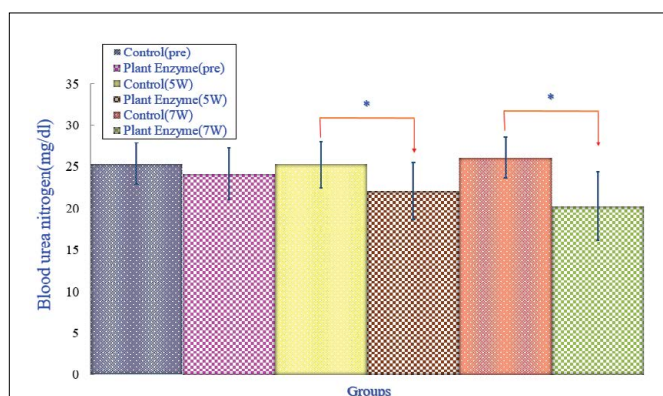
Figure 4: The effects of the plant enzymes of repeated administration on the levels of urine protein creatinine ratio in mice. Changes in the levels were obtained. The results measured represent ± S.D. Urine protein creatinine ratio were also measured (*P<0.05*), *significantly different from the control group (*p <0.05*).

Figure 6: The effects of the plant enzymes of repeated administration on the levels of plasma creatinine of concentration (ng/dl) in mice. Changes in the levels were obtained. The results measured represent ± S.D. Plasma creatinine of concentration (ng/dl) were also measured (*P<0.05*), *significantly different from the control group (*p <0.05*).

Figure 5: The effects of the plant enzymes of repeated administration on the levels of blood urea nitrogen (mg/dl) in mice. Changes in the levels were obtained. The results measured represent ± S.D. Blood urea nitrogen (mg/dl) were also measured (*P<0.05*), *significantly different from the control group (*p<0.05*).

enzyme-administered group relative to that in the control group ($P < 0.05$).

Creatinine analysis: Creatinine is a waste product made from a substance in muscle that is discharged into the urine after being filtered by the kidneys. The amount of creatinine is related to muscle and momentum.

The concentration of creatinine in the blood is an indicator of kidney function. When renal function is impaired, the amount of excretion decreases and the concentration of creatinine in the blood increases. Kidney function was assessed by measuring mice creatinine concentrations in kidney disease. As shown in Figure 6, a decrease in

creatinine concentration was observed from the fifth to the seventh week of the plant enzyme-administration group relative to that in the control group ($P < 0.05$).

DISCUSSION

In this study, we investigated several immunological and physiological mechanisms that regulate renal function recovery after administration of plant enzymes to diabetic nephropathy db/db mice. Recent experimental data have indicated that intestinal microflora significantly affects the outcome of renal disorder [1–3]. Studies have been conducted to investigate controlling the balance of intestinal microflora to suppress the deterioration of renal function in chronic kidney disease [4]. The results of the present study demonstrated that intestinal bacterial flora had a negative effect on kidney disease called uremic toxin production but also had beneficial effects such as short-chain fatty acid production and amino acid metabolism. As a result, it is thought that kidney disease is more likely to deteriorate in patients without a plexus [5]. This finding suggests that controlling the balance of intestinal flora is important in preventing the progression of chronic kidney disease. The accumulation of neutral fat has been reported to be one of the causes of arteriosclerosis and diabetes.

Histological findings of the glomerular tissue of the kidney (histological changes in the kidney). The renal function recovery by plant enzyme administration in a diabetes model mouse (db/db mice) was examined. Microscopic histological findings of tissue samples from the glomerular corpuscular section of the kidney after administration of plant enzymes demonstrated recovery in the plant enzyme-administered group. In renal failure, deterioration

of intestinal barrier function, intestinal mucosal disorder, decrease in intestinal tract function, and accumulation of uremic toxins produced in the intestinal tract are observed. This condition further promotes chronic kidney disease, which is assumed to cause not only kidney death but also cardiovascular disorders [6, 7].

Intestinal immunity (humoral immunity: IgA) and intestinal action. IgA decreases gut-associated lymphoid tissue (GALT) function, including intestinal lymphocyte counts and secretory IgA level [8, 9]. In this study as well, IgA concentration in the intestines was examined to investigate the effect of reducing intestinal immunity and the burden on the kidney caused by intestinal action. We found that IgA concentration increased in the plant enzyme-treated group. Therefore, administration of plant enzymes not only increased GALT function, including intestinal lymphocyte counts and the secretory IgA level, but also decreased the molecular weights of proteins at the stage of nutritional absorption, decreased the burden on the kidneys, and eventually led to kidney function recovery [10, 11].

Mouse blood and urinary protein test of kidney disease (urine protein). Proteins such as albumin and globulin remain in the blood even after the blood is filtered by the renal glomeruli. However, urine protein appears in the urine when the renal glomeruli are injured; therefore, proteinuria was used as an indicator of kidney damage. The assessment of kidney function in diabetic nephropathy db/db mice by measuring urinary protein showed a decrease in the concentration of urinary protein from the fifth to the seventh week in the plant enzyme-administered group relative to that in the control group. Therefore, by administration of plant enzymes, the intestinal function was improved, which led to a reduction in the molecular weights of proteins even at the nutrient absorption stage, a reduction in the burden on the kidneys, and renal function recovery [12–15].

BUN. Proteins ingested into the body are broken down into amino acids; the nitrogen components of the degradation products eventually become urea and are excreted in the urine. Because of this, urea in the blood cannot be excreted outside the body if renal function decreases; the concentration of urea therefore increases, which is why it is one of the indicators of kidney function. Compared with creatinine, the concentration of urea in blood tends to change because of nutritional conditions such as dehydration; hence, it is inferior to creatinine as an indicator of kidney function.

BUN represents the nitrogen content of urea in the blood and is physiologically synonymous with urea. The assessment of kidney function by measuring mouse BUN in kidney disease showed a decrease in the concentration of BUN from the fifth to the seventh week in the plant enzyme-administered group relative to that in the control group.

Therefore, administration of plant enzymes improved intestinal function, reduced the molecular weights of proteins even at the nutrient absorption stage, reduced the burden on the kidneys, and presumably recovered renal function [16–18].

Creatinine. Plasma creatinine is a waste product of creatine, a type of amino acid used in whole body muscle. An almost constant amount goes out of the muscle every day and is excreted out of the body from the kidneys. As renal function decreases, the amount of excretion from the kidney decreases; the creatinine concentration in the blood therefore increases and is an important indicator of kidney function.

Creatinine is a waste product made from a substance in muscle and is discharged into the urine after being filtered by the kidneys. The amount of creatinine is related to muscle and momentum.

The concentration of creatinine in the blood is an indicator of kidney function. When renal function is impaired, the amount of excretion decreases and the concentration of creatinine in the blood increases.

The assessment of kidney function by measuring mouse creatinine in kidney disease showed a decrease in BUN concentration from the fifth to the seventh week in the plant enzyme-administered group relative to that in the control group. Therefore, administration of plant enzymes improved intestinal function, reduced the molecular weights of proteins even at the nutrient absorption stage, reduced the burden on the kidneys, and recovered renal function [19–21].

While the intestinal flora has a negative effect on kidney disease, the production of urinary toxins, it plays a beneficial role such as short-chain fatty acid production and amino acid metabolism, resulting in the absence of the intestinal flora. Kidney disease is more likely to get worse. It is suggested that regulation of intestinal microflora balance is important for preventing the development of chronic kidney disease. This study revealed one of the roles of intestinal flora in the pathogenesis of chronic kidney disease [22].

The mice were treated to induce renal injury and examined how the gut microbiota changed to ensure that certain gut bacteria changed. In this study, it was found that some protective factors for the kidney are produced from intestinal bacteria. Administration of food enzymes activated enterobacteria and increased the factor "D-amino acid" that ameliorates kidney disease [23].

D-serine was produced by intestinal bacteria and was found to reach the kidney via blood. Further, in the kidney, in addition to D-serine derived from intestinal bacteria, the synthesis of D-serine by the kidney also occurs, and the
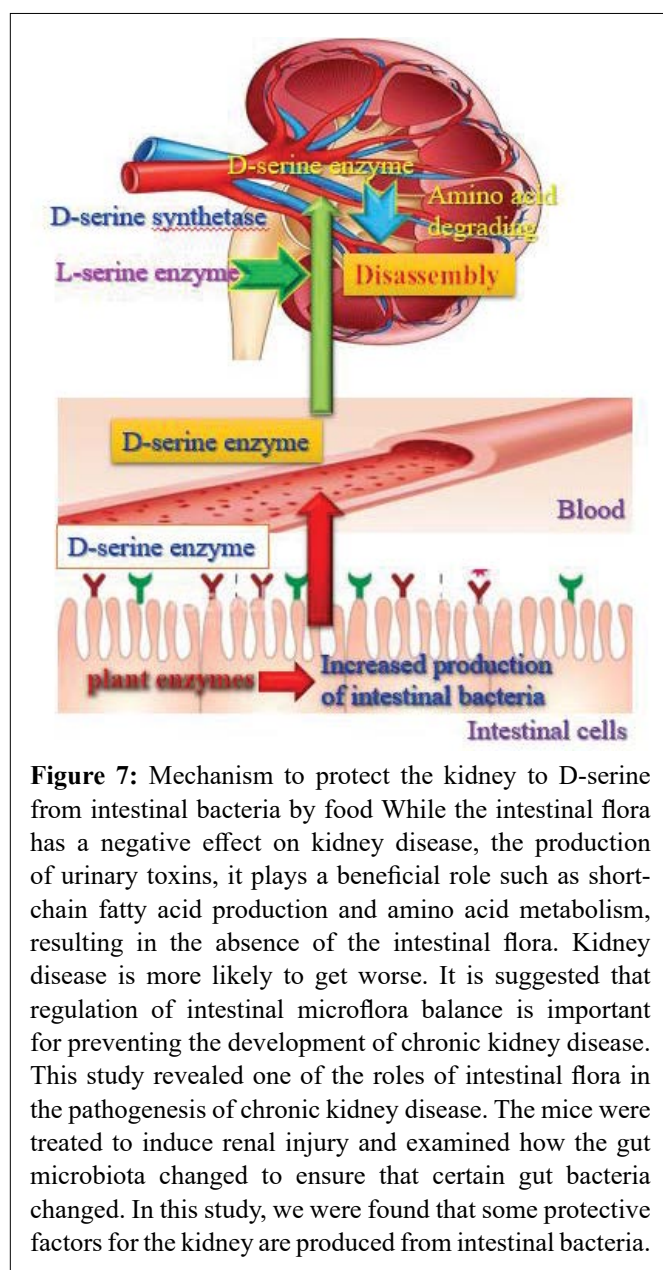
Figure 7: Mechanism to protect the kidney to D-serine from intestinal bacteria by food While the intestinal flora has a negative effect on kidney disease, the production of urinary toxins, it plays a beneficial role such as short-chain fatty acid production and amino acid metabolism, resulting in the absence of the intestinal flora. Kidney disease is more likely to get worse. It is suggested that regulation of intestinal microflora balance is important for preventing the development of chronic kidney disease. This study revealed one of the roles of intestinal flora in the pathogenesis of chronic kidney disease. The mice were treated to induce renal injury and examined how the gut microbiota changed to ensure that certain gut bacteria changed. In this study, we were found that some protective factors for the kidney are produced from intestinal bacteria.

D-serine concentration increases. Therefore, even in human acute kidney injury patients, D-serine in the blood shows a high value as compared with normal persons, and shows a high correlation with creatinine which is an index of kidney disease. In the human body, it is thought that intestinal bacteria produce D-amino acids and have a mechanism that protects the kidneys via blood [24-26] (Figure 7).

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This study revealed one of the roles of intestinal flora in the pathogenesis of chronic kidney disease. The mice were treated to induce renal injury and examined how the gut microbiota changed to ensure that certain gut bacteria changed. In this study, we were found that some protective factors for the kidney are produced from intestinal bacteria.

CONCLUSION

In this study, we investigated whether the administration of plant enzymes could improve the intestinal microflora environment in a chronic renal injury mouse model (db/db mice). The intestinal environment was improved by the plant enzymes, which showed that improvement of intestinal function could improve chronic kidney disease. Decreases in the concentrations of urinary proteins were observed from the fifth to the seventh week in the plant enzyme-administration group relative to the concentrations in the control group.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reason able request.

CONFLICTS OF INTEREST

The authors declare no competing interests regarding the final version of the manuscript.

FUNDING STATEMENT

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