Studies on the acceleration of renal decline in rat models of diabetic kidney disease

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Contents

List of Abbreviations

Abbreviation	Term
ACE	Angiotensin-converting enzyme
ACR	Albumin-to-creatinine ratio
ARB	Angiotensin receptor blocker
BUN	Blood urea nitrogen
CKD	Chronic kidney disease
CVD	Cardiovascular disease
Ccr	Creatinine clearance rate
Cre	Creatinine
DKD	Diabetic kidney disease
DN	Diabetic nephropathy
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
FFA	Free fatty acid

List of Abbreviations (continued)

Abbreviation	Term
FITC	Fluorescein isothiocyanate
GFR	Glomerular filtration rate
HE	Hematoxylin and eosin
Hb	Hemoglobin concentration
HbA1c	Hemoglobin A1c
Hct	Hematocrit
KDIGO	Kidney Disease: Improving Global Outcomes
MRA	Mineralocorticoid receptor antagonist
PAS	Periodic acid-schiff
pCre	Plasma creatinine
RAAS	Renin-angiotensin-aldosterone system
RBC	Red blood cell
S.D.	Standard deviation
SBP	Systolic blood pressure
SD	Sprague Dawley

List of Abbreviations (continued)

Abbreviation	Term
SDT	Spontaneously Diabetic Torii
SGLT	Sodium-glucose co-transporter
SHR	Spontaneously hypertensive rats
SHR/cp	SHR/NDmcr-cp (cp/cp)
STZ	Streptozotocin
TC	Total cholesterol
TG	Triglyceride
UACR	Urinary albumin-to-creatinine ratio
UNx	Unilateral nephrectomy
UP	Urine protein excretion
WKY	Wistar Kyoto

Chapter 1

General Introduction

Chronic kidney disease (CKD) is a risk factor for end-stage renal disease (ESRD) and cardiovascular disease (CVD), with the number of patients increasing worldwide. In addition, declining glomerular filtration rate (GFR: the best overall indicator of the level of kidney function) and albuminuria or proteinuria are both independent risk factors for and associated with CVD, that is, the risk of CVD increases with lower GFR and higher levels of albuminuria or proteinuria. In Japan, it is estimated that approximately 14.6% of the adult population, or 14.8 million people, is affected by CKD (Nagai *et al.*, 2021). Diabetic nephropathy (DN) is the main reason for the introduction of dialysis and the leading cause of end-stage renal failure in Japan. Its proportion is approximately 40% of all nephropathy cases, making it a serious healthcare and economic problem. The prevalence of DN in diabetic patients in Japan is reported to be 42.1% in patients with type 2 diabetes with albuminuria (Yokoyama *et al.*, 2007).

There are numerous underlying causes for the etiology of CKD, including lifestyle-related diseases such as diabetes, hypertension and dyslipidemia. Irrespective of the associated disease, the progression of CKD is characterized by the loss of functional renal cells, such as podocytes, and their subsequent replacement by other cells or extracellular matrix (ECM) or both, epithelial-to-mesenchymal transition (EMT) and other factors resulting in glomerulosclerosis and tubulointerstitial fibrosis.

Thus, the consequences of CKD are glomerulosclerosis and tubulointerstitial fibrosis caused by an imbalance between excessive synthesis and reduced breakdown of the ECM (Nogueira *et al.*, 2017).

Prognosis of CKD by GFR and albuminuria category

To assess diabetic kidney damage, kidney function tests are performed and GFR and urinary albumin-to-creatinine ratio (ACR) are calculated. GFR and ACR groups are created according to the Kidney Disease: Improving Global Outcomes (KDIGO) 2012 Clinical Practice Guidelines classification (Figure 1) (KDIGO Blood Pressure Work Group, 2021). CKD stage is categorized according to GFR as follows: CKD stage G1, G2, G3a, G3b, G4, G5 (≥90, 60–89, 45–59, 30–44, 15–29 and <15 mL/min/1.73 m^2 respectively). On the other hand, CKD stage is classified according to ACR as: A1, A2 and A3 (<30, 30–300 and >300 mg/mg respectively). Finally, CKD risk probability is assigned based on the KDIGO classification of CKD prognosis by GFR and albuminuria categories as follows: low risk (G1 or G2 and A1), moderate risk (G1 or G2 and A2, G3a and A1), high risk (G1 or G2 and A3, G3a and A2, G3b and A1) and very high risk (G3a and A3, G3b and A2 or A3, G4 and A1, A2 or A3, G5 and A1, A2 or A3) (Ale-Chilet *et al.*, 2021). For measurement of GFR, inulin clearance is the gold standard, however, as the measurement is complicated, the estimated glomerular filtration rate (eGFR) based on serum creatinine or cystatin C concentration is generally used. The eGFR is calculated with the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Levey *et al.*, 2009). At present, much of the evidence on clinical decision making in relation to CKD is based solely on GFR.

Therapeutic agents for CKD

For more than two decades, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) have been used to treat DN as well as

hypertension (Kota *et al*., 2012). However, the number of target patients receiving these agents is limited (Parving *et al*., 2001; Keane *et al*., 2003). Losartan, for example, is only indicated for the treatment of DN with elevated serum creatinine and proteinuria (urinary ACR [UACR] >300 mg/g) in patients with type 2 diabetes and a history of hypertension. According to a recent report, ARBs may suppress ACR in advanced CKD patients with G3b (Kim-Mitsuyama *et al.*, 2018). In practice, however, it is expected that losartan will be effective in patients with DN in CKD stage G3a or milder stages.

Recently, sodium-glucose co-transporter (SGLT) 2 inhibitors, and the nonsteroidal mineralocorticoid receptor antagonist (MRA) finerenone all reduced the risk of progression to diabetic kidney disease (DKD). According to the results from the EMPA-KIDNEY, CREDENCE and DAPA-CKD clinical trials, SGLT2 inhibitors showed efficacy in DKD patients with eGFR $\geq$ 20 mL/min/1.73 m^2 (including some patients with stage G4 CKD) (Perkovic *et al.*, 2019; Heerspink *et al.*, 2020; Herrington *et al.*, 2022). Notably, empagliflozin was even effective in non-diabetic patients with CKD (Herrington *et al.*, 2022). Also the MRA finerenone showed benefits in DKD patients with eGFR 25-90 mL/min/1.73 m^2 in the FIDELIO-DKD and FIGARO-DKD clinical trials (Bakris *et al.*, 2020; Pitt *et al.*, 2021). However, the clinical outcome rates for patients treated with ACE inhibitors or ARBs are still in the high range, although slightly lower with the addition of SGLT2 inhibitors or MRA. Thus, it has been suggested that those patients receiving the novel therapies mentioned above as well as those with advanced CKD (CKD stage G4 and G5) or rapidly progressing CKD remain in high unmet therapeutic need (Fried *et al.*, 2022).

Animal models of CKD

The use of animal models such as surgical, chemical and physical models, spontaneous models and genetic models is crucial to our understanding of CKD. For the above reasons, especially the development of animal models of diabetes with a background of various CKD-associated diseases is helpful in the search for mechanisms and pharmacological evaluation of renal function decline.

Various animal models of DKD are known, however, only a few of them develop the complex etiological complications associated with decreased renal function in patients with DKD (Chander *et al*., 2004; Shinozaki *et al*., 2022). As examples of DKD models with complex etiological complications, Zucker diabetic fatty rats, Spontaneously Diabetic Torii (SDT) fatty rats, and uni-nephrectomized db/db mice develop nephropathy with histopathological changes and show no renal function decline as indicated by creatinine clearance rate (Ccr) or GFR during the observation period, but rather an increase in GFR due to glomerular hyperfiltration (Mizuno *et al*., 2006; Rosenthal *et al*., 2010; Sano *et al*., 2021; Maekawa *et al*., 2022). Therefore, currently few pharmacologically available animal models exhibit renal function decline due to the complex etiopathogenic characteristics of the disease that these animals suffer from.

Purpose

To understand the etiology of glomerulosclerosis and renal fibrosis and for the evaluation of new treatments, it is crucial to develop animal models that can assess renal function decline using Ccr or GFR. Here, I attempted to develop animal models with a pathological background of diabetes mellitus that could be used to evaluate decline in renal function. In Chapter 2, I will describe performing unilateral nephrectomy (UNx) in

spontaneously hypertensive rats [(SHR)/NDmcr-cp (cp/cp) (SHR/cp) rats] to establish a long-term model of declining renal function with pathological change. In Chapter 3, I will present my investigation of a short-term model of accelerated renal function decline in SDT fatty rats induced by salt loading via drinking water as well as UNx.

In these two models, changes in renal function-related parameters and metabolic parameters as well as renal histopathology were investigated and evaluated. In addition, losartan, an ARB, was used as a tool for efficacy studies and for comparison of its efficacy in each model with that in patients with CKD.

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				< 30 mg/g < 3 mg/mmol	30-300 mg/g 3-30 mg/mmol	> 300 mg/g >30 mg/mmol
GFR categories (ml/min/1.73 m^2) Description and range	G1	Normal or high	≥ 90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	< 15			

Figure 1. CKD stages and risk prognosis adapted from KDIGO clinical guidelines, 2012.

Prognosis of CKD by GFR and albuminuria categories, as per the KDIGO 2012 classification. Green, low risk (if no other markers of kidney disease, no CKD); yellow, moderately increased risk; orange, high risk; red, very high risk (Ale-Chilet *et al.*, 2021).

Chapter 2

Unilateral nephrectomized SHR/NDmcr-cp rat shows a progressive decline of glomerular filtration with tubular interstitial lesions

Introduction

DKD is characterized by a complicated pathology that involves renal anemia, obesity, hypertension, hyperlipidemia, and hyperglycemia, accompanied by a decline in renal function. The eGFR or Ccr is frequently used to assess renal function in patients with DKD. For over a decade, ACE inhibitors and ARBs such as ramipril and losartan have been used to treat DN other than hypertension (Kota *et al.*, 2012). However, the target patients of these agents are limited to those suffering from DN with hypertension, type 2 diabetes mellitus, and proteinuria (Parving *et al.*, 2001; Keane *et al.*, 2003). In recent years, evidences are accumulating that SGLT2 inhibitors prevent disease progression in established heart failure or CKD, independent of the presence of diabetes (Heerspink *et al.*, 2020; Packer *et al.*, 2020), and it is likely that the basic medication used for CKD as well as DKD patients may be changed in the near future to SGLT2 inhibitors (Tuttle *et al.*, 2021).

Various animal models of DKD are known, however, the complex etiological complications associated with decreased renal function in patients with DKD, develop in only a few of them (Chander *et al.*, 2004; Shinozaki *et al.*, 2022). As an example, in the widely used streptozotocin (STZ)-induced type 1 diabetes model, blood glucose levels are elevated but, unlike in humans, hypertension, albuminuria level, and the loss of renal function are often much less severe (Tesch *et al.*, 2007). Whereas, Zucker diabetic fatty rats, SDT fatty rats, and uni-nephrectomized db/db mice, which are DKD

models with such complications, developed nephropathy with histopathological changes, however, these models did not show renal function decline as indicated by Ccr or GFR during the observation period, but rather showed an increase in GFR due to glomerular hyperfiltration (Mizuno *et al.*, 2006; Rosenthal *et al.*, 2010; Sano *et al.*, 2021; Maekawa *et al.*, 2022). Therefore, there are currently few pharmacologically available animal models that exhibit renal function decline due to the complex etiopathogenic characteristics of the disease that these animals suffer from.

The SHR/cp rat is an obese, type 2 diabetic model of DKD characterized by hyperglycemia, hyperlipidemia, and hypertension with the same hypertensive background as SHR, as well as a genetic mutation in the leptin receptor gene (Ohtomo *et al.*, 2010; Kawai *et al.*, 2012). By utilizing SHR/cp rats, we attempted to establish a DKD model showing renal function decline as assessed by Ccr. To cause the decline in renal function, UNx was performed, and in addition, the efficacy of the ARB (losartan) was evaluated.

Materials and Methods

Animals

Male SHR/cp rats and age-matched Wistar Kyoto (WKY) rats were purchased from Japan SLC (Shizuoka, Japan), and maintained in a specific pathogen-free room at a temperature of 23 ± 3°C and air humidity of 55 ± 15%, on a 12-h/12-h light/dark cycle. This animal study was conducted in accordance with the Japanese Law for the Humane Treatment and Management of Animals (Law No. 105, October 1, 1973). Prior to the initiation of the animal study, an outline of the animal study protocol had been reviewed by the Institutional Animal Care and Use Committee of the Biological/Pharmacological

Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc.

Chemicals

Losartan (≥98% purity) was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). The losartan diet was prepared every week by mixing losartan with standard powdered chow (CRF-1 powder, Oriental Yeast Co., Ltd., Tokyo, Japan). The mixed diet containing 0.02% losartan (approximately 10 mg/kg/day) was fed for 39 weeks from 12 to 51 weeks of age.

Influences of UNx on renal function and related parameters in SHR/cp rats and effect of losartan on the model

The study design is shown in Figure 2. UNx of left kidney for SHR/cp rats at 7 weeks of age (twice as many rats used in the study) was performed as previously described (Katsuda *et al.*, 2014b; Shinozaki *et al.*, 2022). Three weeks later, half of the rats with 24-hour urine protein excretion (UP) closest to the mean of all rats treated with UNx were selected. Then these rats were allocated to two groups with 10 rats in each group so as to balance the means of the UP. The six WKY rats serving as a normal group were not treated with nephrectomy. Two more weeks later, starting at 12 weeks of age, these rats were fed a standard powder chow or mixed diet containing losartan for 39 weeks. The groups were: 1) UNx-treated SHR/cp rats fed a normal diet; 2) UNx-treated SHR/cp rats fed the 10 mg/kg losartan diet; 3) non-nephrectomized WKY rats fed a normal diet.

During the experimental period, body weight was measured sequentially, and blood and urine samples were collected from the tail vein and using metabolic cages,

respectively. As a parameter of renal function, Ccr was measured at 16, 24, 33, 41, 44, 48, and 51 weeks of age after starting the treatment at 12 weeks of age. Ccr was calculated based on plasma and urine creatinine (Cre) levels using the formula: Ccr (mL/min/100 g body weight) = urine Cre (mg/dL) × 24-hour urine volume (mL)/1440 (min) × 1/serum Cre (mg/dL) × 1/body weight (g) × 100. After the last sampling at 51 weeks of age, the rats were euthanized and their right kidneys were excised and processed for histological evaluation. Blood glucose, triglyceride (TG), total cholesterol (TC), plasma creatinine (pCre), blood urea nitrogen (BUN), and urine Cre levels were measured using an automatic biochemical analyzer (Model 7180, Hitachi High-Tech Corporation, Tokyo, Japan). Urinary protein levels were measured using a commercially available kit (Tonein-TPII; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The erythrocyte-related parameters (hemoglobin concentration [Hb], red blood cell [RBC] count, hematocrit [Hct]) were measured using a hematology analyzer (ADVIA® 120, Siemens Healthcare Diagnostics Ltd., Tokyo, Japan).

For histological analysis, the 10% neutral formalin-fixed right kidneys were sectioned and stained with hematoxylin and eosin (HE), periodic acid-schiff (PAS), and Sirius red. PAS staining was used to evaluate the degree of glomerular alteration; HE staining and PAS staining were used to evaluate the degree of tubulointerstitial alteration, and Sirius red staining was used to evaluate the degree of tubulointerstitial fibrosis. The histological evaluation was assessed by the following 7 parameters defined in the preliminary examination: increased mesangial matrix, glomerular crescent formation/adhesion, glomerular sclerosis/atrophy, tubular hyaline casts, tubular dilatation, regeneration/degeneration of tubular epithelium, and tubulointerstitial fibrosis. The severity of each histological change was scored on a 5-point scale ranging

from 0 to 4 (Score 0: normal; Score 1: minimal, solitary [very small] lesion; Score 2: mild, focal [small] lesion; Score 3: moderate, sporadic lesion; and Score 4: marked, diffuse lesion) based on the severity and distribution of the changes.

In preliminary study, hypertension in the model was assessed and the efficacy of losartan for hypertension was investigated. UNx of left kidney for SHR/cp rats at 7 weeks of age was performed as described above, and two weeks later, starting at 9 weeks of age, these rats were fed a standard powder chow or mixed diet containing losartan for 32 weeks. The groups were: 1) UNx-treated SHR/cp rats fed a normal diet; 2) UNx-treated SHR/cp rats fed the 10 mg/kg losartan diet; 3) non-nephrectomized WKY rats fed a normal diet (8 rats in each group). Systolic blood pressure (SBP) levels (mmHg) were measured by the indirect tail cuff method (Katsuda *et al*., 2014b) measured at 9, 14, 18, 23, 30, 35, and 40 weeks of age after starting the treatment at 9 weeks of age.

Statistical analysis

Data are expressed as the mean and standard deviation (S.D.) of the indicated numbers of animals or samples (Figure 2). All statistical analyses were performed using StatLight 2000 (Yukms Co., Ltd, Kanagawa, Japan). In a two-group comparison, the statistical significance was assessed using the Student's *t*-test (for homoscedastic data) or Aspin-Welch's *t*-test (for heteroscedastic data) after homoscedasticity analysis by an *F*-test. For histological scores, the statistical significance was assessed by Wilcoxon rank sum test for two-group comparison. All statistical analyses were two-sided, and statistically significant level was set at $P < 0.05$.

Results

Influences of UNx on renal function and related parameters in SHR/cp rats and the effect of losartan on the model.

A significant decrease in Ccr was observed in UNx-treated SHR/cp rats compared to untreated normal rats (WKY rats) (Figure 3A). In addition, significant increases in BUN levels and 24-hour UP were observed in UNx-treated SHR/cp rats during the experimental period (Figure 3B, 3C). Furthermore, marked reductions of the erythrocyte-related parameters, including RBC count, Hb, and Hct were observed (Figure 3E-G). On the other hand, pCre levels appeared to be similar between UNx-treated SHR/cp rats and WKY rats (Figure 3D).

Investigating the effects of losartan on these renal-related parameters, the results showed that dietary administration of losartan (0.02% losartan) significantly suppressed Ccr decline, hypertension, increases of BUN, UP, and pCre, and also reductions of the erythrocyte-related parameters (RBC count and Hb) in UNx-treated SHR/cp rats (Figure 3, Figure 4).

Effect on metabolic parameters

Higher blood glucose levels were observed in the UNx-treated SHR/cp rats until 24 weeks of age (Figure 5A), thereafter the levels were comparable to those of WKY rats. Plasma TG levels in the UNx-treated SHR/cp rats were elevated compared to WKY rats, whereas TC levels were similar during the experimental period (Figure 5B, 5C). Body weight gains of UNx-treated SHR/cp rats were significant compared to those of WKY rats up to 40 weeks of age, thereafter no further gains were observed (Figure 5D).

Administration of losartan had no effect on these parameters, except that significantly

higher body weight gains were observed at 51 weeks of age (Figure 5A-D).

Effect on renal histopathology

Histopathologically, in UNx-treated SHR/cp rats at 51 weeks of age, some glomerular, tubular, and tubulointerstitial lesions were prominently observed. Administration of losartan reduced glomerular lesions (mesangial matrix increase, adhesion/crescent, sclerosis/atrophy), tubular lesions (hyaline casts, dilatation, regeneration/degeneration of epithelium), and tubulointerstitial lesions (fibrosis) (Figure 6, Figure 7).

Discussion

In SHR/cp rats, UNx was able to induce a Ccr decline, accompanied by histopathological changes such as increased glomerular mesangial matrix, glomerular crescent formation/adhesion, glomerular sclerosis/atrophy, tubular hyaline casts, tubular dilatation, regeneration/degeneration of tubular epithelium, and tubulointerstitial fibrosis (Figure 3A, Figure 6, Figure 7). Furthermore, UNx-treatment of SHR/cp rats caused renal-related parameters such as urinary protein and BUN to increase, in contrast to levels of these parameters in normal rats (WKY rats) (Figure 3B-D). The hallmarks of DKD include decline in renal function as indicated by GFR or Ccr, proteinuria, and impaired renal morphology (increased glomerular basement membrane thickness, mesangial hyperplasia, interstitial fibrosis, glomerular hypertrophy, glomerulosclerosis, podocyte foot process effacement, and arterial hyalinosis) (Alicic *et al.*, 2017). The induction of decreased renal function by UNx in SHR/cp rats was suggested to be reminiscent of the pathological condition in patients with DKD, as indicated above.

Over the past few decades, various animal models of DKD have been developed.

However, complications due to complex etiologies associated with renal dysfunction in DKD patients appear in only a few of them. As an example, in the widely used STZ-induced type 1 diabetes model, blood glucose levels are elevated, however unlike in humans, hypertension, level of albuminuria, and the loss of renal function are often much less severe (Tesch *et al.*, 2007). The Dahl salt-sensitive hypertensive rat is a model of hypertension, however it is not a model for studying the risk factors typical of DKD, such as diabetes, obesity, and abnormal lipid metabolism. SDT Fatty rats and db/db mice, which are known diabetic models, show signs of DKD, such as abnormal lipid metabolism and obesity, however, during the lifetime of the animals, renal function is not influenced or in a hyperfiltration state, and infrequently declines as measured by Ccr (Gartner, 1978; Cohen *et al.*, 2001; Nangaku *et al.*, 2005). To promote renal dysfunction by reducing the number of nephrons, 5/6 nephrectomy and UNx in mice and rats are widely used to generate experimental models of CKD (Katsuda *et al.*, 2014b; Racanicchi *et al.*, 2015; O'Sullivan *et al.*, 2019). Therefore, to accelerate the decline of renal function, we performed UNx in SHR/cp rats, and this model exhibited a Ccr decline and might mimic the pathogenesis of DKD in patients.

In the present study, we performed UNx in the rats, which allowed us to establish a model that would lead to steady Ccr decline, hypertension and worsened renal function parameters, accompanied by renal histopathological changes. It was considered that the decreased number of nephrons due to nephrectomy causes an increased single nephron GFR, and the persistent hypertension due to hyperfiltration leads to glomerulosclerosis and enhances medullary hypoxia, resulting in further reduction in the number of nephrons and hypoxia due to interstitial fibrosis (Heyman *et al.*, 2008; Heyman *et al.*, 2019; Heyman *et al.*, 2020). Furthermore in our model, it was possible to observe renal

anemia (Figure 3E-G), which is associated with a decline in renal function. Renal anemia could reflect impaired erythropoietin (Epo) production by Epo-producing cells in the renal cortex and outer medulla region, and it could be correlated with declining renal function (Suzuki, 2015). In addition, plasma TG levels were elevated in SHR/cp rats treated with UNx in this study (Figure 5B). Plasma TGs could accumulate as lipid droplets and become toxic within cells such as renal tubular epithelial cells (Bobulescu, 2010; Afshinnia *et al.*, 2019). It has also been reported that TGs and free fatty acids (FFAs) bound to albumin accumulated in the proximal tubules could cause damage and induce inflammation (Bobulescu, 2010). Furthermore, in the preliminary, our model is characterized by hypertension (Figure 4).

In this study, decrease in blood glucose levels were observed in these rats (Figure 5A). The reason for the decrease might be related to the upward trend in the plasma insulin concentration of UNx-treated SHR/cp rats, as observed in a preliminary study (data not shown). However, initial duration of high glucose exposure has been suggested to cause long-term negative effects on the kidney, whereas its physiological mechanisms are not yet well defined (Roy *et al.*, 1990; Cooper *et al.*, 2010; Thomas, 2014). Possibilities include epigenetic programming, remodeling, and persistent post-translational modifications such as advanced glycation end-products (Cooper *et al.*, 2010). Further understanding of the molecular basis would certainly provide new targets for interventions to reduce the symptom burden of DKD patients, and our model might be helpful in identifying them.

This study confirmed the effect of losartan, which is widely used for the treatment of DN (Tuttle *et al.*, 2020). The results showed that dietary administration of losartan attenuated the Ccr decline and attenuated renal anemia induced by UNx in SHR/cp rats

(Figure 3). Even the results of histopathological evaluation were substantially improved by losartan. These results suggest that this model might mimic the pre-phase of the later stages of DKD, when ACE inhibitors and ARBs are effective in clinical treatment.

In preclinical research, the lack of animal models that mimic the pathophysiological characteristics of patients with DKD has been a hurdle to precisely addressing clinical needs (Noshahr *et al.*, 2020). Animal models might offer new insights into the development and progression of nephropathy in patients with DKD, and help us better understand the etiology of the disease. In addition, animal models could be used to explain how novel therapies might function, identify alternative pathways other than current therapies, and even help validate potentially adverse side effects.

In patients with DKD, risk factors such as dyslipidemia, hyperuricemia, and hypertension, in addition to diabetes mellitus, are considered to be complex pathophysiological determinants of the condition. Therefore, we considered it noteworthy that our model is also characterized by these risk factors in addition to diabetes. In contrast, it is unclear which of these risk factors, including the diabetes mellitus, contribute more specifically to the pathogenesis of DKD.

Previously, the ACE inhibitors and ARBs, two antihypertensive drugs that slow the progression of DN, have been extensively studied in numerous experimental DN models. However, not all of the typical features of DN are exhibited in many of these models. As an example, the mouse model has several limitations. Indeed, the classical DN model exhibits only early-stage DKD features such as moderate albuminuria, glomerular hypertrophy, and slight expansion of the mesangial matrix (Soler *et al.*, 2012). Glomerulosclerosis, tubular atrophy, or tubulointerstitial fibrosis is rarely observed in these animals. The present model of DKD developed by

uninephrectomizing SHR/cp rats is a novel animal model that exhibits many of the same features observed in patients with DKD and therefore might be helpful in better defining the mechanisms involved in this disease.

In recent years, preclinical and clinical studies have accumulated evidence for the efficacy of non-steroidal MRAs and SGLT2 inhibitors in treating CKD and DN (Zinman *et al.*, 2015; Neal *et al.*, 2017; Norgaard *et al.*, 2019; Perkovic *et al.*, 2019; Heerspink *et al.*, 2020; Patel *et al.*, 2021).

In the future, we expect to use our model to evaluate the effects of MRA or SGLT2 inhibitor alone or in combination and to understand the characteristics and limitations of the model by comparing the pathology of the DKD in our animal model with that of DKD in patients, which will allow us to further characterize pathogenic factors other than the current ones used as therapeutic targets in our model. Ultimately, we would like to establish our model as useful in evaluating the effects of many other agents with various mechanisms of action.

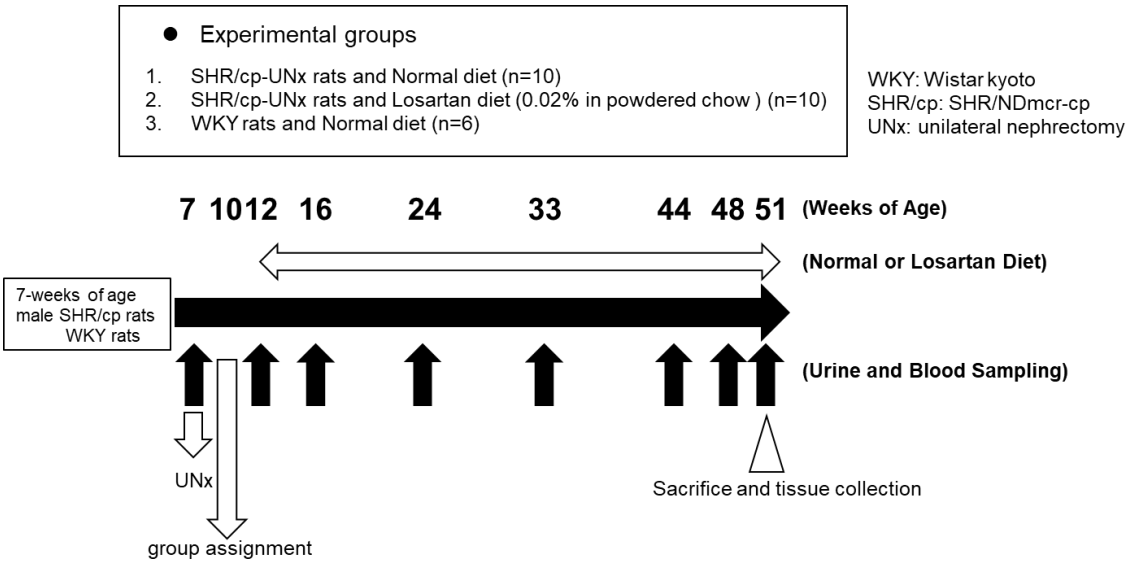

Figure 2. Experimental design.

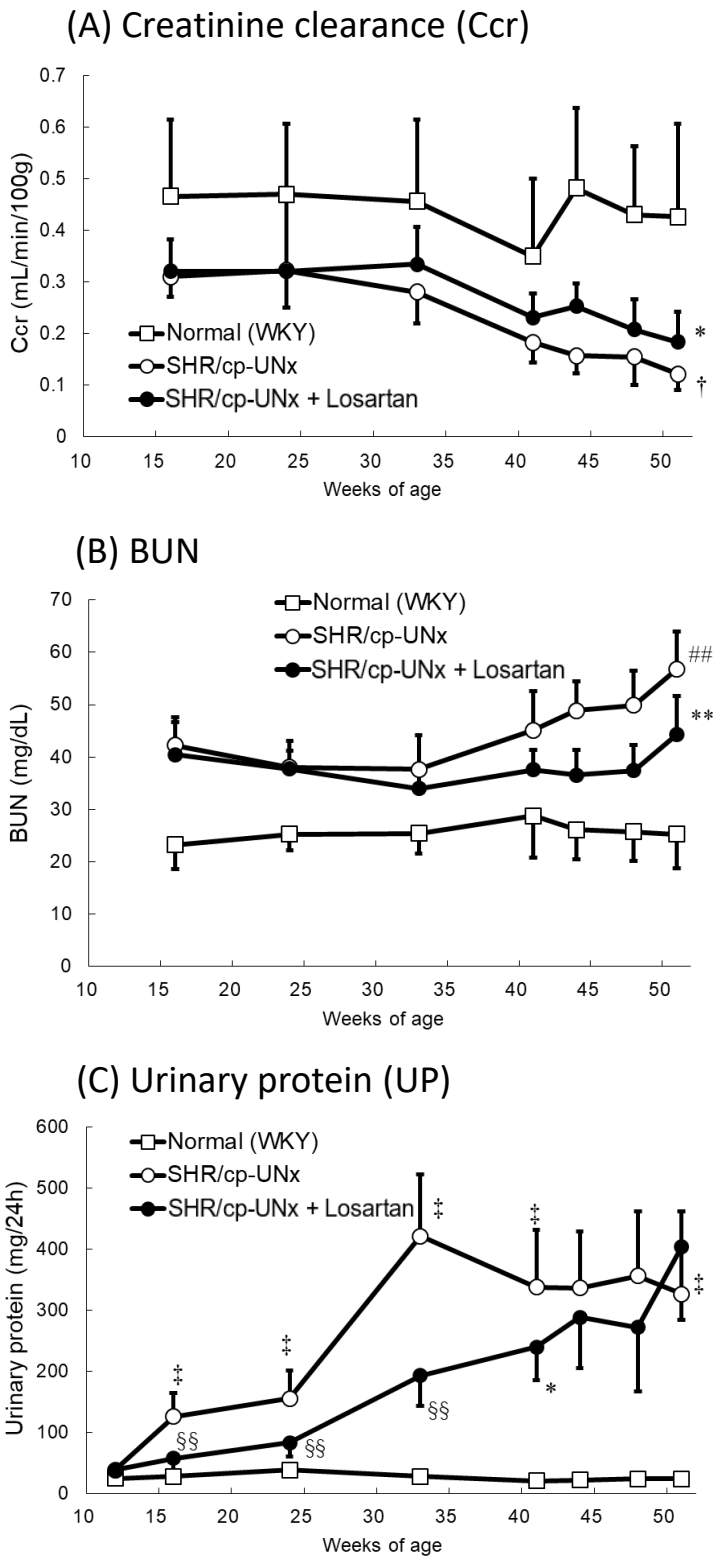

Figure 3. Influences of UNx on renal function and related parameters in SHR/cp rats and effect of losartan on the rats.

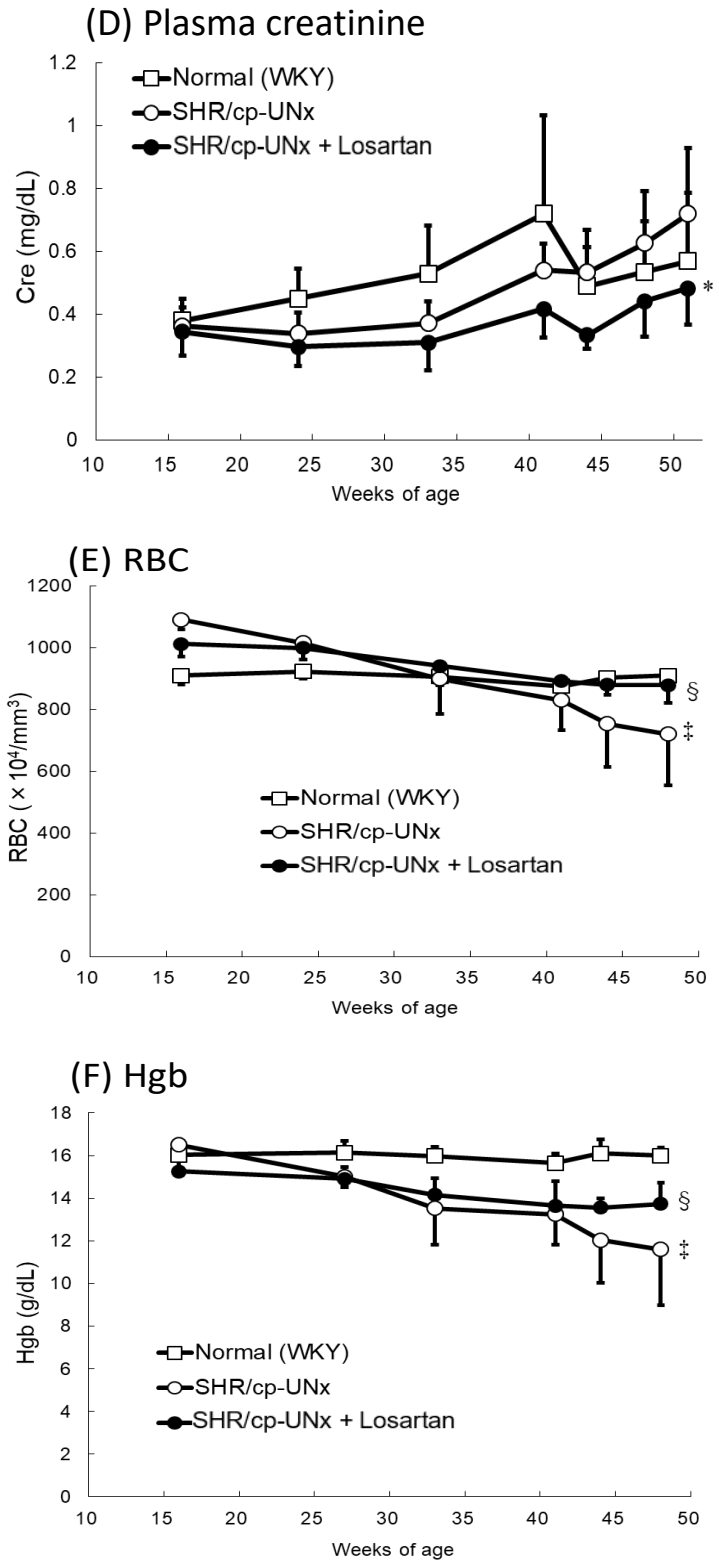

Figure 3. Influences of UNx on renal function and related parameters in SHR/cp rats and effect of losartan on the rats. (continued)

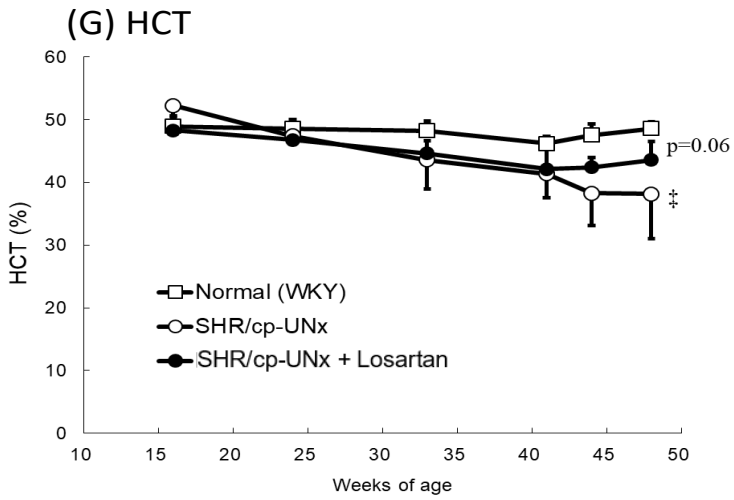

Figure 3. Influences of UNx on renal function and related parameters in SHR/cp rats and effect of losartan on the rats. (continued)

Renal-related parameters (A-D) and erythrocyte-related parameters (E-G). Data points and bars represent the mean and S.D. (n = 6-10).

$P < 0.01$ vs. Normal WKY rats group (Student's *t*-test)

†, ‡ $P < 0.05$, $P < 0.01$ vs. Normal WKY rats group (Welch's test)

*, ** $P < 0.05$, $P < 0.01$ vs. SHR/cp-UNx rats group (Student's *t*-test)

§, §§ $P < 0.05$, $P < 0.01$ vs. SHR/cp-UNx rats group (Welch's test)

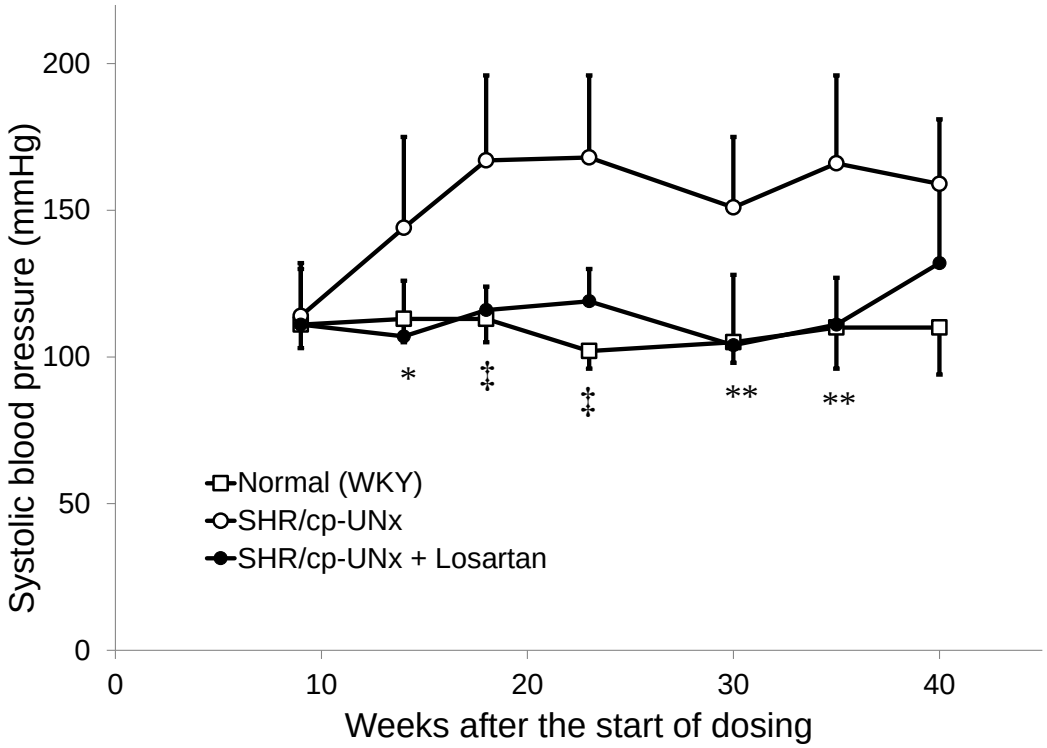

Figure 4. Preliminary study demonstrating hypertension in SHR/cp-UNx rats and the efficacy of losartan against hypertension.

UNx of the left kidney was performed as described above, in SHR/cp rats at 7 weeks of age, and two weeks later, starting at 9 weeks of age, these rats were fed a standard powder chow or mixed diet containing losartan for 32 weeks. The groups were: 1) UNx-treated SHR/cp rats fed a normal diet; 2) UNx-treated SHR/cp rats fed the 10 mg/kg losartan diet (mixed diet containing 0.02% losartan); 3) non-nephrectomized WKY rats fed a normal diet (8 rats in each group).

‡ P <0.01 vs. Normal WKY rats group (Welch's test)

*, ** P <0.05, P <0.01 vs. SHR/cp-UNx rats group (Student's t-test)

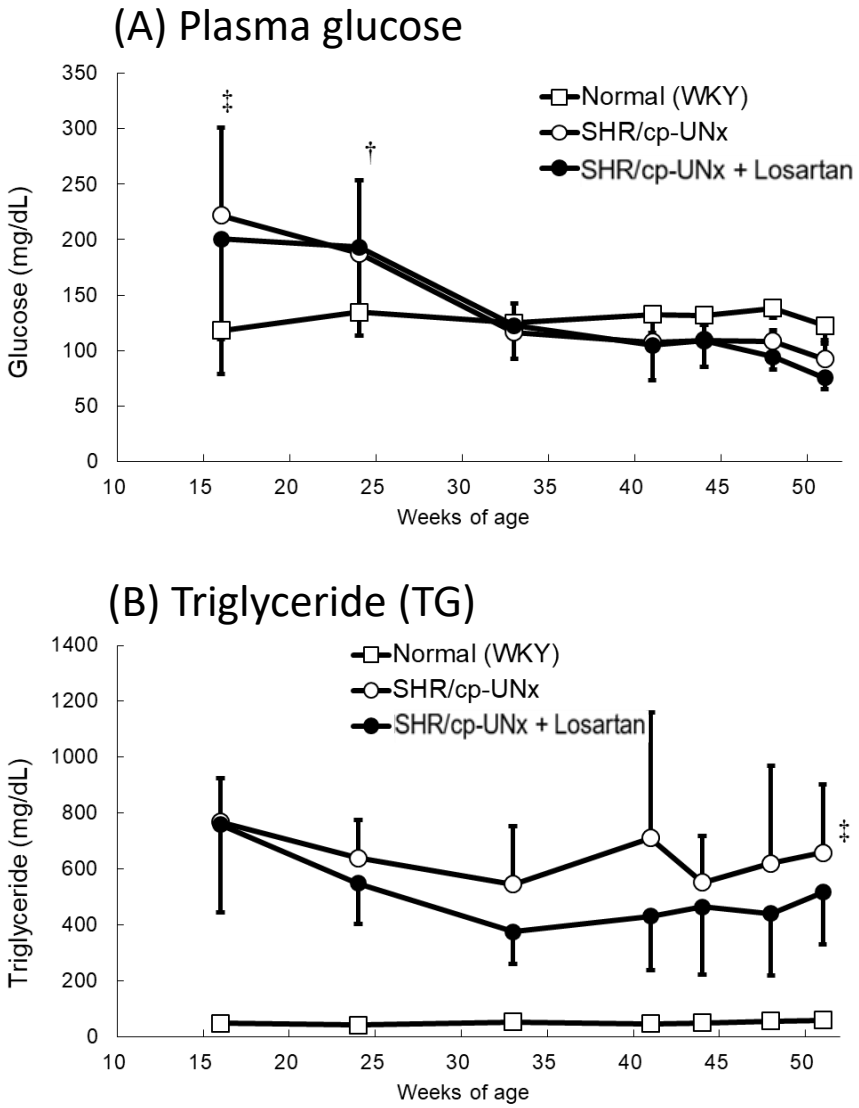

Figure 5. Effect on metabolic parameters.

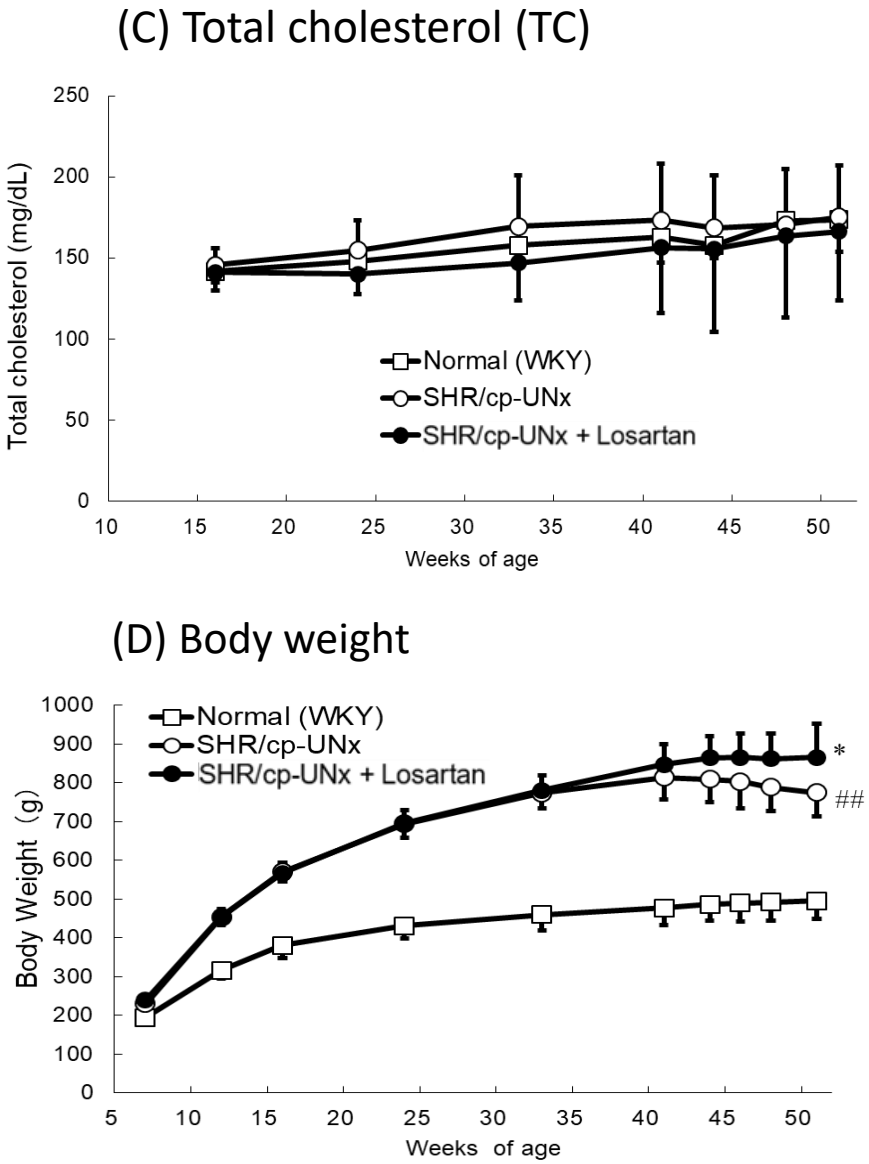

Figure 5. Effect on metabolic parameters. (continued)

A: Blood glucose. B: Triglyceride (TG). C: Total cholesterol (TC). D: Body weight.

Data points and bars represent the mean and S.D. (n = 6).

$P < 0.01$ vs. Normal WKY rats group (Student's *t*-test)

†, ‡ $P < 0.05$, $P < 0.01$ vs. Normal WKY rats group (Welch's test)

* $P < 0.05$ vs. SHR/cp-UNx rats group (Student's *t*-test)

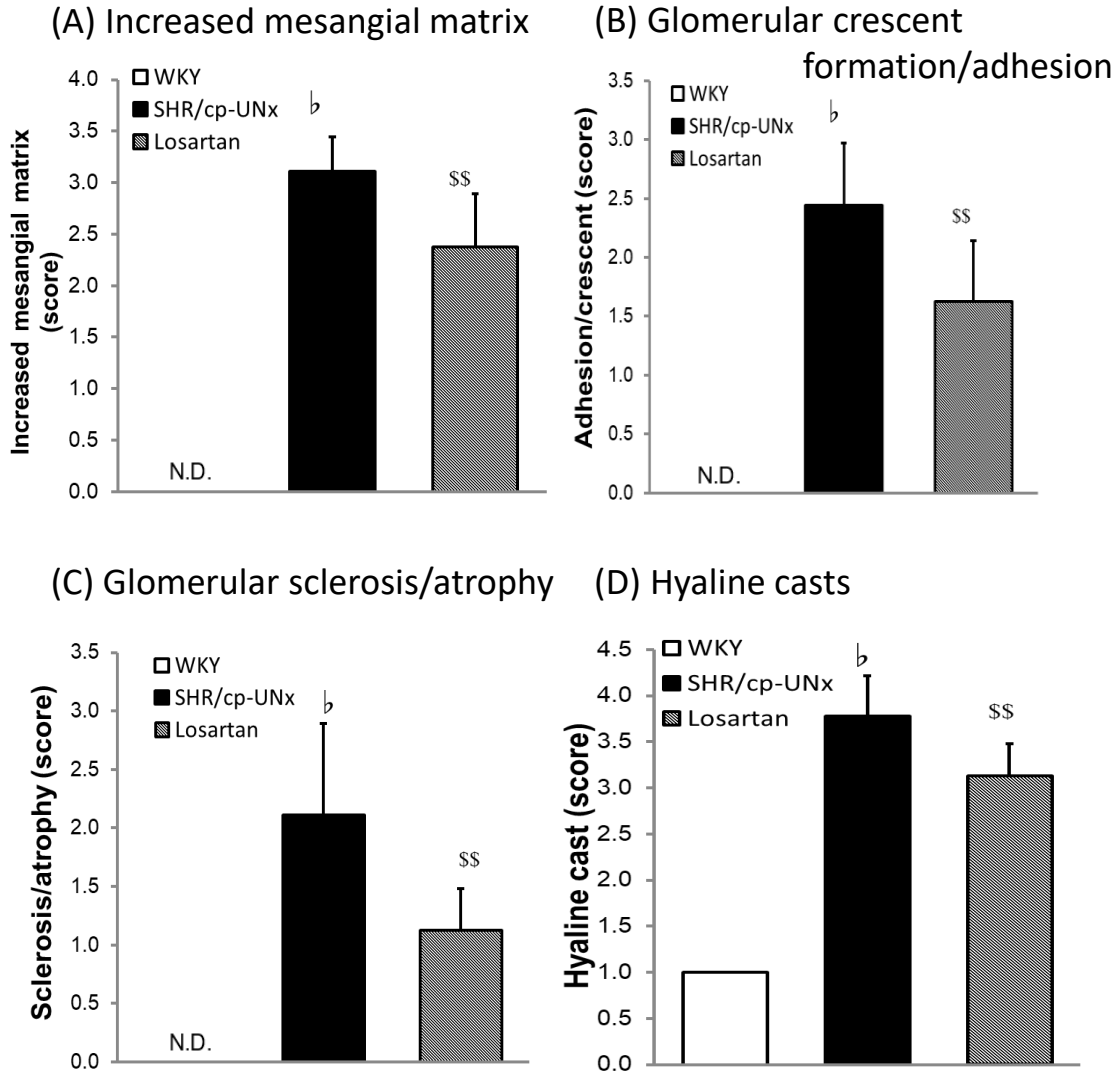

Figure 6. Effects on renal histopathology.

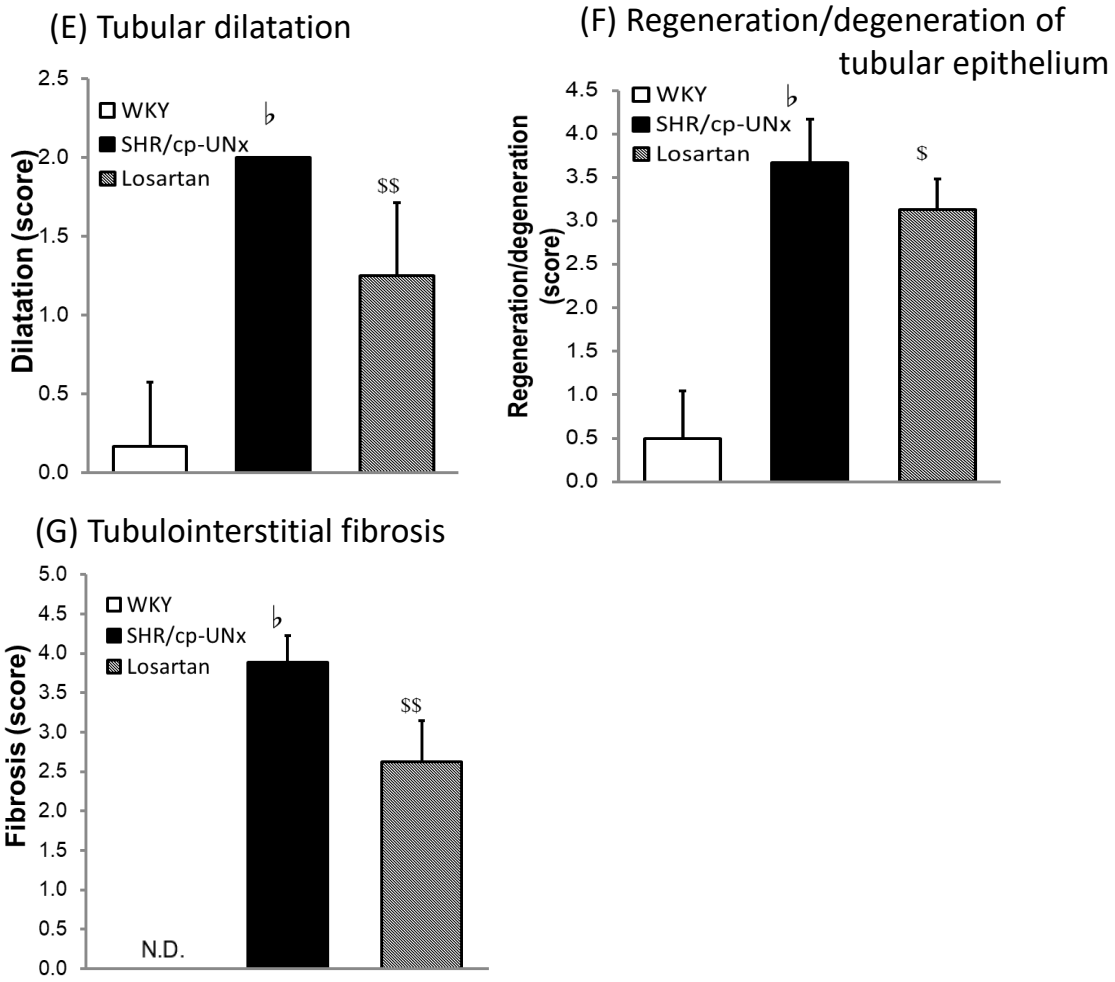

Figure 6. Effects on renal histopathology. (continued)

A: Increase in mesangial matrix. B: Adhesion/crescent. C: Sclerosis/necrosis/atrophy. D: Hyaline casts. E: Dilatation. F: Regeneration/degeneration in epithelium. G: Fibrosis.

Data points and bars represent the mean and S.D. (n = 6-10).

♭ $P < 0.05$ vs. Normal WKY rats group (Wilcoxon rank sum test)

$, $$ $P < 0.05$, $P < 0.01$ vs. SHR/cp-UNx rats group (Wilcoxon rank sum test)

N.D., not detected

(A) Sirius Red stain

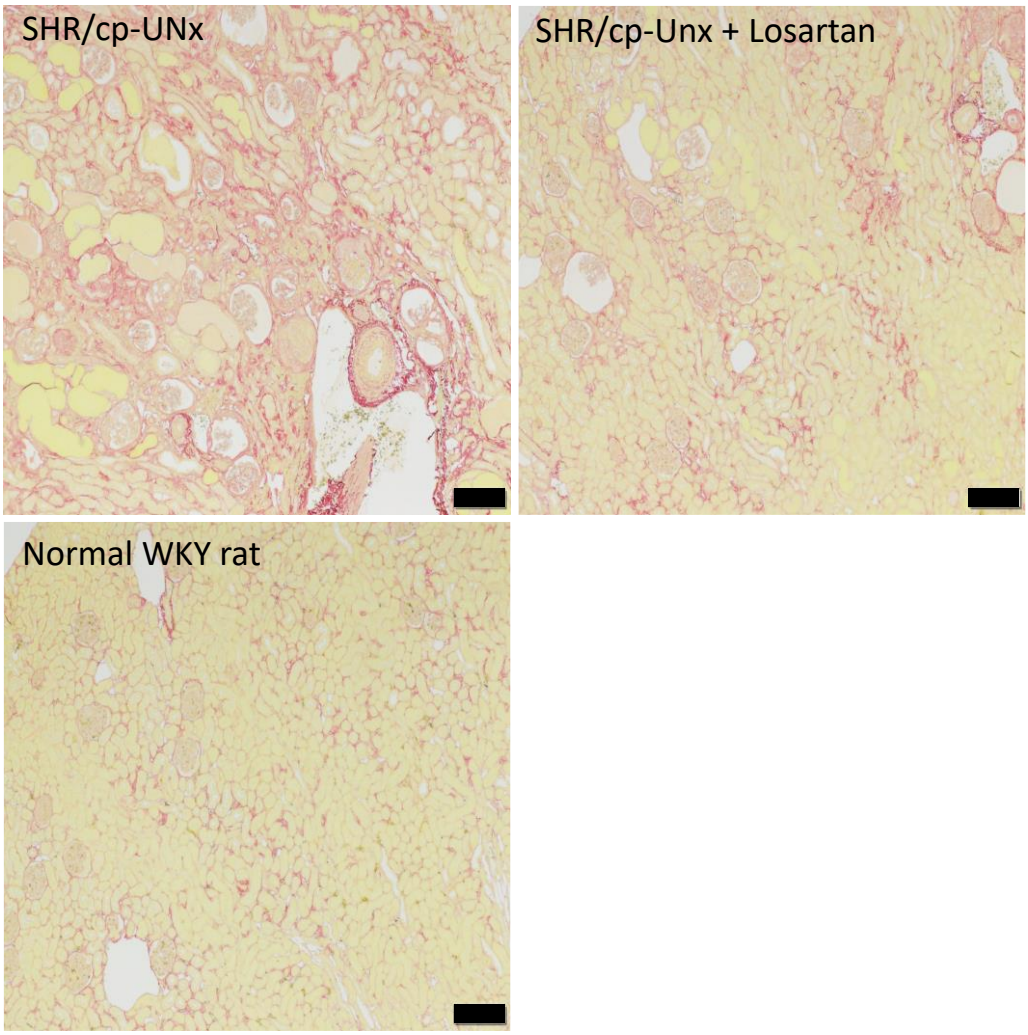

Figure 7. Representative images of kidney sections.

(B) PAS stain

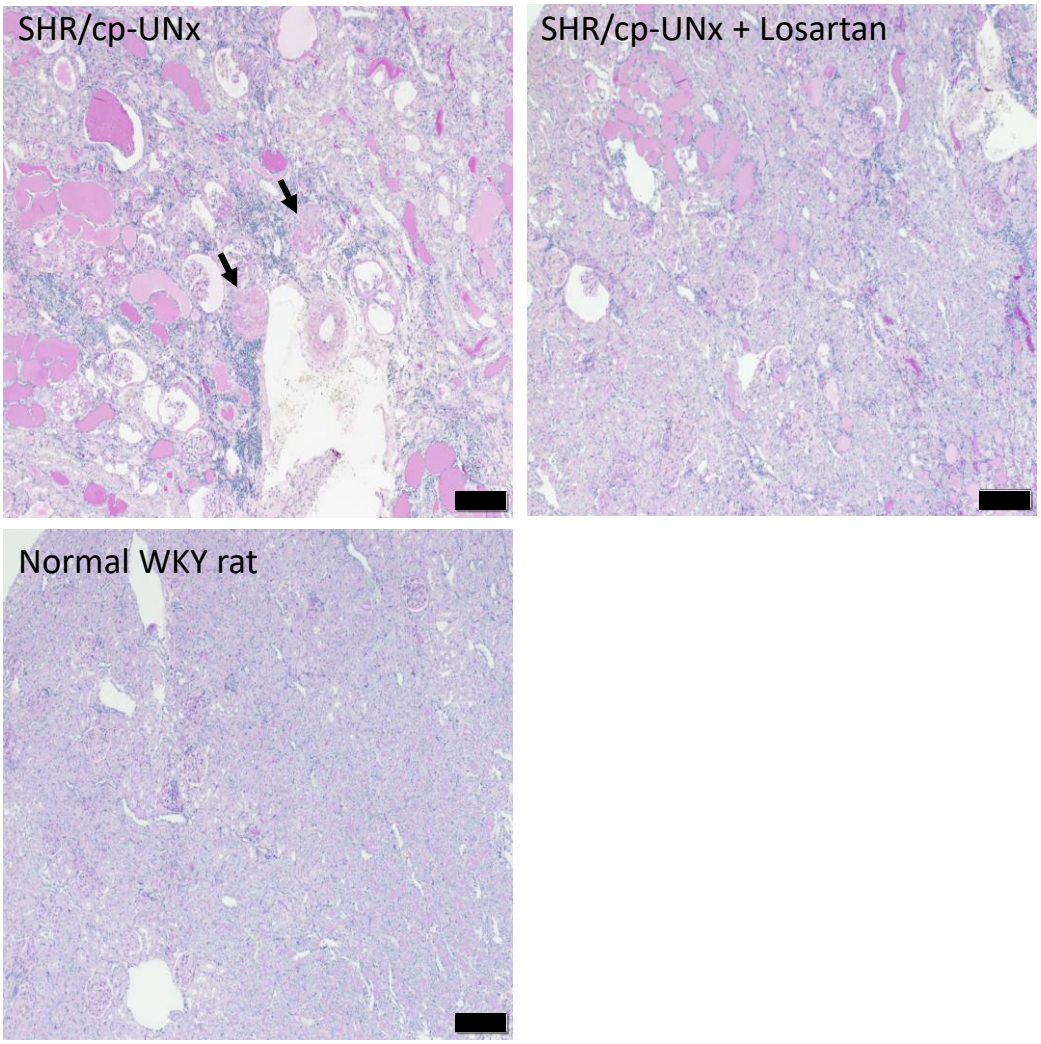

Figure 7. Representative images of kidney sections. (continued)

A: Representative images of Sirius Red staining showing tubulointerstitial fibrosis in UNx-treated SHR/cp rat.

B: Representative images of PAS staining showing glomerular sclerosis (arrows).

Black scale bar: 200 μm.

Chapter 3

Salt loading with unilateral nephrectomy accelerates decline in glomerular filtration rate in the hypertensive, obese, type 2 diabetic SDT fatty rat model of diabetic kidney disease

Introduction

DKD is characterized by a complicated pathology that involves hyperglycemia, hyperlipidemia, obesity, and hypertension, accompanied by a decline in GFR. The eGFR is frequently used to assess renal function in patients with DKD. That is, an eGFR decline indicates a decrease in renal function. For more than a decade, ACE inhibitors or ARBs such as ramipril or losartan have been used for the treatment of DN (Kota *et al.*, 2012). However, the target populations of these agents are limited to patients suffering from DN with type 2 diabetes, hypertension, elevated pCre, and proteinuria (Parving *et al.*, 2001; Keane *et al.*, 2003). In recent years, evidence of the efficacy of SGLT2 inhibitors in patients with DKD has been accumulating (Tuttle *et al.*, 2020), and it is likely that the treatment strategy will be changed in the near future so that SGLT2 inhibitors can become the basic medication used for DKD patients (Tuttle *et al.*, 2021).

Various animal models of DKD are known, however, the complex etiological complications associated with decreased renal function in patients with DKD, develop in only a few of them. As an example, the widely used STZ-induced type 1 diabetes model, unlike humans, shows elevated blood glucose levels but not hyperlipidemia or hypertension. Whereas, endothelial nitric oxide synthase (eNOS) (-/-) db/db mice, SHR/cp rats, and Zucker diabetic fatty rats, which are hypertensive DKD models with

such complications, must be more than 6 months old before nephropathy develops with histopathological changes (Chander *et al.*, 2004; Nangaku *et al.*, 2005; Zhao *et al.*, 2006; Mohan *et al.*, 2008; Rosenthal *et al.*, 2010; Giani *et al.*, 2012; Harlan *et al.*, 2018). Furthermore, these animal models do not exhibit a GFR decline during this period, but rather they show an increase in GFR due to glomerular hyperfiltration (Tang *et al.*, 2020). Therefore, there are currently no pharmacologically available animal models of DKD that show a GFR decline, which is a surrogate for renal function in patients with DKD.

The SDT fatty rat is an obese, type 2 diabetic model of DKD characterized by hyperglycemia, hyperlipidemia, and hypertension (Ishii *et al.*, 2010; Kemmochi *et al.*, 2013; Ohta *et al.*, 2014; Katsuda *et al.*, 2015; Toriniwa *et al.*, 2018). By utilizing SDT fatty rats, we attempted to establish an accelerated hypertensive DKD model in which GFR decline, and these characteristics develop rapidly. To accelerate the decrease in renal function, salt loading with or without UNx was performed, and in addition, the efficacy of ARBs (losartan, in particular) was evaluated.

Materials and Methods

Animals

Male SDT fatty rats and age-matched Sprague Dawley (SD) rats were purchased from CLEA Japan, Inc. (Tokyo, Japan), and maintained in a specific pathogen-free room at a temperature of 23 ± 3°C and air humidity of 55 ± 15%, on a 12-h/12-h light/dark cycle. This animal study was conducted in accordance with the Japanese Law for the Humane Treatment and Management of Animals (Law No. 105, October 1, 1973).

Chemicals

Losartan (≥98% purity) was purchased from LKT Laboratories, Inc. The losartan diet was prepared every week by mixing losartan with standard powdered chow (CE-2, CLEA Japan Inc.), adjusting the mixture ratio based on the body weight and average dietary intake of the rats. The mixed diet containing approximately 0.015% losartan (10 mg/kg/day) was fed for 10 weeks.

Effects of salt loading with or without UNx on GFR

The study design is shown in Figure 8A. Animals at 9 weeks old were divided into 5 treatment groups based on the measurements of GFR: 1) SDT fatty rats drinking normal water and not nephrectomized (without UNx), 2) SDT fatty rats salt loaded by drinking 0.3% salt water and nephrectomized (with UNx), 3) SDT fatty rats salt loaded by drinking 0.6% salt water without UNx, 4) SDT fatty rats salt loaded by drinking 0.8% salt water without UNx, or 5) SD rats not salt loaded by drinking normal water without UNx. UNx (left kidney) for the rats in the 0.3% salt loaded group was performed as previously described (Katsuda *et al.*, 2014b). In order to establish a model with a decrease in GFR, the salt concentration used for salt loading was investigated. Our previous study showed that 0.3% salt loading alone nor UNx treatment alone did not decrease creatinine clearance (Ohta *et al.*, 2014), so the combination of the 0.3% salt water with UNx group was set up. In the following week, these groups started to the salt-loading treatment by drinking water containing 0, 0.3, 0.6, or 0.8% salt for 13 weeks. During the experimental period, body weight was measured sequentially, and blood and urine samples were collected from the tail vein and using metabolic cages, respectively. As a parameter of renal function, GFR was measured before group

assignment and at 2, 6, 10, and 13 weeks after salt loading. GFR was determined by measuring the plasma clearance of fluorescein isothiocyanate (FITC)-labeled inulin after a single bolus injection as previously described (Hinojosa-Laborde *et al.*, 2015). After the last sampling at week 13, the rats were euthanized and their right kidneys were excised and processed for histological evaluation. Plasma glucose, pCre, BUN, and urine Cre levels were measured using an automatic biochemical analyzer (Model 7180). Plasma insulin levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan). Urine albumin levels were measured using a commercially available ELISA kit (Shibayagi Co., Ltd., Gunma, Japan). For histological analysis, the 10% neutral formalin-fixed right kidneys were sectioned and stained with HE, PAS, and Sirius red. PAS staining was used to evaluate the degree of glomerular alteration; HE staining and PAS staining were used to evaluate the degree of tubulointerstitial alteration, and Sirius red staining was used to evaluate the degree of interstitial fibrosis. The histological evaluation was assessed by the following four parameters defined in the preliminary examination: interstitial fibrosis, glomerular hypertrophy, mesangial hyperplasia, and interstitial infiltration of inflammatory cells. Preliminary observation of the entire cortical to medullary regions was performed by using renal specimens and 4 parameters described above that showed prominent changes were examined in this study. The severity of each histological change was scored on a 5-point scale ranging from 0 to 4 (Score 0: within normal limits; Score 1: minimal, solitary [very small] lesion; Score 2: slight, focal [small] lesion; Score 3: moderate, scattered lesion; and Score 4: severe, marked, extensive lesion) based on the severity and extent of the change. For evaluation of glomerular change, glomerular sizes in the cortical area were

analyzed. To measure glomerular size, one section per rat was photographed under a light microscope (BX51, Olympus Corporation, Tokyo, Japan) using the 4× objective lens and analyzed using ImageJ software (Rasband WS, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018) as previously described (Sano *et al.*, 2021). Six animals per group were used for measurement of glomerular size. The glomerular area measurement was performed by randomly divided into sections of the entire kidney from the cortical surface to the juxtamedullar, and all glomeruli within the area and overlapping the upper and right edges were counted. Approximately 50 to 100 glomeruli were counted per animal.

Effects of losartan on GFR decline in SDT fatty rats undergoing 0.3% salt loading with UNx

The study design is shown in Figure 8B. SDT fatty rats were salt-loaded by drinking 0.3% salt water and subjected to UNx based on the results of the study showing the fastest GFR decline (Figure 9A).

UNx was done in the SDT fatty rats at 9 weeks old. In the following week, group assignment was performed based on the GFR, and then 0.3% salt loading was started in all rats except SD rats (control group). The groups were: 1) SDT fatty rats fed a normal diet, salt-loaded by drinking 0.3% salt water, and nephrectomized (with UNx); 2) SDT fatty rats fed the 10 mg/kg losartan diet, salt-loaded by drinking 0.3% salt water, and with UNx; 3) SD rats drinking normal water and not nephrectomized (without UNx). Furthermore, a standard powder chow or mixed diet containing losartan (10 mg/kg/day, approximately 0.015%) was fed for 10 weeks. GFR was measured before and at 2, 5, and 10 weeks after salt loading by intravenous injection of FITC-sinistrin as previously

described (Ellery *et al.*, 2015). After the last measurement of GFR, the rats were euthanized, and their right kidneys were excised and processed for histological evaluation as described above.

Statistical analysis

Data are expressed as the mean and S.D. of the indicated numbers of animals or samples. All statistical analyses were performed using Statlight 2000. In a two-group comparison, the statistical significance was assessed using Student's *t*-test (for homoscedastic data) or Aspin-Welch's *t*-test (for heteroscedastic data) after homoscedasticity analysis by an *F*-test. In a multi-group comparison, the statistical significance was assessed using Dunnett's test (for homoscedastic data) or Steel's test (for heteroscedastic data) after homoscedasticity analysis by Bartlett's test. For histological scores, the statistical significance was assessed by Wilcoxon rank sum test for two-group comparison and Steel's test for a multi-group comparison. All statistical analyses were two-sided, and statistically significant level was set at $P < 0.05$.

Results

Effect of salt loading with or without unilateral UNx on renal function

A significant increase in GFR was observed in untreated SDT fatty rats (SDT fatty rats provided with normal drinking water) compared to SD rats, and the increase persisted throughout the experiment. On the other hand, a significant decrease in GFR was observed in the rats undergoing 0.6% salt loading without UNx (0.6% salt), 0.8% salt loading without UNx (0.8% salt), or 0.3% salt loading with UNx (0.3% salt/UNx), compared to the untreated SDT fatty rats. Furthermore, the GFR was significantly lower

in the rats with 0.3% salt/UNx at week 13 than in SD rats. In addition, elevated UACR was observed in the untreated SDT fatty rats, and was further increased by 0.3% salt/UNx, 0.6% salt, or 0.8% salt (Figure 9).

Effect on renal related and metabolic parameters

During the experimental period, higher pCre and BUN levels were observed in rats treated with 0.3% salt/UNx compared to other treated SDT fatty rats; chronic hyperglycemia was observed in untreated SDT fatty rats compared to SD rats, however, lower plasma glucose levels were observed in rats treated with 0.3% salt/UNx, 0.6% salt, or 0.8% salt, and were accompanied by a decrease in hemoglobin A1c (HbA1c) levels at week 12. Plasma TC and TG levels, which are elevated in SDT fatty rats, were increased by salt loading (Figure 10).

Effect on renal histology

Histopathologically, tubulointerstitial fibrosis was prominently observed in the untreated SDT fatty rats at week 13. Moreover, treatment with 0.8% salt and 0.3% salt/UNx resulted in increased interstitial fibrosis (Figure 11A, Figure 12). Glomerular hypertrophy, mesangial hyperplasia, and interstitial infiltration of inflammatory cells were induced by treatment with 0.6% salt, 0.8% salt, and 0.3% salt/UNx (Figure 11B-D). A significant increase in glomerular size was observed in untreated SDT fatty rats compared to SD rats. Furthermore, SDT fatty rats treated with 0.8% salt had significantly larger glomerular size than untreated SDT fatty rats. The average size (μm^2) of glomeruli in the SD rats, untreated SDT fatty rats, 0.6% salt treated rats, 0.8% salt treated rats, and 0.3% salt/UNx treated rats were (7594 ± 484, 12427 ± 1365, 14746

± 1695, 16030 ± 4192, and 15401 ± 1359), respectively. Disruption of the glomerulus was observed in rats treated with 0.8% salt (data not shown).

Effect of losartan on GFR decline and renal histology in rats treated with 0.3% salt loading and UNx

Next, we investigated the effect of losartan on GFR decline in rats treated with 0.3% salt/UNx. The results showed that dietary administration of losartan (10 mg/kg/day) significantly suppressed GFR decline in the rats treated with 0.3% salt/UNx (Figure 13). However, no efficacy of losartan diet on SBP levels was observed (Figure 14).

Histopathologically, it was found that 0.3% salt/UNx caused glomerular lesions (hypertrophy, adhesion, increase in mesangial matrix), tubular lesions (cast formation, regeneration, expansion) and tubulointerstitial lesions (fibrosis, inflammatory cell infiltration). However, no improvement was evident with administration of losartan, and in several cases, limited improvement in tubular dilatation and cast formation was detected (data not shown).

Discussion

The graphical summary is shown in Figure 15.

In SDT fatty rats, 0.3% salt loading by drinking water with UNx (0.3% salt/UNx), or 0.8% salt loading alone, was able to induce an early decline in GFR as well as an elevated UACR, accompanied by histopathological changes such as kidney fibrosis, glomerular hypertrophy, and inflammatory cell infiltration (Figure 9A-C, Figure 11, Figure 12). Furthermore, 0.3% salt/UNx caused renal-related parameters such as pCre and BUN to increase, in contrast to 0.8% salt loading alone (Figure 10A, 10B). The

hallmarks of DKD include GFR decline, albuminuria, and impaired renal morphology (increased glomerular basement membrane thickness, mesangial hyperplasia, interstitial fibrosis, glomerular hypertrophy, glomerulosclerosis, podocyte foot process effacement, and arterial hyalinosis) (Alicic *et al.*, 2017). The induction of decreased renal function by 0.3% salt/UNx in SDT fatty rats was suggested to be reminiscent of the pathological condition in patients with hypertensive DKD, as indicated above.

Recently, induced renal injury by dietary salt loading in the Dahl salt-sensitive hypertensive rat has been used as an animal model to evaluate the efficacy of agents for antihypertensive treatment (Bayorh *et al.*, 2006; Buss *et al.*, 2006). Although the Dahl salt-sensitive hypertensive rat is a model of hypertension, it is not a model to study risk factors specific to DKD, such as diabetes mellitus, abnormal lipid metabolism, and obesity. SHR/cp rats and db/db mice, which are known diabetic models, show signs of DKD, such as abnormal lipid metabolism and obesity, however, during the lifetime of the animals, renal function is not influenced or in a hyperfiltration state, and infrequently declines as measured by creatinine clearance (Gartner, 1978; Cohen *et al.*, 2001; Nangaku *et al.*, 2005). Therefore, the SDT fatty rat treated with salt loading alone exhibits a more rapid GFR decline and this model might mimic the pathogenesis of DKD in patients.

On the other hand, salt loading might not cause hyperfiltration since it would not induce contraction of the efferent arteriole by inhibiting the activation of renin-angiotensin system in the renal glomerulus (Charytan *et al.*, 2012). This could lead to a more rapid and direct detection of the effects of various risk factors on renal function. However, since salt loading is likely to cause an increase in body fluid volume, renal-related parameters in the blood, such as pCre and BUN, may not be elevated even

though these parameters usually increase with a decrease in renal function. Therefore, it is preferable to influence renal function by using a lower salt concentration for loading.

To further accelerate the decline of renal function, we performed UNx in addition to salt loading. By promoting renal dysfunction via reducing the number of nephrons, UNx and 5/6 nephrectomy in rats and mice are widely used as experimental models of CKD (Katsuda *et al.*, 2014b; Racanicchi *et al.*, 2015; O'Sullivan *et al.*, 2019).

In the present study, we performed UNx as well as 0.3% salt loading in rats, which allowed us to establish a model in which even loading a low concentration of salt would lead to steady GFR decline, hypertension and worsened renal function parameters, accompanied by renal histopathological changes. It was considered that the decrease in the number of nephrons due to nephrectomy causes an increase in the single nephron GFR, and the sustained hypertension due to salt loading accelerates glomerulosclerosis and intensifies medullary hypoxia, resulting in further reduction in the number of nephrons and hypoxia caused by interstitial fibrosis (Heyman *et al.*, 2008; Heyman *et al.*, 2019; Heyman *et al.*, 2020). Furthermore, in this study, plasma TC and TG levels were elevated in rats treated with 0.3% salt/UNx as well as 0.6% salt loading alone or 0.8% salt loading alone (Figure 10E, 10F). In a healthy condition, both TG and TC are known to be absorbed by the renal cells such as tubular epithelial cells and used as energy source or lipid membrane components. However, in CKD condition, the intracellular factors involved in their utilization are decreased, and these could accumulate as lipid droplets and exert cytotoxic effects (Bobulescu, 2010; Afshinnia *et al.*, 2019). It has also been reported that TGs and FFAs bound to albumin accumulated in the proximal tubules could cause damage and induce inflammation (Bobulescu, 2010). Furthermore, our model is characterized by hypertension. In the preliminary

study, SBP levels (mmHg) of SD rats and 0.3% salt/UNx treated rats were 105.3 ± 23.8 and 209.6 ± 24.2 after 5 weeks of treatment, and 126.8 ± 12.5 and 191.4 ± 35.4 after 10 weeks of treatment, respectively, measured by the indirect tail cuff method (Katsuda *et al.*, 2014b).

Unfortunately, it was not possible to identify the lesion as a prominent change due to the depth of the renal area observed. In addition, because the changes appeared in each nephron, we considered it difficult to observe pathological shift from a cortical layer to a deeper layer. Additionally, decrease in blood glucose levels were observed in these rats (Figure 10C). The reason for the decrease in the levels might be related to the downward trend in food intake of SDF Fatty rats treated with salt loading (Katsuda *et al.*, 2014a). On the other hand, there is also a possibility that sodium loading might impair the function of SGLT1 in the intestine, however, these should be the themes for our future study.

Next, we confirmed the effect of losartan, which is widely used for the treatment of DN (Tuttle *et al.*). The results showed that dietary administration of losartan attenuated the GFR decline induced by 0.3% salt loading and UNx in rats (Figure 13). However, the results of SBP levels and histopathological evaluation showed that losartan was less effective (Figure 14, histopathological data not shown). The average size (μm^2) of glomeruli was comparable to the normal diet, showing no efficacy (normal diet: 14475 ± 3016, losartan diet: 17173 ± 3539). Previous reports suggest that it could be that the ARBs acted as neuroprotective agents to prevent GFR decline as another function of losartan other than reducing efferent artery constriction (Saavedra, 2012; Villapol *et al.*, 2015). These results suggest that this model might mimic the later stages of DKD,

where ACE inhibitors and ARBs are less effective in clinical care. Whereas, the GFR of SD rats appeared spontaneously decreased in Figure 13, it was considered that there was a possibility of detecting the aging-related decrease to some degree (Baylis *et al.*, 1998).

In preclinical studies, the lack of animal models that mimic the pathophysiological characteristics of patients with DKD has been an obstacle to address the clinical needs precisely (Noshahr *et al.*, 2020). Animal models might provide new-insights into the development and progression of nephropathy in patients with DKD, and help us better understand the etiology of the disease. In addition, animal models could be used to explain how novel therapies might function, identify alternative pathways for these therapies, and even help to validate the onset of potential side effects.

In patients with DKD, risk factors such as hypertension, dyslipidemia, and hyperuricemia, in addition to diabetes mellitus, are considered to be complex pathological determinants. Thus, we considered that our model is also characterized by including these risk factors in addition to diabetes. On the other hand, it is unknown which of these factors, including hypertension, contributes to the pathogenesis of the patients. In this study, plasma TG and TC levels were elevated in our hypertensive DKD model (data not shown). The dyslipidemia in CKD patients is characterized by elevated TG and TC, therefore, in our model, we intended to mention these elevations. For the mechanism of TG and TC elevation in our model, the article by M Lee *et al*. that studied the response of Adipocytes to salt loading (Lee *et al.*, 2019) might be informative. This study reported that high salt increased the expression of adipogenic/lipogenic genes and, inversely, decreased the gene of lipolysis.

In the past, ACE inhibitors and ARBs, two groups of anti-hypertensive medications

that slow the progression of DN, have been extensively studied in various experimental DN models. However, not all the typical DN features develop in many of these models. For example, the mouse model has several limitations, and only the early stages of DKD develop in this model. In fact, the classical model of DN only exhibits the features of early stage DKD: moderate albuminuria, glomerular hypertrophy, and slight expansion of the mesangial matrix (Soler *et al.*, 2012). Glomerulosclerosis, tubular atrophy, or interstitial fibrosis is rarely presented in these animals. The model presented herein, which is produced by the combination of UNx and salt loading, is a novel animal model that exhibits many of the features observed in patients with DKD and therefore might be helpful in characterizing the mechanisms involved in this disease. Recently, it was reported that, in hypertensive DKD mouse model, under conditions in which spironolactone and esaxerenone showed similar reduction in blood pressure, esaxerenone elicited a greater attenuation of albuminuria, glomerular injury, tubulointerstitial fibrosis, and renal inflammation than spironolactone (Bhuiyan *et al.*, 2019).

In the future, we expect to use our model to evaluate the effects of Dipeptidyl peptidase IV (DPP-IV) inhibitor/SGLT2 inhibitor alone or in combination with ACE inhibitors or ARBs and to understand the characteristics and limitations of this model by comparing this model to patients with DKD, which will allow us to further characterize the pathogenic factors other than hypertension in our model. It was reported that ARB reduced proteinuria even though it was not effective for blood pressure (Nishiyama *et al.*, 2004). In addition, ARBs are neuroprotective agents, therefore, it is possible that ARBs have a renoprotective effect through this action (Villapol *et al.*, 2015). As mentioned above, we would like to establish the position of our model by evaluating the

efficacy of agents with other mechanisms of action other than the antihypertensive effect of ARBs on our model in the future.

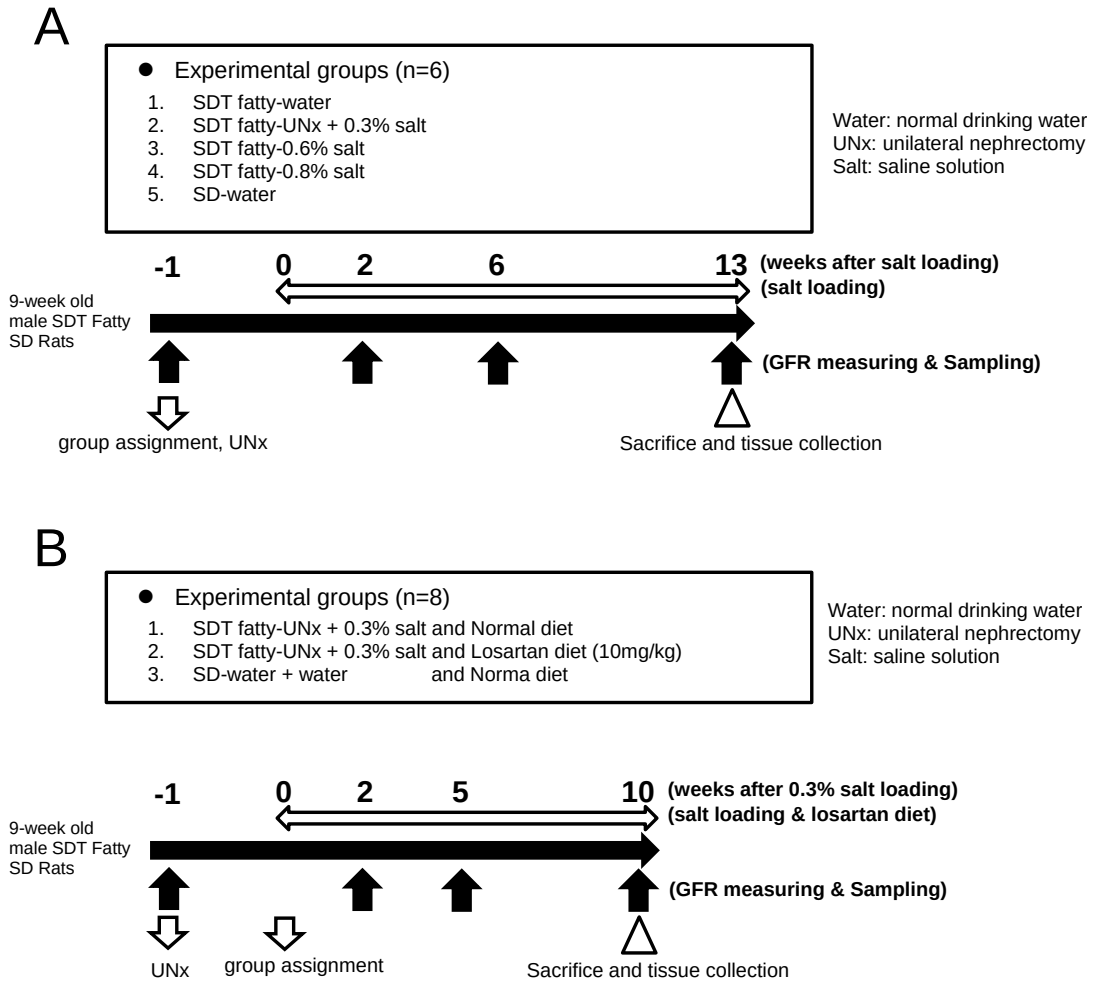

Figure 8. Experimental design.

A. Effects of salt loading with or without UNx on renal function

B. Effect of losartan on GFR decline

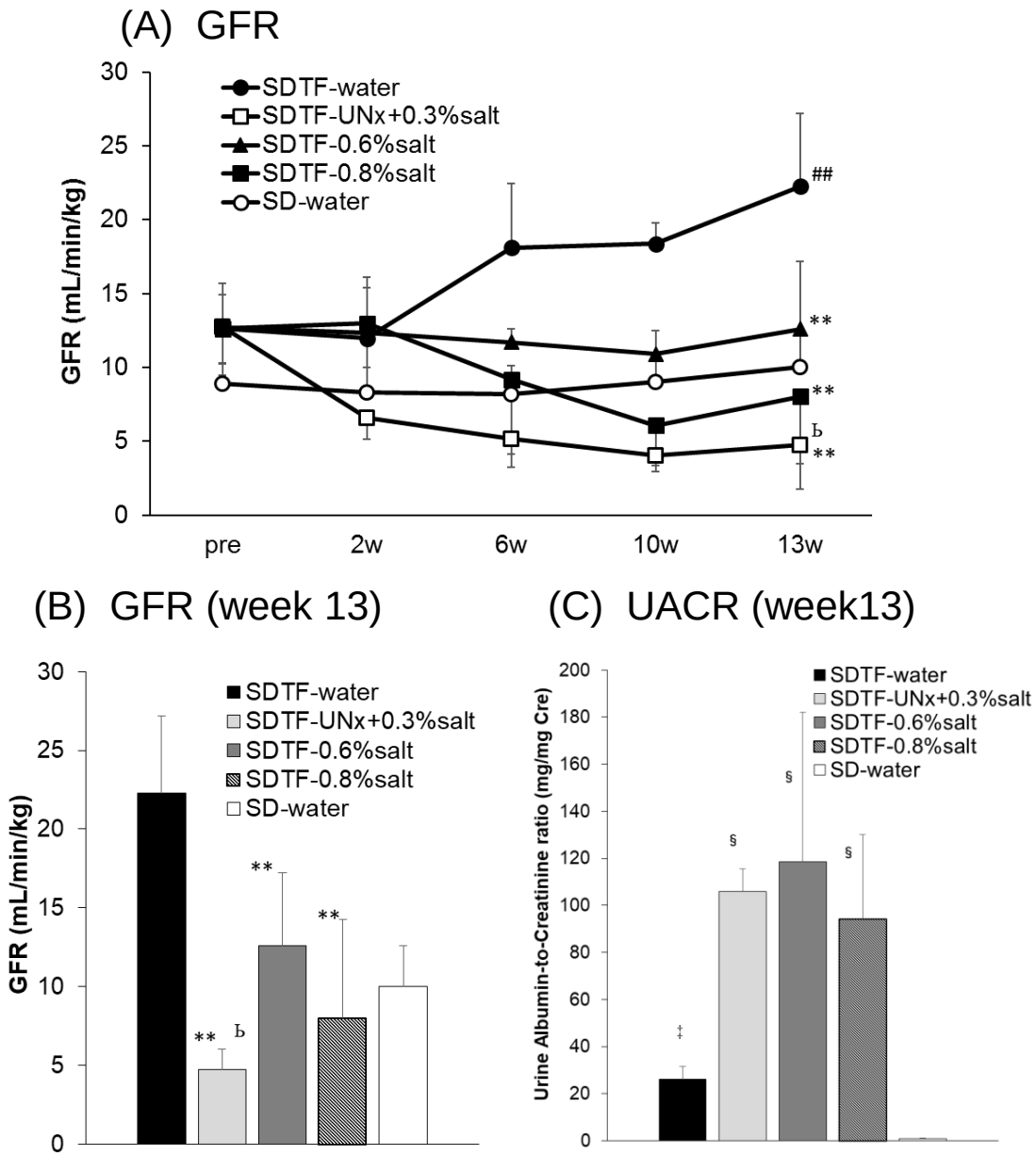

Figure 9. Effects of salt loading with or without UNx on GFR and urinary albumin level.

A: GFR changes during the experiment period. B: GFR at week 13.

C: UACR at week 13

Data points and bars represent the mean and S.D. (n = 6).

$P < 0.01$ vs. SD-water group (Student's *t*-test)

** $P < 0.01$ vs. SDTF-water group (Dunnett's test)

[b] $P < 0.05$ vs. SD-water group (Steel's test)

‡ $P < 0.01$ vs. SD-water group (Welch's test)

§ $P < 0.05$ vs. SDTF-water group (Steel's test)

SDTF, SDT Fatty

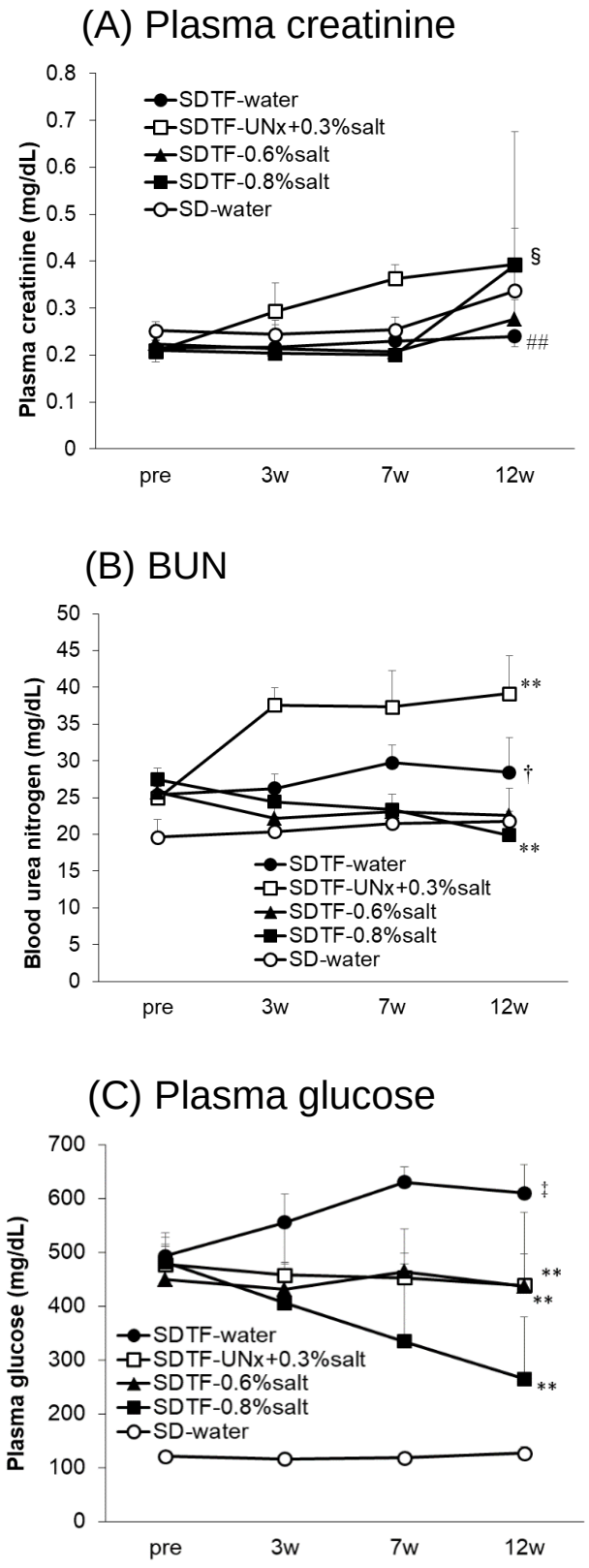

Figure 10. Effects on renal-related and metabolic parameters.

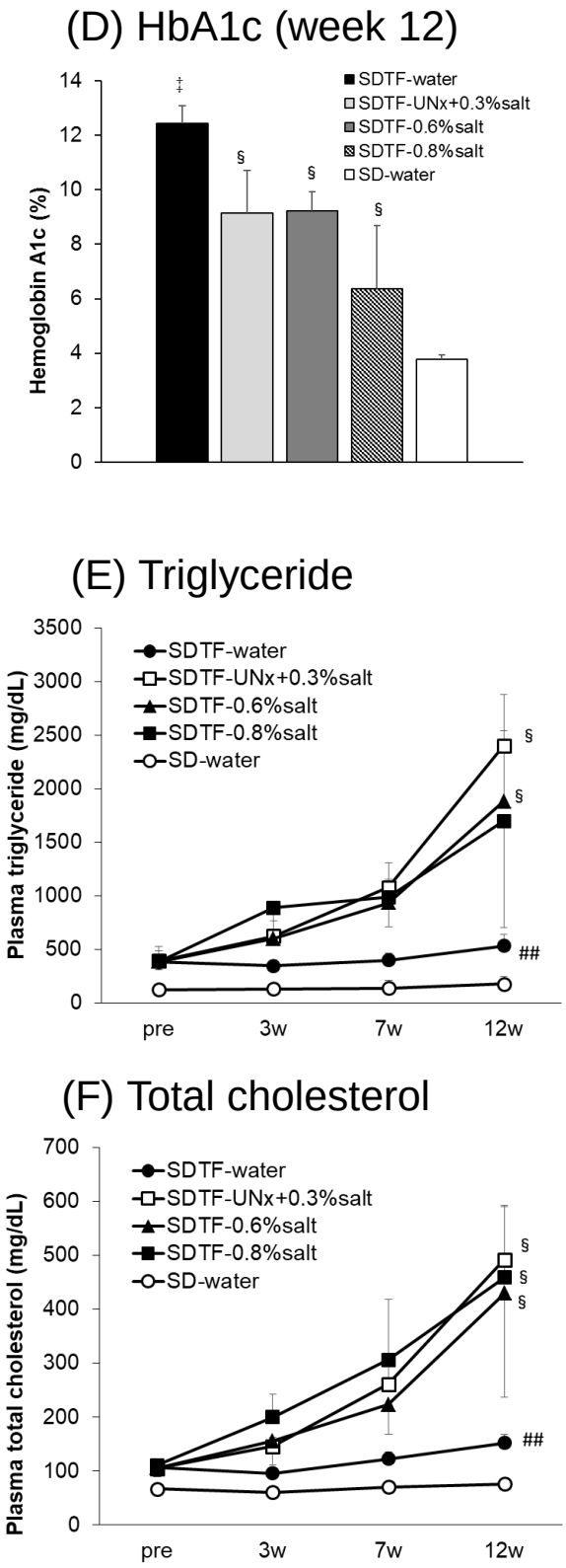

Figure 10. Effects on renal-related and metabolic parameters. (continued)

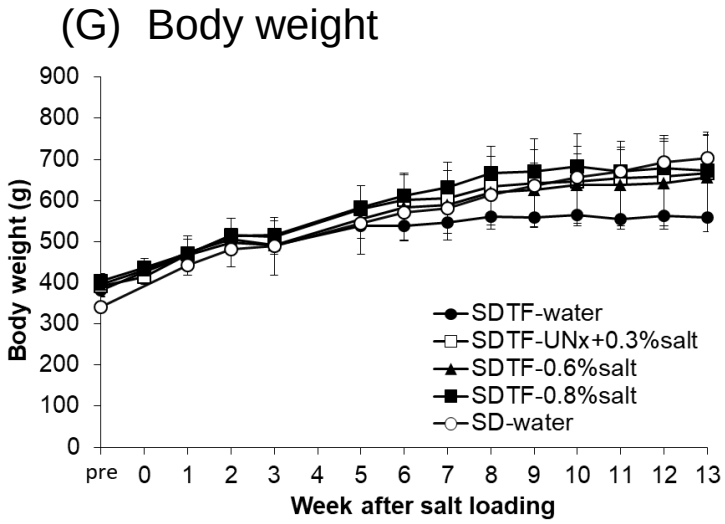

Figure 10. Effects on renal-related and metabolic parameters. (continued)

A: Plasma creatinine (pCre). B: Blood urea nitrogen (BUN). C: Plasma glucose. D: HbA1c at week 12. E: Plasma triglyceride. F: Plasma total cholesterol. G: Body weight. Data points and bars represent the mean and S.D. (n = 6).

$P < 0.01$ vs. SD-water group (Student's *t*-test)

†, ‡ $P < 0.05$, $P < 0.01$ vs. SD-water group (Welch's test)

** $P < 0.01$ vs. SDTF-water group (Dunnett's test)

§ $P < 0.05$ vs. SDTF-water group (Steel's test)

SDTF, SDT Fatty

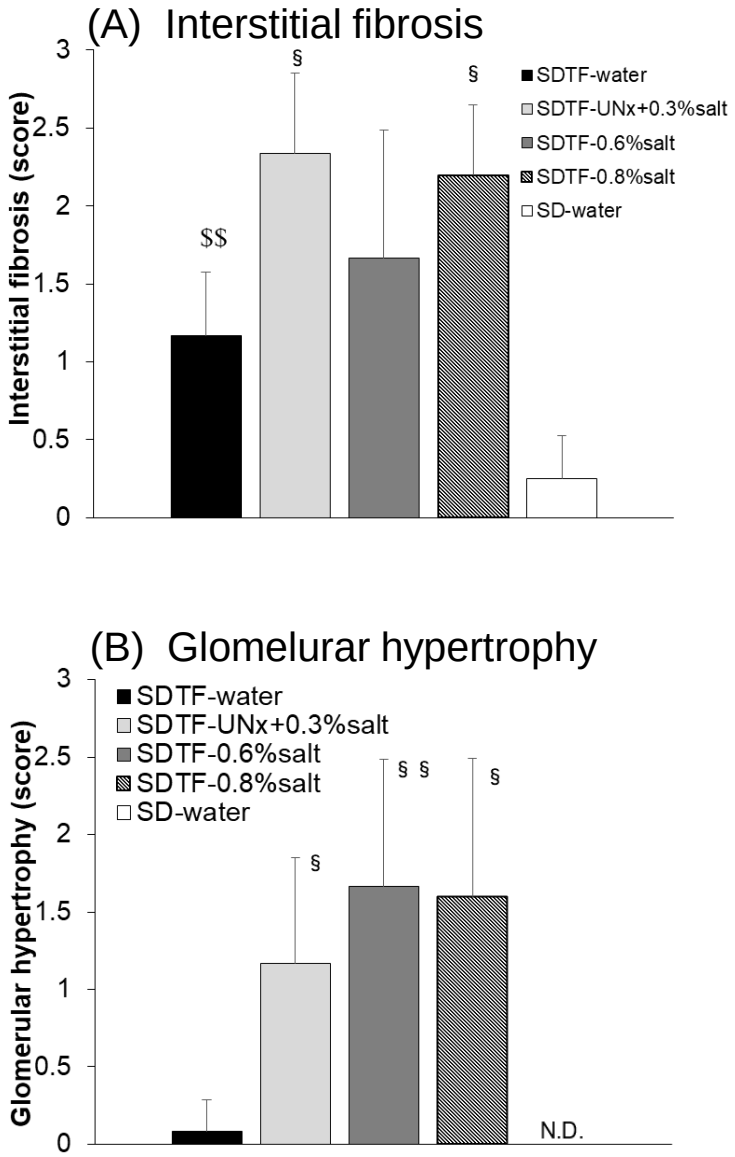

Figure 11. Effects of salt loading with or without UNx on renal histopathology.

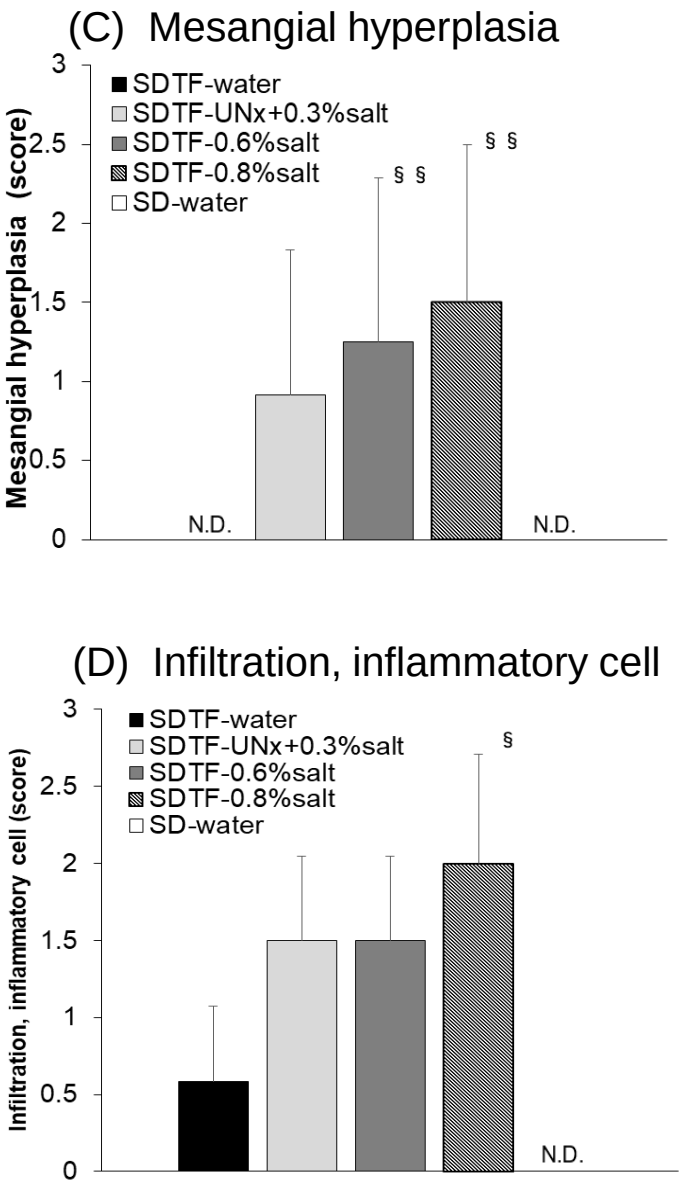

Figure 11. Effects of salt loading with or without UNx on renal histopathology. (continued)

A: Interstitial fibrosis. B: Glomerular hypertrophy. C: Mesangial hyperplasia. D: Interstitial infiltration of inflammatory cells

Data points and bars represent the mean and S.D. (n = 6).

$P < 0.01$ vs. SD-water group (Student's *t*-test)

*, ** $P < 0.05$, $P < 0.01$ vs. SDTF-water group (Dunnett's test)

$$, $P < 0.01$ vs. SD-water group (Wilcoxon rank sum test)

§, §§ $P < 0.05$, $P < 0.01$ vs. SDTF-water group (Steel's test)

SDTF, SDT Fatty; N.D., not detected

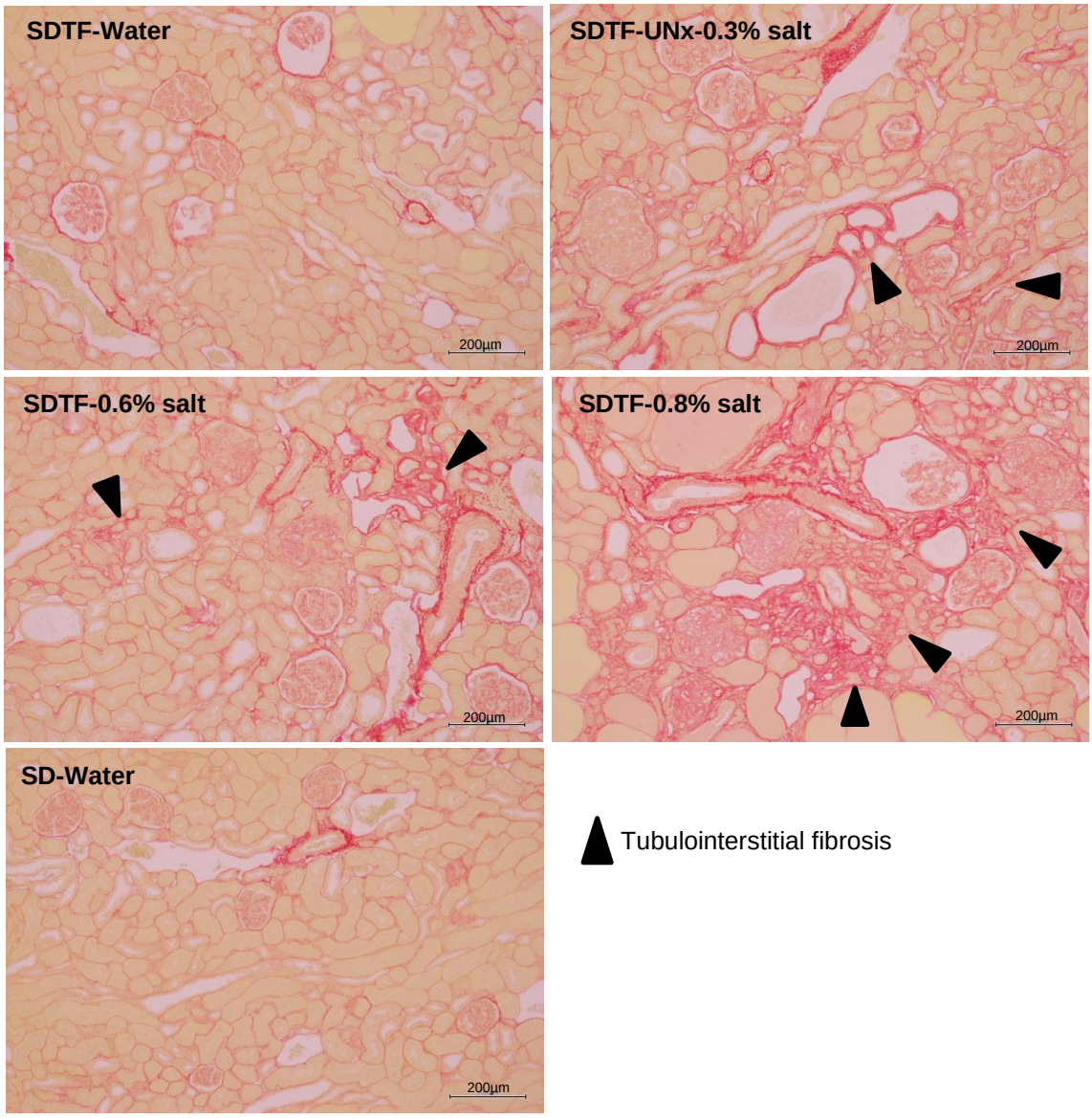

Figure 12. Representative images of Sirius red stain.

Representative images of Sirius red staining showing tubulointerstitial fibrosis.

Scale bar: 200 µm.

SDTF, SDT Fatty

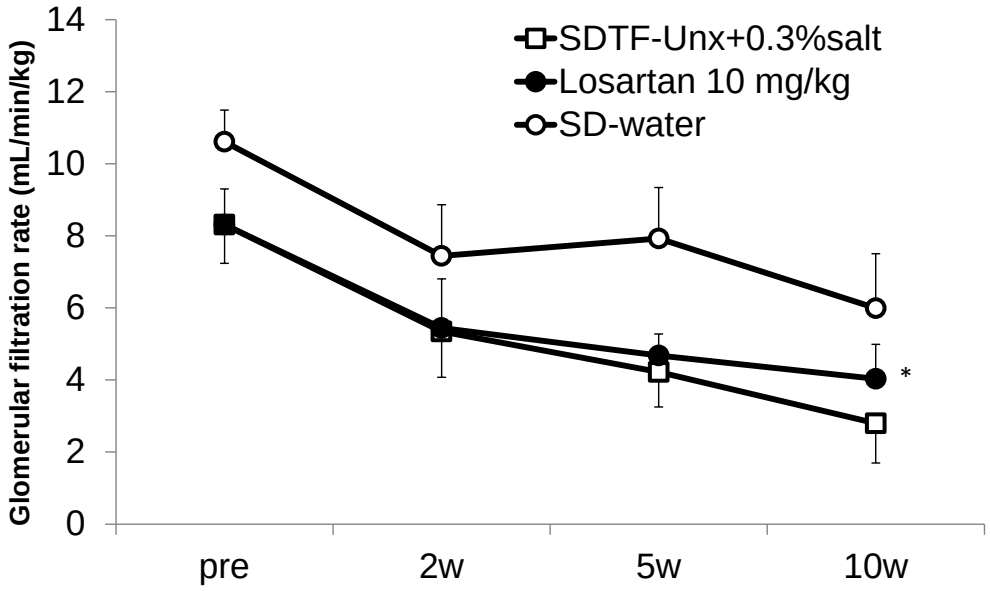

Figure 13. Effect of losartan diet on GFR decline in 0.3% salt-loaded UNx-SDTF rats.

Data points and bars represent the mean and S.D. (n = 6).

* *P* <0.05 vs. SDTF-0.3% salt/UNx group (Dunnett's test)

SDTF, SDT Fatty

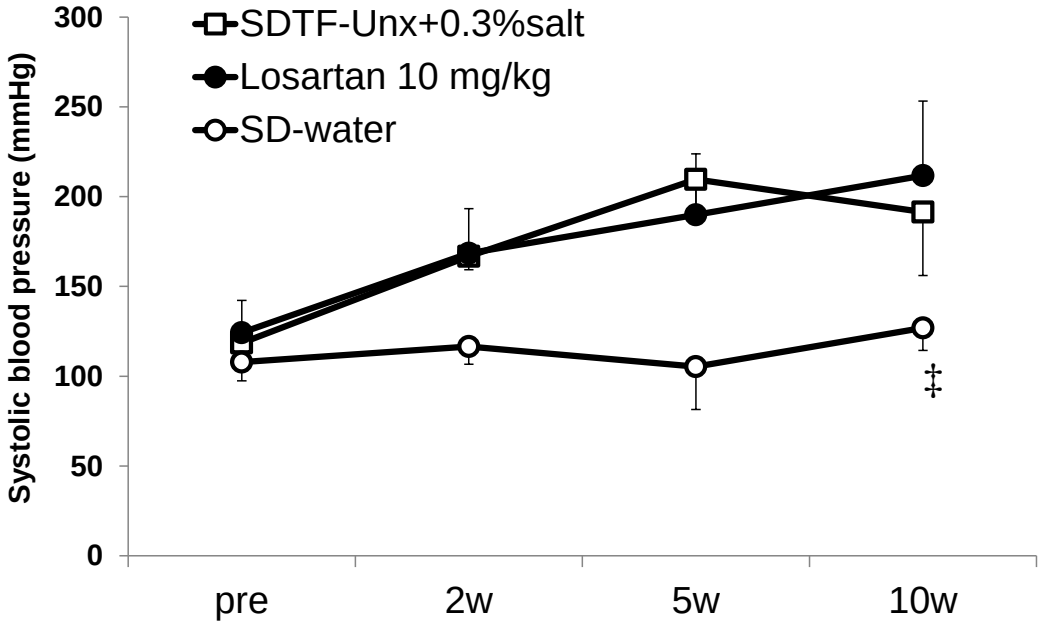

Figure 14. Effect of losartan diet on SBP levels in 0.3% salt-loaded UNx-SDTF rats.

Data points and bars represent the mean and S.D. (n = 6).

‡ P <0.01 vs. SD-water group (Welch's test)

SDTF, SDT Fatty

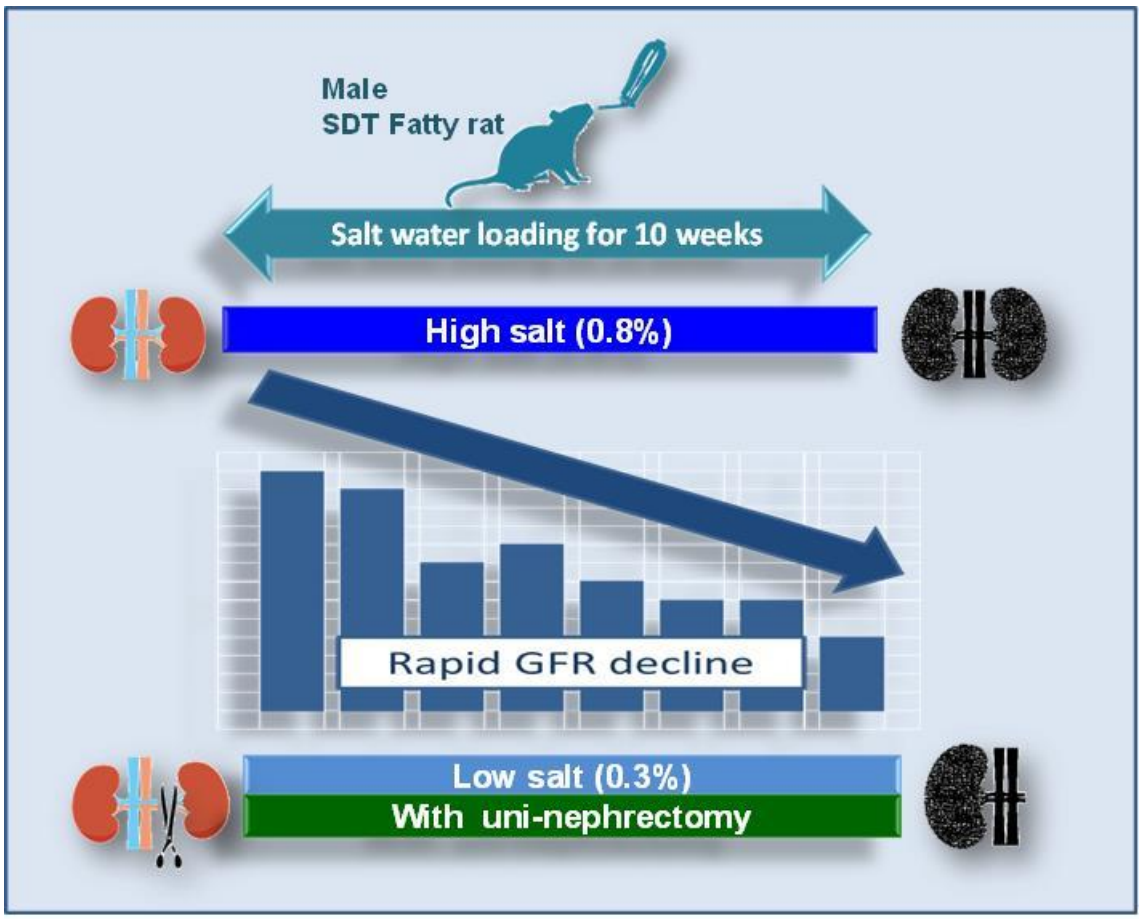

Figure 15. Graphical Summary.

0.3% salt loading by drinking water containing sodium chloride (Low salt) with UNx or 0.8% salt loading alone (High salt) caused a rapid GFR decline and impaired renal morphology.

Chapter 4

General Discussion

Proteinuria might be a valuable marker in the assessment of renal function. However, in recent years, as some patients with decline in renal function without proteinuria have been reported (Thomas *et al.*, 2009; Thomas *et al.*, 2015; Afkarian *et al.*, 2016), proteinuria is not a universally accepted marker. Therefore, it would be potentially valuable to be able to utilize GFR and Ccr as assessment parameters as well as proteinuria in the DKD animal model. In addition, for the evaluation of novel therapeutics or analysis of the pathogenesis of DKD, the development of animal models that show Ccr or GFR decline comparable to Ccr or GFR decline in patients with DKD is crucial. To achieve these aims, I established a long-term model using UNx-SHR/NDmcr-cp rats as described in Chapter 2 and a short-term model using 0.3% salt-loaded UNx-SDT fatty rats as described in Chapter 3.

These models are intrinsically characterized by leptin receptor dysfunction and in addition they are also obese, type 2 diabetic models of DKD with hyperglycemia, hyperlipidemia, and hypertension (Masuyama *et al.*, 2005; Nangaku *et al.*, 2005). Furthermore, UNx performed on SHR/NDmcr-cp rats and 0.3% salt loading along with UNx in SDT fatty rats resulted in declining renal function as measured by Ccr and GFR (Figure 3, Figure 9), and histopathological similarity to patients with CKD, with glomerular lesions such as glomerulosclerosis and glomerular hypertrophy, tubular lesions such as tubular dilatation and tubular epithelium regeneration or degeneration, and tubulointerstitial fibrosis (Figures 5,6,11,12). In particular, glomerulosclerosis and tubulointerstitial fibrosis are characteristic findings shared by patients with CKD, suggesting a final common pathway in the later stages (Fogo, 2007).

On the other hand, losartan was effective in UNx-SHR/NDmcr-cp rats but less effective in 0.3% salt-loaded UNx-SDT fatty rats (Figures 5,6,14,15), suggesting that these two models are substantially different. During the course of the study, 0.3% salt-loaded UNx-SDT fatty rats showed consistently very high levels of blood glucose and metabolic parameters, while UNx-SHR/NDmcr-cp rats exhibited lower levels blood glucose at later stages and metabolic parameters compared to the above 0.3% salt-loaded model (Figure 5,11), which might have influenced the difference in the nature of the pathological conditions between the two models. In addition, since salt loading has been reported not to induce contraction of the efferent arteriole by inhibiting the activation of renin-angiotensin system in the renal glomerulus, it was also suggested that was responsible for the reduced efficacy of losartan in the 0.3% salt-loaded UNx-SDT fatty model (Charytan *et al*., 2012).

The characteristics of the individual models are described below.

Models for pharmacological evaluation

Characteristics of the UNx-SHR/NDmcr-cp rat model

- Hyperlipidemia and hypertension are noted, however, the increase in blood glucose is relatively mild and returns to normal levels in the later phase of the model.
- Prolonged poor health causes chronic renal decline in this model.
- The pathological findings may be similar to those in patients with CKD.
- Efficacy of losartan is detectable, suggesting a role for renin-angiotensin -aldosterone system (RAAS) in the pathogenesis of the disease.

Meanwhile, due to the slow development of disease in the chronic model, evaluation periods of at least 25 weeks after UNx are required before histopathological changes

become evident.

Characteristics of the 0.3% salt-loaded UNx-SDT fatty rat model

- Elevated hyperlipidemia and hypertension are observed, as well as a significant increase in blood glucose.
- Decline in renal function is rapid, requiring approximately 10 weeks.
- The pathological findings are prominent glomerular lesions such as glomerular hypertrophy as well as tubulointerstitial fibrosis.
- There is less involvement of the RAAS in the pathogenesis of the model.

From the results of the evaluation of the ARB losartan with Ccr or GFR as indices, it is concluded that the UNx-SHR/NDmcr-cp rat might serve as a model for up to stage G3a and the 0.3% salt-loaded UNx-SDT fatty rat might serve as a model for stage G3b or later.

Future studies should be performed in which each model is employed to evaluate recently approved agents such as SGLT2 inhibitors and MRAs and to analyze their efficacy using various parameters, including pathological findings. By comparing the efficacy of these new agents in these animal models with that in patients and by accumulating further data, the detailed similarities and dissimilarities between the pathology of the animal models and that of the patients can be clarified and better characterization of these DKD models can be achieved. Moreover, future work should repeat the evaluation of agents with different mechanisms of action and compare the results with the clinical efficacy profile in patients. By accumulating the data from these studies, it should be possible to more accurately predict the efficacy of new candidate

medicines in patients with DKD from the results in animal models.

Conclusion

In conclusion, I have developed two animal models that showed decline of glomerular filtration.

First, I found that UNx in SHR/cp rats could induce a decline of glomerular filtration, and that UNx-SHR/cp rats might be a potentially useful model corresponding to CKD stage G3a in patients, by showing that treatment of these rats with losartan improved the histopathology of the kidney and prevented renal function decline.

Furthermore, I demonstrated that UNx and 0.3% salt water loading in SDT fatty rats rapidly reduced glomerular filtration rate, and losartan treatment in these rats had limited efficacy, indicating that salt-loaded UNx-SDT fatty rats might be a valuable model for CKD stage G3b and later.

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