# Inhibition of sodium-glucose cotransporter 2 ameliorates renal injury in a novel medaka model of nonalcoholic steatohepatitis-related kidney disease

Takuro Nagoya[1], Kenya Kamimura[1] (ID), Ryo Goto[1], Yoko Shinagawa-Kobayashi[1], Yusuke Niwa[1], Atsushi Kimura[1], Norihiro Sakai[1], Masayoshi Ko[1], Hiroshi Nishina[2] and Shuji Terai[1]

1 Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, Japan
2 Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Japan

**Correspondence**
K. Kamimura and Shuji Terai, Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, 1-757 Asahimachi-dori, Chuo–ku, Niigata, Niigata, 9518510, Japan
Fax: +81 (25) 227-0776
Tel: +81 (25) 227-2207
E-mail: kenya-k@med.niigata-u.ac.jp

Takuro Nagoya and Kenya Kamimura contributed equally to this work

The effect of sodium-glucose cotransporter 2 inhibitor (SGLT2I) on nonalcoholic steatohepatitis (NASH) has been reported, but there are few studies on its effect on NASH-related renal injury. In this study, we examined the effect of SGLT2I using a novel medaka fish model of NASH-related kidney disease, which was developed by feeding the d-rR/Tokyo strain a high-fat diet. SGLT2I was administered by dissolving it in water of the feeding tank. SGLT2I ameliorates macrophage accumulation and oxidative stress and maintained mitochondrial function in the kidney. The results demonstrate the effect of SGLT2I on NASH-related renal injury and the usefulness of this novel animal model for research into NASH-related complications.

Nonalcoholic fatty liver disease (NAFLD) is closely associated with obesity, diabetes, hyperlipidemia, hypertension, and insulin resistance [1–3] and frequently complicated with various metabolic diseases including chronic kidney disease and cardiovascular disease which can adversely impact prognosis. As insulin resistance is a major risk factor for disease progression, a variety of antidiabetic therapies are expected to provide benefit in NAFLD [4–6]. Among these potential therapeutic approaches, sodium-glucose cotransporter 2 inhibitors (SGLT2Is), which reduce hyperglycemia by suppressing glucose reabsorption in proximal tubules and improving

insulin resistance, glucotoxicity, and lipotoxicity [7–9], were shown to be effective in ameliorating NAFLD progression in basic [10–13] and clinical [14–24] studies. In addition, we recently showed the preventive effect of tofogliflozin (Tofo), a highly specific SGLT2I [13], on fatty infiltration and fibrotic changes in the liver of high-fat diet (HFD)-fed medaka fish (*Oryzias latipes*) nonalcoholic steatohepatitis (NASH) model [25]. This model provided ease of maintenance of animals under the same condition (the same concentrations of the chemical compound dissolved in water) and high fecundity. Intriguingly, a recent study reported that the

**Abbreviations**
8-OHdG, 8-hydroxy-2′-deoxyguanosine; GPX1, glutathione peroxidase 1; HFD, high-fat diet; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OPA1, optic atrophy type 1; SGLT2I, sodium-glucose cotransporter 2 inhibitor; Tofo, tofogliflozin.

HFD-fed medaka exhibited enlarged glomeruli, dilated glomerular capillaries, and expanded mesangium, changes comparable to changes observed in humans with metabolic syndrome-related glomerulopathy [26]. To date, only a few studies demonstrated the beneficial effect of SGLT2Is on chronic renal injury in NASH animal models and elucidated the underlying mechanisms [27–31]. The mechanisms include the renal inflammation, fibrosis, ER stress, apoptosis and lipid accumulation due to the increased renal expression of reactive oxygen species related to oxidative stress [11,28] and TGF-β1, type IV collagen, and fibronectin [11]. Therefore, we examined whether NASH-related renal injury could be ameliorates by the SGLT2I Tofo and assessed renal changes, inflammation, oxidative stress, and renal mitochondrial damage in the medaka model of NASH. We found that Tofo ameliorates the accumulation of macrophages and oxidative stress and maintained mitochondrial function in the renal tubules of medaka fish in the HFD-induced renal injury model. These results demonstrate the beneficial effect of an SGLT2I in NASH-related renal injury and further reveal the utility of the medaka model of NASH in examining these effects and evaluation of other potential therapeutic compounds for NASH-related complications.

## Materials and methods

### Animals and diets

All animal experiments were conducted in full compliance with the regulations of the Institutional Animal Care and Use Committee at Niigata University (Niigata, Japan) that approved the study protocol. All animals received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' by the National Academy of Sciences and published by the National Institute of Health. The d-rR/Tokyo strain of medaka fish (strain ID: MT837) was supplied by NBRP Medaka (https://shigen.nig.ac.jp/medaka/). All animals were 4 months old and maintained in plastic tanks containing 2 L tap water in plastic tanks under fluorescent light from 8 AM to 8 PM. The water temperature was maintained at $25 \pm 1$ °C. The medaka NASH model was developed by feeding the fish an HFD described in a previous study [25,32]. Briefly, each tank was supplied with a control diet (Hikari Labo M-450; Kyorin, Hyogo, Japan) or HFD (HFD32; CLEA Japan, Tokyo, Japan), at a rate of 20 mg·day$^{-1}$ per fish to be consumed within 14 h.

### Tofogliflozin administration

Tofogliflozin (Kowa Co. Ltd., Tokyo, Japan) was prepared at a final concentration of 0.5 mg·L$^{-1}$ in the tank containing the fish to be treated, as described previously [25]; this concentration is the $C_{max}$ of 500 ng·mL$^{-1}$ in humans treated with a standard Tofo dose of 20 mg. Briefly, Tofo was dissolved in dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) to a concentration of 100 mg·mL$^{-1}$ and then added to the water of the plastic tank at a final concentration of 0.5 mg·L$^{-1}$. The same amount of dimethyl sulfoxide was administered to the tank of the HFD group as vehicle control.

### Histological analyses

Kidney tissue samples were collected at the indicated time points, fixed in 10% formalin, and embedded in paraffin. Sections that were 10 μm in thickness were stained using the standard periodic acid/Schiff method. Mouse anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody (ab48508, 1 : 100 dilution; Abcam, Cambridge, UK), rabbit anti-glutathione peroxidase 1 (GPX1) antibody (ab22604, 1 : 1000 dilution; Abcam), rabbit anti-F4/80 antibody (ab111101, 1 : 100 dilution; Abcam), rabbit anti-optic atrophy type 1 (OPA1) antibody (ab157457, 1 : 500 dilution; Abcam), Vectastain Elite ABC rabbit IgG kit (PK-6101; Vector Laboratories, Burlingame, CA, USA), Vectastain Elite ABC mouse IgG kit (PK-6102; Vector Laboratories), and DAB chromogen tablets (Muto Pure Chemicals, Tokyo, Japan) were used for immunohistochemical staining. Images were randomly captured from each tissue section, and the measurement of the kidney and glomerular sizes and the quantitative analysis of areas and/or cells stained positively for 8-OHdG, GPX1, OPA1, and F4/80 were performed using the IMAGEJ software (version 1.6.0_20; National Institutes of Health, Bethesda, MD, USA) as reported previously [33]. Liver tissue samples were collected at the appropriate time points, fixed in 10% formalin, and embedded in paraffin. Sections (10 μm) were stained with standard hematoxylin and eosin.

### Biochemical analyses

Blood samples were collected from the animals following a 12-h fasting period for all instances, as previously reported [25]. Fish were kept on ice for 1 min; thereafter, they were bled by cutting the ventral portion of the tail fin. Blood was collected in a heparinized microcapillary tube (VC-H075H; Terumo, Tokyo, Japan) and centrifuged at 1200 *g* for 12 min at 22 °C. Blood glucose levels were analyzed using a glucometer (Glucocard G Black, GT-1830; Arkray, Kyoto, Japan).

### Statistical analyses

Data were analyzed using one-way or two-way analysis of variance with repeated measures followed by Bonferroni's

multiple comparison test. A *P* value ≤ 0.05 was considered to indicate statistical significance.

## Results

### Effect of Tofo on blood glucose and kidney size in the medaka model of NASH

We first evaluated the effect of Tofo on changes in blood glucose levels over time in chow-fed (chow), HFD-fed (HFD), and HFD-fed and Tofo-treated (HFD + Tofo) groups (Fig. 1A). Over 12 weeks, whereas blood glucose levels did not show significant changes in the chow group over 12 weeks, the HFD group exhibited a significant increase from the control

level of $36.4 \pm 4.4$ mg·dL$^{-1}$ to $102.8 \pm 78.3$, $132.2 \pm 112.3$, and $146.0 \pm 121.8$ mg·dL$^{-1}$ in 4, 8, and 12 weeks, respectively. Importantly, the blood glucose levels were modestly decreased to $78.3 \pm 19.7$, $112.3 \pm 22.3$, and $121.8 \pm 54.2$ mg·dL$^{-1}$ in 4, 8, and 12 weeks, respectively, in the HFD + Tofo group ($P < 0.05$ in 4 weeks) (Fig. 1A). The liver tissue showed successful development of significant fatty infiltration in the liver (Fig. 1B) and that Tofo ameliorates the changes (Fig. 1C) as previously reported [25]. The HFD group exhibited enlargement of the renal size from the control level of $737.7 \pm 101.3$ μm to $1645.5 \pm 109.8$ μm following 12 weeks of HFD feeding, whereas the HFD + Tofo group showed milder enlargement to $1343.3 \pm 92.5$ μm ($P < 0.01$)
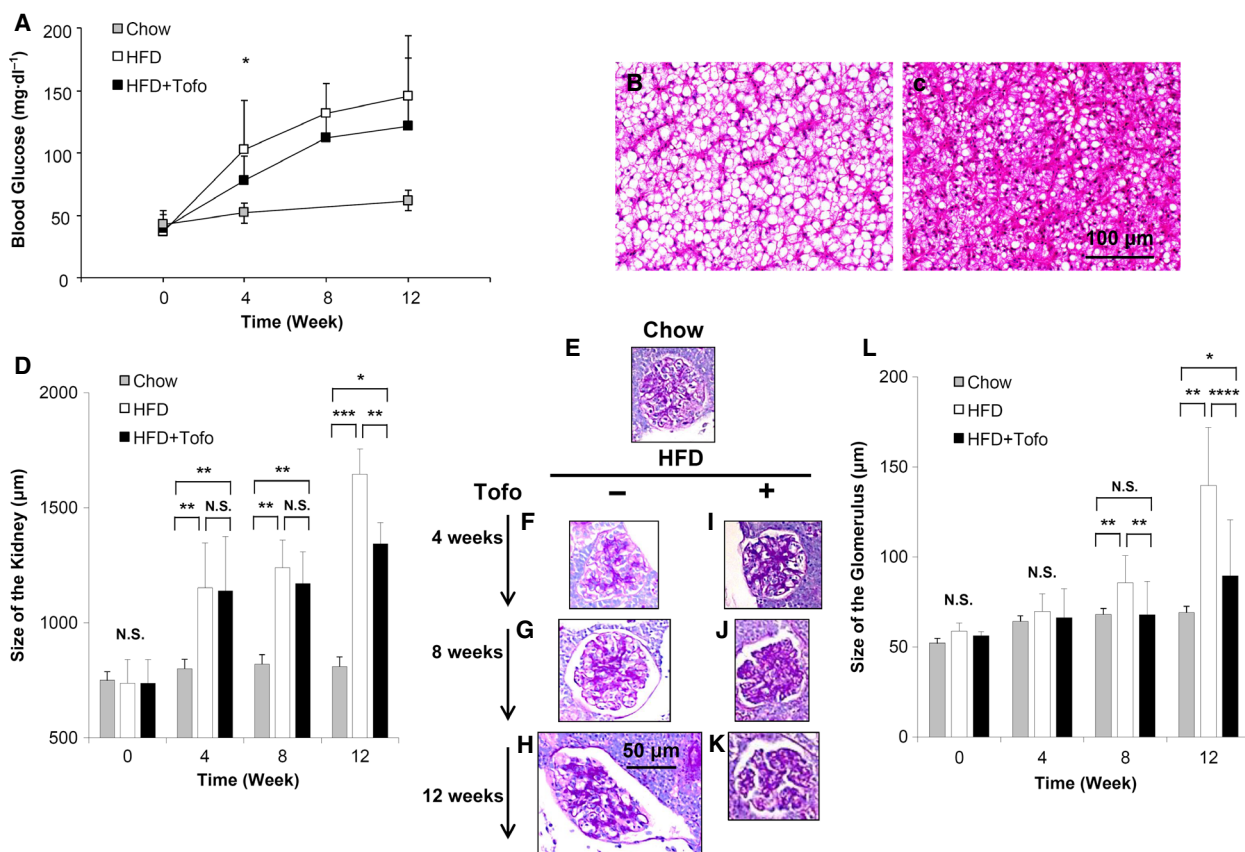


**Fig. 1.** Effects of Tofo on blood glucose, renal size, histological changes, and the glomerular size in the kidney of the medaka model of NASH. (A) The time-dependent changes in the blood glucose serum biochemical markers. The values represent mean ± SD values (*n* = 15 for each group). Two-way ANOVA followed by Bonferroni's multiple comparison test. *P < 0.05. Representative microscopic findings of medaka liver tissues stained with hematoxylin and eosin staining from medaka fed with HFD (B) and HFD + Tofo (C) for 12 weeks. Scale bar represents 100 μm. (D) The time-dependent changes in the renal size in each group. The values represent mean ± SD values (*n* = 15 for each group). *P < 0.05, **P < 0.01, ***P < 0.001, and NS, no statistical significance. One-way ANOVA followed by Bonferroni's multiple comparison test. Representative microscopic findings of medaka kidney tissues stained with the periodic acid/Schiff method. (E) Chow-fed medaka. (F–K) Medaka fed with HFD and HFD + Tofo for 4, 8, and 12 weeks. Scale bar represents 50 μm. (L) Quantitative analysis of glomerular size in the medaka kidneys. The values represent mean ± SD values (*n* = 15 for each group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and NS, no statistical significance. One-way ANOVA followed by Bonferroni's multiple comparison test.

(Fig. 1D). Mild and statistically nonsignificant changes in renal size were seen over the 12-week period in the chow group. And the differences between Chow and HFD showed the evidence of successful development of NASH-related kidney disease. These results confirmed that in the HFD-induced NASH model in medaka, Tofo was effective in reducing elevated blood glucose level and renal swelling induced by elevated blood glucose.

## Effect of Tofo on glomerular size in the medaka model of NASH

To determine whether glomerular enlargement, a characteristic of HFD-induced renal injury associated with mesangial expansion and glomerular capillary dilatation, was suppressed by Tofo, we assessed time-dependent changes in glomerular size in our medaka fish model of NASH. The HFD group exhibited a significant, time-dependent increase in glomerular size to 140 μm compared with the glomerular size of 60 μm in the chow group over 12 weeks (Fig. 1E–H), and this increase was milder when Tofo was administered ($P < 0.05$) (Fig. 1I–K). No significant changes in the glomerular size were seen in the chow group, and no difference in expansion of the renal matrix was seen in the HFD and HFD + Tofo groups (Fig. 1L). These results suggested that Tofo slowed down HFD-induced glomerular enlargement in the medaka model of NASH.

## Effect of Tofo on oxidative stress in the kidney of the medaka model of NASH

To further elucidate the mechanism underlying the effect of Tofo on HFD-induced renal injury in the medaka model of NASH, we assessed the expression levels of 8-OHdG and GPX1 in the renal tissues using immunohistochemical analyses (Fig. 2). The HFD group showed increases in the percentage of 8-OHdG-positive areas in the renal tissue in a time-dependent manner, from $8.1 \pm 0.5\%$ in the control group (Fig. 2A) to $12.0 \pm 0.8\%$, $17.8 \pm 0.5\%$, and $18.7 \pm 1.0\%$ (Fig. 2B–D) at 4, 8, and 12 weeks in the HFD group, respectively. The percentage of 8-OHdG-positive areas was significantly suppressed to $9.2 \pm 0.3\%$, $10.8 \pm 1.1\%$, and $11.8 \pm 1.1\%$ at 4, 8, and 12 weeks, respectively, in the HFD + Tofo group (Fig. 2E–H). Conversely, the percentage of GPX1-positive areas was significantly decreased from the control level of $6.4 \pm 0.6\%$ (Fig. 2I) to $4.4 \pm 0.5\%$, $3.3 \pm 0.6\%$, and $3.1 \pm 0.3\%$ (Fig. 2J–L) at 4, 8, and 12 weeks in the HFD group, respectively, and was

significantly suppressed to $5.6 \pm 0.3\%$, $5.3 \pm 0.4\%$, and $4.2 \pm 0.1\%$ by Tofo administration (Fig. 2M–P). The differences between Chow and HFD (Fig. 2H,P) suggested that the HFD-induced renal injury led to oxidative stress represented by the accumulation of 8-OHdG and the decrease in GPX1-positive staining, both of which were effectively alleviated by Tofo, although the effect is milder in later stage of 12 weeks.

## Accumulation of F4/80-positive cells in the kidney of the medaka model of NASH

To examine the HFD-induced inflammation in the kidney, we next determined whether there was macrophage accumulation in the kidneys of medaka fish in the HFD-induced NASH model and whether this could be ameliorates by Tofo. Therefore, we determined the number of cells positively stained with F4/80. While no increase with aging in 12 weeks was observed in the chow group (50–60 cells per high-power field) (Fig. 3A), the HFD group showed increases in the F4/80-positive cells over time to $110.1 \pm 16.9$, $187.6 \pm 14.5$, and $152.6 \pm 25.6$ at 4, 8, and 12 weeks, respectively (Fig. 3B–D). This increase was significantly suppressed to $77.5 \pm 12.7$, $118.5 \pm 25.4$, and $125.7 \pm 26.8$ at 4, 8, and 12 weeks, respectively, by Tofo administration (Fig. 3E–H). The differences between Chow and HFD (Fig. 3H) provided evidence that HFD-induced inflammation and oxidative stress led to an increase in the number of macrophages, which was suppressed by Tofo administration in our animal model although the effect is milder in later stage of 8 and 12 weeks.

## Effect of Tofo on mitochondrial function in renal tubules in the medaka model of NASH

To determine whether HFD led to mitochondrial damage, we assessed the kidney tissue samples in the medaka model of NASH by OPA1 staining. While no change in the percentage of OPA1-stained areas was observed in the chow group, with 6%–7% OPA1-positive areas in the control (Fig. 4A), the HFD group exhibited significant decreases in the percentage of OPA1-positive areas to $5.5 \pm 0.6\%$, $4.2 \pm 0.2\%$, and $3.9 \pm 0.3\%$ at 4, 8, and 12 weeks, respectively (Fig. 4B–D). These decreases were reversed with significant increases in the percentage of OPA1-positive areas to $6.4 \pm 0.5\%$, $6.2 \pm 0.3\%$, and $5.2 \pm 0.3\%$ at 4, 8, and 12 weeks, respectively, by Tofo administration (Fig. 4E–H). The differences between Chow and HFD (Fig. 4H) suggested that HFD-induced renal injury was associated with damaged mitochondrial
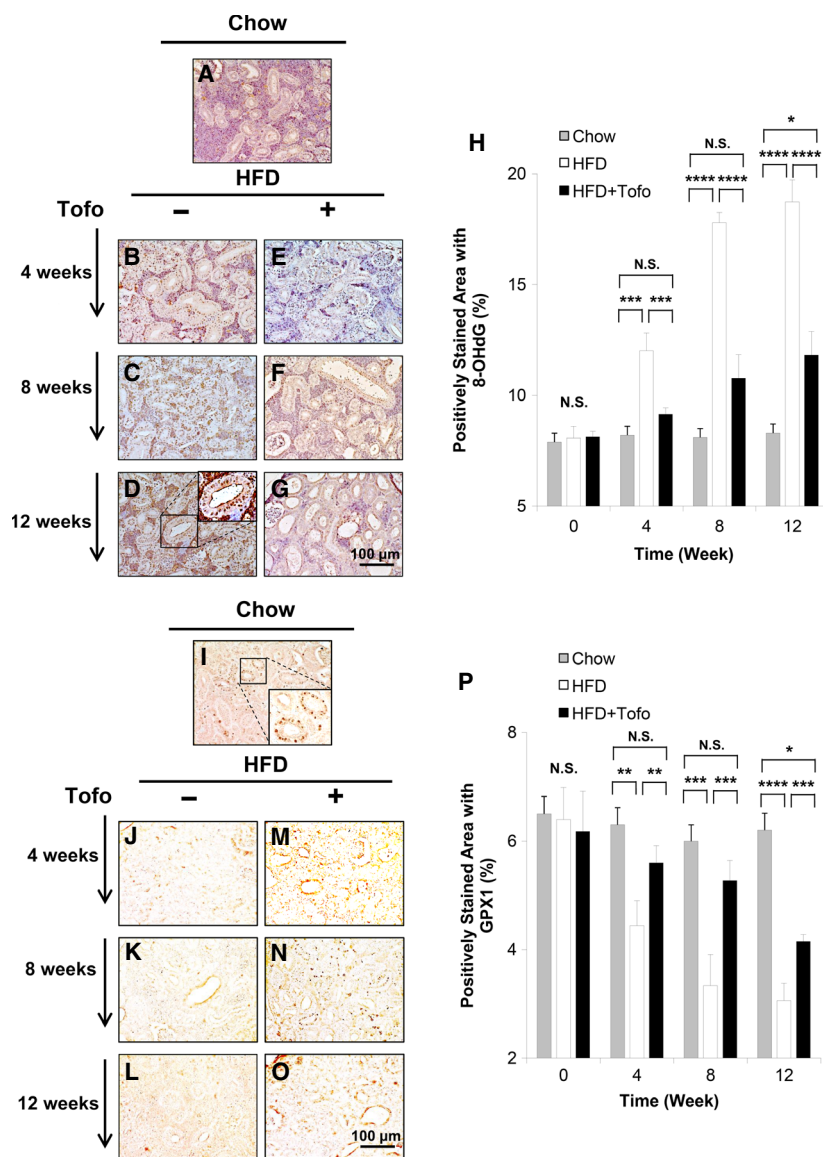
**Fig. 2.** Effects of Tofo on the level of 8-OHdG and GPX1 in the kidney of the medaka model of NASH. Representative microscopic findings of medaka kidney tissues stained with anti-8-OHdG antibody. (A) Chow-fed medaka. (B–G) Medaka fed with HFD and HFD + Tofo for 4, 8, and 12 week. Scale bar represents 100 μm. (H) Quantitative analysis of positively stained area with anti-8-OHdG antibody in the medaka kidneys. The values represent mean ± SD values ($n = 15$ for each group). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, and NS, no statistical significance. One-way ANOVA followed by Bonferroni's multiple comparison test. Representative microscopic findings of medaka kidney tissues stained with anti-GPX1 antibody. (I) Chow-fed medaka. (J–O) Medaka fed with HFD and HFD + Tofo for 4, 8, and 12 weeks. Scale bar represents 100 μm. (P) Quantitative analysis of positively stained area with anti-GPX1 antibody in the medaka kidneys. The values represent mean ± SD values ($n = 15$ for each group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and NS, no statistical significance. One-way ANOVA followed by Bonferroni's multiple comparison test.

function in the renal tubules represented by OPA1 expression, a key GTPase necessary for mitochondrial fusion and maintenance of mitochondrial homeostasis, which was suppressed by Tofo administration in our animal model although the effect is milder in later stage of 12 weeks.

## Discussion

With increasing incidence, NAFLD is becoming the most common chronic liver disease. Disease progression is related to obesity, diabetes, hyperlipidemia, hypertension, and insulin resistance [1–3], and chronic renal injury and cardiovascular disease are common complications that aggravate each other and are

associated with bad prognosis in patients with NAFLD/NASH [34]. These complications are known to be induced by inflammation and oxidative stress, which aggravate glucotoxicity and lipotoxicity of metabolic syndrome [27,34]. Specifically, NASH-related renal injury shares several features with diabetic kidney disease and is associated with renal changes including glomerular enlargement, focal segmental glomerulosclerosis, and renal tubular damage [35–37]. The causative factors underlying these features of chronic renal injury include changes in adipokine levels such as a reduction in adiponectin, which mediates fatty acid metabolism by increasing peroxisome proliferative-activated receptor-α expression and inducing AMP-activated protein kinase phosphorylation [38].

**Fig. 3.** Effects of Tofo on the accumulation of macrophages in the kidney of the medaka model of NASH. Representative microscopic findings of medaka kidney tissues stained with anti-F4/80 antibody. (A) Chow-fed medaka. (B–G) Medaka fed with HFD and HFD + Tofo for 4, 8, and 12 weeks. Scale bar represents 100 μm. (H) Quantitative analysis of the number of positively stained cells per high-power field with anti-F4/80 antibody in the medaka kidneys. The values represent mean ± SD values (*n* = 15 for each group). **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, and NS, no statistical significance. One-way ANOVA followed by Bonferroni's multiple comparison test.
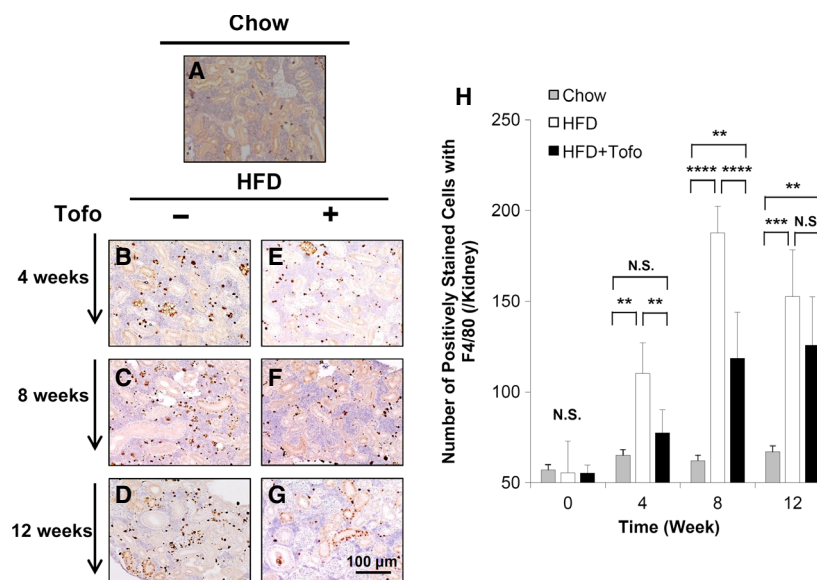


**Fig. 4.** Effects of Tofo on the expression of OPA1 in the renal tubules in the kidney of the medaka model of NASH. Representative microscopic findings of medaka kidney tissues stained with anti-OPA1 antibody. (A) Chow-fed medaka. (B–G) Medaka fed with HFD and HFD + Tofo for 4, 8, and 12 weeks. Scale bar represents 100 μm. (H) Quantitative analysis of the number of positively stained cells with anti-OPA1 antibody in the renal tubules in the medaka kidneys. The values represent mean ± SD values (*n* = 15 for each group). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, and NS, no statistical significance. One-way ANOVA followed by Bonferroni's multiple comparison test.
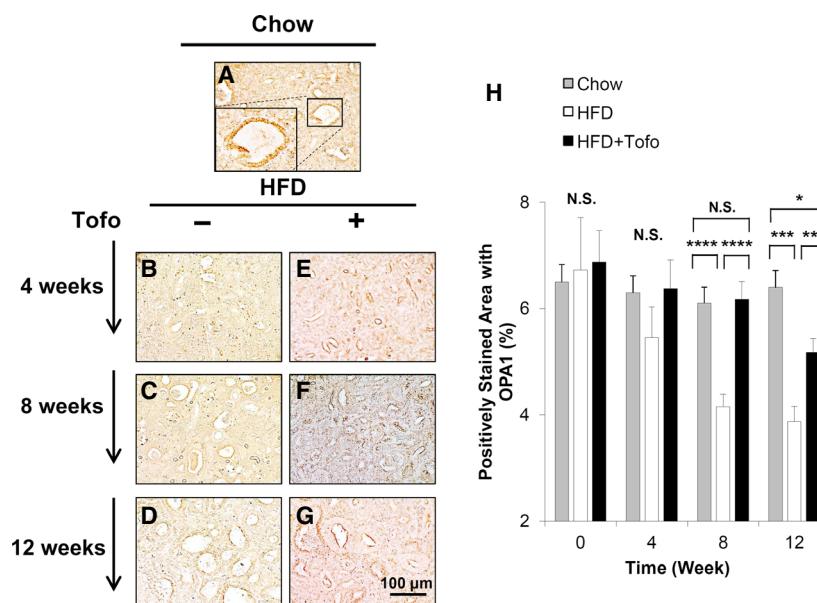
Basic and clinical studies that provide evidence for these mechanisms used renal histological analyses in HFD-induced diabetic nephropathy models and reported inflammation, macrophage activation in glomerular as well as interstitial lesions, accumulation of oxidative stress [39–41], and mitochondrial damage in renal tubules [29,37]. However, few studies have investigated the effect of SGLT2Is on NASH-related renal injury in an animal model. Therefore, we have examined the renal inflammation, oxidative stress, and mitochondrial function in a medaka model of HFD-induced renal injury that recapitulates changes

in NASH-associated renal injury [26], because of the ease of maintenance of these animals under the same concentrations of chemical compounds dissolved in water and their high fecundity. In this model, we previously reported that Tofo attenuated the fatty and fibrotic changes in the liver [25]. In the current study, we examined the effect of Tofo, a selective SGLT2I, on NASH-related renal injury by assessing macroscopic and microscopic changes in the kidneys, markers of inflammation and oxidative stress, and mitochondrial function in the renal tubules. Comparison of the Chow and HFD groups with regard to the

levels of blood glucose, sizes of the kidneys and glomerulus (Fig. 1), and levels of 8-OHdG (Fig. 2), GPX1 (Fig. 2), F4/80 (Fig. 3), and OPA1 (Fig. 4) indicated the successful development of NASH-related kidney diseases. Additionally, after demonstrating the effect of Tofo on blood glucose levels in the medaka model of NASH, we showed that Tofo slowed glomerular enlargement that led to renal swelling induced by HFD. However, our results indicated that Tofo did not affect the mesangial expansion, which is consistent with the findings of a previous report [11,28,31]. In addition, in our medaka model of NASH, we showed that these changes associated with Tofo administration were related to the amelioration of macrophage accumulation and oxidative stress in the kidney as well as the maintenance of mitochondrial function in the renal tubules in HFD-induced renal injury in this medaka model of NASH. Our study appeared to have a limitation in the analysis of renal function (including proteinuria and the estimated glomerular filtration rate) because the urinary volume of medaka has been reported to be 1 mL·h$^{-1}$·kg$^{-1}$ and urine is difficult to collect appropriately [42]. The comparison of the Chow and HFD + Tofo groups demonstrated that the effect of Tofo became mild with continuous feeding of HFD for 12 weeks, indicating that further analyses regarding the doses and combinations with other antidiabetic drugs, lipid-lowering drugs, etc., will help improve its effectiveness.

In conclusion, we showed that the highly specific SGLT2I Tofo prevented the progression of NASH-related renal injury by reducing oxidative stress and macrophage accumulation and maintaining mitochondrial function in the renal tubules. Additionally, the medaka model might be a useful platform for cost-effective evaluation of the efficacy of therapeutic approaches in NASH-related complications. Therefore, further analyses involving testing of various medicines in the medaka NASH model will help reveal better combinations of medicines for the treatment and prevention of NASH and its complications.

## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

TS, NH, and KK conceived and supervised the study; KK and NT designed experiments; NT, GR, SKY, NY, KS, SN, KM, and KK performed experiments; and NT, KK, and TS analyzed the data and wrote the manuscript.

## References

1 NCD Risk Factor Collaboration (2016) Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. *Lancet* **387**, 1377–1396.

2 Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L and Wymer M (2016) Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64**, 73–84.

3 Neuschwander-Tetri BA (2017) Non-alcoholic fatty liver disease. *BMC Med* **15**, 45.

4 Lallukka S and Yki-Jarvinen H (2016) Non-alcoholic fatty liver disease and risk of type 2 diabetes. *Best Pract Res Clin Endocrinol Metab* **30**, 385–395.

5 Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, Buzzigoli E, Sironi AM, Cersosimo E, Ferrannini E *et al.* (2007) Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology* **133**, 496–506.

6 Sumida Y and Yoneda M (2018) Current and future pharmacological therapies for NAFLD/NASH. *J Gastroenterol* **53**, 362–376.

7 Bailey CJ (2011) Renal glucose reabsorption inhibitors to treat diabetes. *Trends Pharmacol Sci* **32**, 63–71.

8 Abdul-Ghani MA, Norton L and DeFronzo RA (2012) Efficacy and safety of SGLT2 inhibitors in the treatment of type 2 diabetes mellitus. *Curr Diab Rep* **12**, 230–238.

9 Tahara A, Takasu T, Yokono M, Imamura M and Kurosaki E (2016) Characterization and comparison of sodium-glucose cotransporter 2 inhibitors in pharmacokinetics, pharmacodynamics, and pharmacologic effects. *J Pharmacol Sci* **130**, 159–169.

10 Komiya C, Tsuchiya K, Shiba K, Miyachi Y, Furuke S, Shimazu N, Yamaguchi S, Kanno K and Ogawa Y

(2016) Ipragliflozin improves hepatic steatosis in obese mice and liver dysfunction in type 2 diabetic patients irrespective of body weight reduction. *PLoS ONE* **11**, e0151511.

11 Tang L, Wu Y, Tian M, Sjöström CD, Johansson U, Peng X-R, Smith DM and Huang Y (2017) Dapagliflozin slows the progression of the renal and liver fibrosis associated with type 2 diabetes. *Am J Physiol Endocrinol Metab* **313**, E563–E576.

12 Obata A, Kubota N, Kubota T, Iwamoto M, Sato H, Sakurai Y, Takamoto I, Katsuyama H, Suzuki Y, Fukazawa M *et al.* (2016) Tofogliflozin improves insulin resistance in skeletal muscle and accelerates lipolysis in adipose tissue in male mice. *Endocrinology* **157**, 1029–1042.

13 Suzuki M, Honda K, Fukazawa M,Fukazawa M, Ozawa K, Hagita H, Kawai T, Takeda M, Yata T, Kawai M *et al.* (2012) Tofogliflozin, a potent and highly specific sodium/glucose cotransporter 2 inhibitor, improves glycemic control in diabetic rats and mice. *J Pharmacol Exp Ther* **341**, 692–701.

14 Ohki T, Isogawa A, Toda N and Tagawa K (2016) Effectiveness of ipragliflozin, a sodium-glucose cotransporter 2 inhibitor, as a second-line treatment for non-alcoholic fatty liver disease patients with type 2 diabetes mellitus who do not respond to incretin-based therapies including glucagon-like peptide-1 analogs and dipeptidyl peptidase-4 inhibitors. *Clin Drug Investig* **36**, 313–319.

15 Ito D, Shimizu S, Inoue K, Saito D, Yanagisawa M, Inukai K, Akiyama Y, Morimoto Y, Noda M and Shimada A (2017) Comparison of ipragliflozin and pioglitazone effects on nonalcoholic fatty liver disease in patients with type 2 diabetes: a randomized, 24-week, open-label, active-controlled trial. *Diabetes Care* **40**, 1364–1372.

16 Seko Y, Sumida Y, Sasaki K, Itoh Y, Iijima H, Hashimoto T, Ishii S and Inagaki N (2018) Effects of canagliflozin, an SGLT2 inhibitor, on hepatic function in Japanese patients with type 2 diabetes mellitus: pooled and subgroup analyses of clinical trials. *J Gastroenterol* **53**, 140–151.

17 Merovci A, Solis-Herrera C, Daniele G, Eldor R, Fiorentino TV, Tripathy D, Xiong J, Perez Z, Norton L, Abdul-Ghani MA *et al.* (2014) Dapagliflozin improves muscle insulin sensitivity but enhances endogenous glucose production. *J Clin Invest* **124**, 509–514.

18 Honda Y, Imajo K, Kato T, Kessoku T, Ogawa Y, Tomeno W, Kato S, Mawatari H, Fujita K, Yoneda M *et al.* (2016) The selective SGLT2 inhibitor ipragliflozin has a therapeutic effect on nonalcoholic steatohepatitis in mice. *PLoS ONE* **11**, e0146337.

19 Nakano S, Katsuno K, Isaji M, Nagasawa T, Buehrer B, Walker S, Wilkison WO and Cheatham B (2015) Remogliflozin etabonate improves fatty liver disease in diet-induced obese male mice. *J Clin Exp Hepatol* **5**, 190–198.

20 Tahara A, Kurosaki E, Yokono M,Yamajuku D, Kihara R, Hayashizaki Y, Takasu T, Imamura M, Li Q, Tomiyama H *et al.* (2014) Effects of sodium-glucose cotransporter 2 selective inhibitor ipragliflozin on hyperglycaemia, oxidative stress, inflammation and liver injury in streptozotocin-induced type 1 diabetic rats. *J Pharm Pharmacol* **66**, 975–987.

21 Nishimura N, Kitade M, Noguchi R,Namisaki T, Moriya K, Takeda K, Okura Y, Aihara Y, Douhara A, Kawaratani H *et al.* (2016) Ipragliflozin, a sodium-glucose cotransporter 2 inhibitor, ameliorates the development of liver fibrosis in diabetic Otsuka Long-Evans Tokushima fatty rats. *J Gastroenterol* **51**, 1141–1149.

22 Hirose S, Nakajima S, Iwahashi Y, Seo A, Takahashi T and Tamori Y (2016) Impact of the 8-week administration of tofogliflozin for glycemic control and body composition in Japanese Patients with Type 2 diabetes mellitus. *Intern Med* **55**, 3239–3245.

23 Iwahashi Y, Hirose S, Nakajima S, Seo A, Takahashi T and Tamori Y (2017) Evaluation of metabolic parameters and body composition in Japanese patients with type 2 diabetes mellitus who were administered tofogliflozin for 48 weeks. *Diabetol Int* **8**, 205.

24 Kaku K, Watada H, Iwamoto Y,Utsunomiya K, Terauchi Y, Tobe K, Tanizawa Y, Araki E, Ueda M, Suganami H *et al.* (2014) Efficacy and safety of monotherapy with the novel sodium/glucose cotransporter-2 inhibitor tofogliflozin in Japanese patients with type 2 diabetes mellitus: a combined Phase 2 and 3 randomized, placebo-controlled, double-blind, parallel-group comparative study. *Cardiovasc Diabetol* **13**, 65.

25 Goto R, Kamimura K, Shinagawa-Kobayashi Y, Sakai N, Nagoya T, Niwa Y, Ko M, Ogawa K, Inoue R and Yokoo T *et al.* (2019) Inhibition of sodium glucose cotransporter 2 (SGLT2) delays liver fibrosis in a medaka model of nonalcoholic steatohepatitis (NASH). *FEBS Open Bio* **9**, 643–652.

26 Ichimura K, Kawashima Y, Nakamura T, Powell R, Hidoh Y, Terai S, Sakaida I, Kodera Y, Tsuji T, Ma J-X *et al.* (2013) Medaka fish, *Oryzias latipes*, as a model for human obesity-related glomerulopathy. *Biochem Biophys Res Commun* **431**, 712–717.

27 Yasui K, Sumida Y, Mori Y, Mitsuyoshi H, Minami M, Itoh Y, Kanemasa K, Matsubara H, Okanoue T and Yoshikawa T (2011) Nonalcoholic steatohepatitis and increased risk of chronic kidney disease. *Metabolism* **60**, 735–739.

28 Jaikumkao K, Pongchaidecha A, Chueakula N, Thongnak LO, Wanchai K, Chatsudthipong V, Chattipakorn N and Lungkaphin A (2018)

Dapagliflozin, a sodium-glucose co-transporter-2 inhibitor, slows the progression of renal complications through the suppression of renal inflammation, endoplasmic reticulum stress and apoptosis in prediabetic rats. *Diabetes Obes Metab* **20**, 2617–2626.

29 Takagi S, Li J, Takagaki Y, Kitada M, Nitta K, Takasu T, Kanasaki K and Koya D (2018) Ipragliflozin improves mitochondrial abnormalities in renal tubules induced by a high-fat diet. *J Diabetes Investig* **9**, 1025–1032.

30 Jaikumkao K, Pongchaidecha A, Chueakula N, Thongnak L, Wanchai K, Chatsudthipong V, Chattipakorn N and Lungkaphin A (2018) Renal outcomes with sodium glucose cotransporter 2 (SGLT2) inhibitor, dapagliflozin, in obese insulin-resistant model. *Biochim Biophys Acta Mol Basis Dis* **1864**, 2021–2033.

31 Nagata T, Fukuzawa T, Takeda M, Fukazawa M, Mori T, Nihei T, Honda K, Suzuki Y and Kawabe Y (2013) Tofogliflozin, a novel sodium-glucose co-transporter 2 inhibitor, improves renal and pancreatic function in db/db mice. *Br J Pharmacol* **170**, 519–531.

32 Matsumoto T, Terai S, Oishi T, Kuwashiro S, Fujisawa K, Yamamoto N, Fujita Y, Hamamoto Y, Furutani-Seiki M, Nishina H *et al.* (2010) Medaka as a model for human nonalcoholic steatohepatitis. *Dis Model Mech* **3**, 431–440.

33 Vrekoussis T, Chaniotis V, Navrozoglou I, Dousias V, Pavlakis K, Stathopoulos EN and Zoras O (2009) Image analysis of breast cancer immunohistochemistry-stained sections using ImageJ: an RGB-based model. *Anticancer Res* **29**, 4995–4998.

34 Papademetriou M, Athyros VG, Geladari E, Doumas M, Tsioufis C and Papademetriou V (2018) The co-existence of NASH and chronic kidney disease boosts cardiovascular risk: are there any common therapeutic options? *Curr Vasc Pharmacol* **16**, 254–268.

35 Furuichi K, Shimizu M, Hara A, Toyama T and Wada T (2018) Diabetic nephropathy: a comparison of the clinical and pathological features between the CKD risk classification and the classification of diabetic nephropathy 2014 in Japan. *Intern Med* **57**, 3345–3350.

36 Furuichi K, Shimizu M, Okada H, Narita I and Wada T (2018) Clinico-pathological features of kidney disease in diabetic cases. *Clin Exp Nephrol* **22**, 1046–1051.

37 Yamamoto T, Takabatake Y, Takahashi A, Kimura T, Namba T, Matsuda J, Minami S, Kaimori J-Y, Matsui I, Matsusaka T *et al.* (2017) High-fat diet-induced lysosomal dysfunction and impaired autophagic flux contribute to lipotoxicity in the kidney. *J Am Soc Nephrol* **28**, 1534–1551.

38 Kim Y and Park CW (2019) Mechanisms of adiponectin action: implication of adiponectin receptor agonism in diabetic kidney disease. *Int J Mol Sci* **20**, 1782.

39 Furuta T, Saito T, Ootaka T, Soma J, Obara K, Abe K and Yoshinaga K (1993) The role of macrophages in diabetic glomerulosclerosis. *Am J Kidney Dis* **21**, 480–485.

40 Hirata K, Shikata K, Matsuda M, Akiyama K, Sugimoto H, Kushiro M and Makino H (1998) Increased expression of selectins in kidneys of patients with diabetic nephropathy. *Diabetologia* **41**, 185–192.

41 Scheen AJ (2019) Beneficial effects of SGLT2 inhibitors on fatty liver in type 2 diabetes: a common comorbidity associated with severe complications. *Diabetes Metab* **45**, 213–223.

42 Lin SC, Liou CH and Shiau SY (2000) Renal threshold for urinary glucose excretion by tilapia in response to orally administered carbohydrates and injected glucose. *Fish Physiol Biochem* **23**, 127–132.