ORIGINAL ARTICLE

Failure to confirm a sodium–glucose cotransporter 2 inhibitor-induced hematopoietic effect in non-diabetic rats with renal anemia

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ABSTRACT

Aims/Introduction: Clinical studies have shown that treatment with inhibitors of sodium–glucose cotransporter 2 (SGLT2) significantly increases the hematocrit in patients with type 2 diabetes. To investigate whether SGLT2 inhibitors directly promote erythropoietin production independently on blood glucose reduction, the hematopoietic effect of the specific SGLT2 inhibitor, luseogliflozin, was examined in non-diabetic rats with renal anemia.

Materials and Methods: Renal anemia was induced by treatment with adenine (200 or 600 mg/kg/day, orally for 10 days) in non-diabetic Wistar–Kyoto or Wistar rats, respectively. Luseogliflozin (10 mg/kg bodyweight) or vehicle (0.5% carboxymethyl cellulose) was then administered for 6 weeks. The hematocrit and the hemoglobin (Hb), blood urea nitrogen, plasma creatinine, and plasma erythropoietin levels were monitored.

Results: Treatment with adenine decreased the hematocrit and the Hb level, which were associated with increases in the blood urea nitrogen and plasma creatinine levels. In Wistar–Kyoto rats treated with 200 mg/kg/day adenine, administration of luseogliflozin induced glycosuria, but did not change the blood urea nitrogen, plasma creatinine levels, hematocrit, Hb or plasma erythropoietin levels. Similarly, luseogliflozin treatment failed to change the hematocrit or the Hb levels in Wistar rats with renal anemia induced by 600 mg/kg/day of adenine. Plasma erythropoietin concentrations were also not different between luseogliflozin- and vehicle-treated rats. Similarly, in human erythropoietin-producing cells derived from pluripotent stem cells, luseogliflozin treatment did not change the erythropoietin level in the medium.

Conclusions: These data suggest that SGLT2 inhibitor fails to exert hematopoietic effects in non-diabetic conditions.

INTRODUCTION

Several animal studies have found renoprotective effects of sodium–glucose cotransporter 2 (SGLT2) inhibitors in diabetic kidney disease (DKD)[1,2]. Furthermore, large clinical trials, such as Empagliflozin Cardiovascular Outcome Event Trial in Type 2 diabetes Mellitus Patients Outcome (EMPA-REG OUTCOME) study, The CANagliflozin cardioVascular Assessment Study (CANVAS) program and the Canagliflozin on Renal and Cardiovascular Outcomes in Participants With Diabetic Nephropathy (CREDENCE) study have showed that treatment with an SGLT2 inhibitor slowed the progression of DKD in patients with type 2 diabetes[3–5]. Reductions in blood glucose levels, bodyweight and blood pressure can partially explain the

renoprotective mechanism of SGLT2 inhibitors; however, the full mechanistic details remain under debate[6].

Sano et al.[7,8] hypothesized that the renoprotective effect of SGLT2 inhibitors is associated with their hematopoietic effects. Indeed, a meta-analysis of 14 randomized controlled trials showed that treatment with SGLT2 inhibitors significantly elevated the hematocrit and the hemoglobin (Hb) levels in patients with type 2 diabetes[9]. Inagaki et al.[10] showed that treatment with canagliflozin for 52 weeks significantly increased the hematocrit by 2.1–2.3% from baseline in type 2 diabetes patients who had normal kidney function. Additionally, Yale et al.[11] showed that canagliflozin administration for 26 weeks increased the hematocrit by 4.8–6.0% from baseline in chronic kidney disease (CKD) patients with type 2 diabetes. These clinical data suggest that renal dysfunction potentiates an SGLT2 inhibitor-induced elevation of the hematocrit in patients with type 2 diabetes.

Lambers et al.[12] showed that treatment with dapagliflozin for 2 weeks significantly increased the hematocrit, and this change was accompanied by an increase in the plasma erythropoietin level. The reticulocyte count was also increased, followed by increases in the hematocrit of 2.2% at 12 weeks of treatment. These results suggest that an SGLT2 inhibitor-induced enhancement of erythropoietin production contributes to increasing the hematocrit in patients with type 2 diabetes. However, it is still not clear whether SGLT2 inhibitors directly stimulate erythropoietin production or not.

To address this clinical question directly, we aimed to examine the effects of an SGLT2 inhibitor on the hematocrit and on the Hb, and plasma erythropoietin levels in non-diabetic rats with renal anemia. Our studies were carried out in non-diabetic animals, because changes in the plasma glucose levels are known to influence kidney erythropoietin production[13]. The hematopoietic effects of the SGLT2 inhibitor, luseogliflozin, were examined in non-diabetic adenine-treated Wistar–Kyoto and Wistar rats, which are models for renal anemia with tubulointerstitial fibrosis, respectively[14]. The effect of an SGLT2 inhibitor on erythropoietin production was also examined in human induced pluripotent stem cell (hiPSC)-derived erythropoietin-producing cells, which were recently established in our laboratory[15].

METHODS

Animals
All experimental procedures were approved by the local institutional committee at Kagawa University and Osaka City General Hospital. Five-week-old male Wistar–Kyoto and Wistar rats were purchased from Japan SLC Inc. (Shizuoka, Japan).

Drugs
Adenine and carboxymethyl cellulose were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively. Luseogliflozin was provided by Taisho Pharmaceuticals Co., Ltd. (Tokyo, Japan).

Experimental protocols
Protocol 1: Wistar–Kyoto rats with renal anemia
Our preliminary experiments showed that the administration of adenine at a dose of 200 mg/kg for 10 days significantly decreased the hematocrit and the Hb levels in Wistar–Kyoto rats, producing a state representative of renal anemia (data not shown). Accordingly, vehicle (0.5% carboxymethyl cellulose) or adenine (200 mg/kg/day) was administered for 10 days by oral gavage (n = 4 or 16, respectively). Thereafter, the vehicle-treated rats continued treatment with the vehicle for 6 more weeks, as a control. The adenine-treated animals were subsequently administered with vehicle (n = 8) or luseogliflozin (10 mg/kg/day, per oral, n = 8) for 6 weeks. All rats were euthanized by administering an overdose of sodium pentobarbital (250 mg/kg, intraperitoneal injection) at the end of the observation period.

Protocol 2: Wistar rats with renal anemia
We followed further studies in Wistar rats treated with 600 mg/kg/day of adenine, a model representing severe renal anemia comparable to Wistar–Kyoto rats with renal anemia to examine the effect of an SGLT2 inhibitor on hematopoiesis in different species. Vehicle (0.5% carboxymethyl cellulose) or adenine (600 mg/kg/day, per oral) was administered for 10 days in Wistar rats (n = 4 or 12, respectively)[14]. Thereafter, the vehicle-treated rats continued treatment with the vehicle for 6 more weeks, as a control. The adenine-treated rats were subsequently administered with vehicle (n = 6) or luseogliflozin (10 mg/kg/day, per oral, n = 6) for 6 weeks. All rats were euthanized by administering an overdose of sodium pentobarbital (250 mg/kg, intraperitoneal injection) at the end of the observation period.

Sample collection and blood pressure measurement
Blood samples were collected from the tail vein at 0, 2 and 6 weeks after vehicle or luseogliflozin treatment. Urine samples were collected for 24 h using metabolic cages. After the rats had been euthanized, the left kidney tissues were harvested, then fixed in 10% neutral-buffered formalin and embedded in paraffin for histological studies.

Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography (model BP-98A; Soft-ron Co., Tokyo, Japan). The mean value of the lowest three readings was recorded[16].

Biochemical and hematological parameters
The hematocrit and the blood urea nitrogen (BUN), plasma creatinine (Cre), Hb, and plasma glucose levels were all measured by using an automated analyzer (Abbott Point of Care, Chicago, IL, USA). Plasma erythropoietin levels were measured by an enzyme-linked immunosorbent assay kit (BioLegend, San Diego, CA, USA). Glucose levels in the urine were measured by using an automated analyzer (Hitachi High-Technology, Tokyo, Japan).

Histological analysis

Tissue samples were cut into 2-µm thick sections and stained with azan reagent, as described previously[17]. The area of positive azan staining was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA), and the affected area was divided by the total area of the microscopic field[18]. A total of 20 consecutive microscopic fields were examined.

Cell culture

HiPSC line 253G4 was used, as previously described[19]. The differentiation of hiPSC-derived erythropoietin-producing cells was carried out based on the method used in our previous work[15]. Briefly, hiPSCs were dissociated, then seeded with stage 1 medium. On day 7, the medium was changed to stage 2 medium. In the present study, hiPSC-derived erythropoietin-producing cells were treated with vehicle (2-hydroxylpropyl-β-cyclodextrin; $n = 5$), 100 nmol/L luseogliflozin ($n = 4$), 500 nmol/L luseogliflozin ($n = 4$) or 50 µmol/L of FG-4592 ($n = 3$; Cayman, Ann Arbor, MI, USA). We previously showed that treatment with 100 nmol/L luseogliflozin sufficiently blocks SGLT2 *in vitro*[20]. Previous work also showed that treatment with 50 µmol/L FG-4592, a selective hypoxia-inducible factor prolyl hydroxylase inhibitor, consistently increased the erythropoietin levels in the medium[15]. Therefore, we utilized FG-4592 as a positive control. After 40 h of incubation, the culture medium was collected to measure human erythropoietin levels by the enzyme-linked immunosorbent assay method in accordance with the manufacturer's protocol (Erythropoietin ELISA; ALPCO, Boston, MA, USA).

Previous studies have shown that hiPSC-derived erythropoietin-producing cells did not grow under glucose conditions <25 mmol/L[15]. Another study was carried out to examine the effect of luseogliflozin under different glucose concentrations (50 mmol/L). HiPSC-derived erythropoietin-producing cells were grown for 5 days, and were incubated in stage 2 medium containing knockout Dulbecco's modified Eagle's medium with 25 or 50 mmol/L glucose. After 40 h of treatment with luseogliflozin (100 nmol/L) was administered, the culture medium was collected to measure erythropoietin levels.

Statistical analysis

Values are presented as the mean ± standard error of the mean. A one-way analysis of variance followed by Turkey's multiple comparison test was used for cross-sectional one-factor data from multiple groups (i.e., urinary glucose excretion, urine volume, water intake, water balance gap and the degree of renal interstitial fibrosis evaluated by azan staining). Longitudinal data (i.e., blood pressure, heart rate, blood glucose, hematocrit, Hb level, plasma erythropoietin level, BUN level, plasma Cre level and bodyweight) were analyzed by a two-way analysis of variance followed by the Bonferroni post-hoc test to determine differences between groups. Differences with values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of an SGLT2 inhibitor in Wistar–Kyoto rats with renal anemia (protocol 1)

Wistar–Kyoto rats treated with 200 mg/kg/day of adenine followed by the administration of luseogliflozin for 6 weeks had a significantly higher level of urinary glucose excretion (476 ± 89 mg/day), as compared with vehicle-treated rats (2 ± 1 mg/dL). However, the plasma postprandial glucose levels (161 ± 1 mg/dL) in the luseogliflozin-treated rats were not different from those of the vehicle-treated rats (157 ± 3 mg/dL).

Treatment with adenine significantly reduced the bodyweight of rats as compared with vehicle treatment, but luseogliflozin treatment did not affect the bodyweight during the observation period (Table 1). Adenine-treated Wistar–Kyoto rats showed a

Table 1 | Hemodynamic effect of luseogliflozin in adenine (200 mg/kg/day)-treated Wistar–Kyoto rats

	Control	Adenine (200 mg/kg) + vehicle	Adenine (200 mg/kg) + luseogliflozin
Bodyweight at 0 week (g)	272 ± 3	179 ± 3*	173 ± 5
Bodyweight at 2 weeks (g)	347 ± 13	283 ± 8*	273 ± 16
Bodyweight at 6 weeks (g)	367 ± 12	309 ± 7*	306 ± 12
Urine volume at 2 weeks (mL/day)	29.4 ± 7.2	72.6 ± 2.1*	69.7 ± 2.5
Water intake at 2 weeks (mL/day)	44.3 ± 6.5	85.8 ± 2.5*	86.8 ± 1.7
Food intake at 2 weeks (g/day)	23.3 ± 0.1	12.2 ± 0.8*	21.8 ± 1.2†
Blood pressure at 1 week (mmHg)	118 ± 4	127 ± 2	131 ± 5
BUN level at 0 week (mg/dL)	22 ± 1	67 ± 4*	72 ± 5
BUN level at 6 weeks (mg/dL)	22 ± 1	56 ± 2*	50 ± 2
Plasma Cre level at 0 week (mg/dL)	0.30 ± 0.01	0.95 ± 0.05*	1.03 ± 0.06
Plasma Cre level at 6 weeks (mg/dL)	0.40 ± 0.01	0.74 ± 0.02*	0.74 ± 0.02

Values are the mean ± standard error of the mean. †*$P < 0.05$, adenine + vehicle versus control. †$P < 0.05$, adenine + luseogliflozin versus adenine + vehicle. Bodyweight at 0, 2 and 6 weeks after luseogliflozin treatment in adenine (200 mg/kg/day)-treated rats, respectively. Urine volume, water intake, food intake and blood pressure after luseogliflozin treatment in adenine (200 mg/kg/day)-treated rats. Blood urea nitrogen (BUN) and plasma creatinine (Cre) levels at 0 and 6 weeks after luseogliflozin treatment in adenine (200 mg/kg/day)-treated rats, respectively. ‡

higher urine volume, accompanied by a higher water intake, compared with vehicle-treated rats (Table 1). However, subsequent luseogliflozin administration did not affect the urine volume or water intake. Food intake in luseogliflozin-treated rats was greater compared with that of adenine-treated Wistar–Kyoto rats (Table 1). As compared with control rats, the blood pressure in adenine-treated Wistar–Kyoto rats trended higher; however, this difference was not statistically significant. Treatment with luseogliflozin did not affect the blood pressure (Table 1).

At 2 weeks after treatment, the hematocrit ($31 \pm 1\%$) and Hb level (10.7 ± 0.1 g/dL) in Wistar–Kyoto rats treated with adenine were remarkably lower as compared with control rats (hematocrit $49 \pm 2\%$ and Hb 16.5 ± 0.5 g/dL; Figure 1a,b). However, in the adenine-treated Wistar–Kyoto rats, subsequent luseogliflozin treatment did not induce any measurable change

(a) Hematocrit (%)

○— Control
●— Adenine (200 mg/kg) + vehicle
▲·· Adenine (200 mg/kg) + luseogliflozin

(b) Hemoglobin level (g/dL)

○— Control
●— Adenine (200 mg/kg) + vehicle
▲·· Adenine (200 mg/kg) + luseogliflozin

(c) Plasma erythropoietin level (pg/mL)

○— Control
●— Adenine (200 mg/kg) + vehicle
▲·· Adenine (200 mg/kg) + luseogliflozin

Figure 1 | Effect of luseogliflozin on the hematocrit and hemoglobin, and plasma erythropoietin levels in non-diabetic Wistar–Kyoto rats with renal anemia. Wistar–Kyoto rats were treated with adenine (200 mg/kg/day) for 10 days to induce mild renal anemia. (a–b) the hematocrits (a) and hemoglobin levels (b) at 0, 2 and 6 weeks after luseogliflozin treatment. (c) The plasma erythropoietin levels before and after luseogliflozin treatment in adenine (200 mg/kg/day)-treated Wistar–Kyoto rats. *$P < 0.05$, adenine + vehicle vs control.

in the hematocrit ($38 \pm 1\%$) or the Hb level (12.9 ± 0.4 g/dL), as compared with vehicle treatment (hematocrit $38 \pm 0.4\%$ and Hb 13.0 ± 0.1 g/dL; Figure 1a,b). Treatment with adenine resulted in a significantly lower plasma erythropoietin level (8.19 ± 0.96 pg/mL), as compared with control treatment (48.8 ± 5.4 pg/mL; Figure 1c). However, in adenine-treated Wistar–Kyoto rats, those additionally treated with luseogliflozin did not have a higher plasma erythropoietin level (5.95 ± 0.56 pg/mL) than those treated with vehicle (6.73 ± 0.60 pg/mL; Figure 1c).

We also investigated whether luseogliflozin causes hemoconcentration by analyzing the water balance gap (calculated as the difference between water intake and urine volume). The water balance gap was higher in Wistar–Kyoto rats treated with adenine than in control rats at 2 weeks after treatment (vehicle-treated rats 36.0 ± 3.9 mL/day vs control rats 15.3 ± 2.1 mL/day). However, among the adenine-treated Wistar–Kyoto rats, those additionally treated with luseogliflozin did not show a difference in the water balance gap (37.1 ± 1.8 mL/day), as compared with those further treated with vehicle.

Adenine-treated Wistar Kyoto rats had higher BUN (69 ± 3 mg/dL) and plasma Cre levels (0.99 ± 0.04 mg/dL), as compared with control rats (BUN 22 ± 1 mg/dL, Cre 0.30 ± 0.01 mg/dL; Table 1). After 6 weeks of subsequent vehicle administration, the BUN and plasma Cre levels were slightly lower in these adenine-treated Wistar–Kyoto rats (BUN 56 ± 2 mg/dL and Cre 0.74 ± 0.02 mg/dL). Luseogliflozin administration for 6 weeks did not change the BUN (50 ± 2 mg/dL) or plasma Cre (0.74 ± 0.02 mg/dL) levels (Table 1). Similarly, luseogliflozin-treated rats did not show any difference in the renal interstitial area of positive azan staining compared with vehicle-treated rats (Figure 2).

Effect of an SGLT2 inhibitor in Wistar rats with renal anemia (protocol 2)

To examine the effect of an SGLT2 inhibitor on hematopoiesis in different species, Wistar rats treated with 600 mg/kg/day of adenine were used[14]. Consistent with the data produced by protocol 1, the administration of luseogliflozin for 2 weeks in Wistar rats treated with 600 mg/kg/day of adenine caused significantly more urinary glucose excretion (175 ± 18 mg/day), but no differences in the plasma postprandial glucose level (122 ± 5 mg/dL), as compared with vehicle administration (urinary glucose excretion 30 ± 1 mg/day, plasma postprandial glucose level 122 ± 2 mg/dL).

Treatment with adenine resulted in significantly lower bodyweight, as compared with control rats, but subsequent luseogliflozin treatment did not alter the bodyweight during the observation period (Table 2). Adenine-treated Wistar rats showed a higher urine volume that was accompanied by a higher level of water intake compared with control rats (Table 2). However, luseogliflozin treatment did not affect the urine volume or water. Treatment with luseogliflozin did not
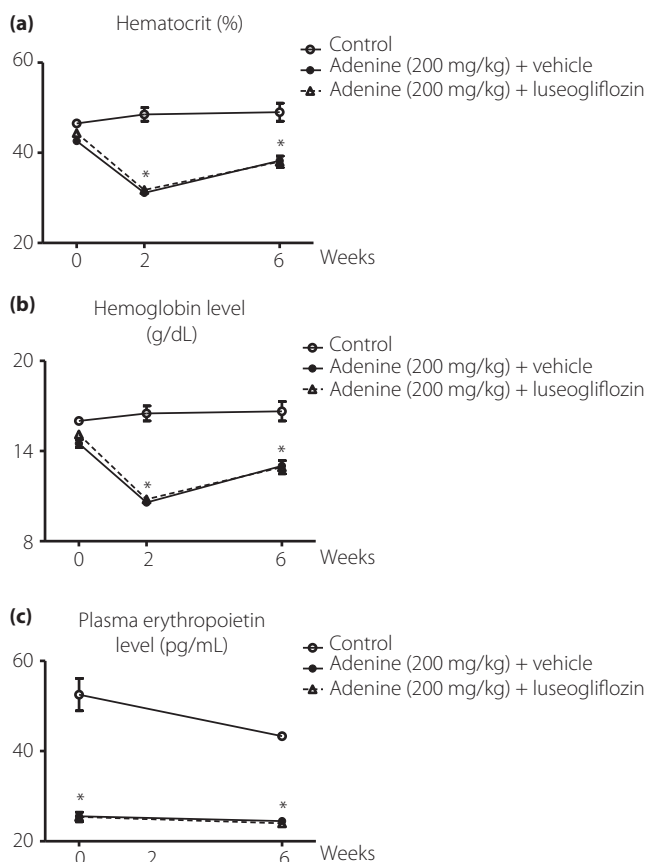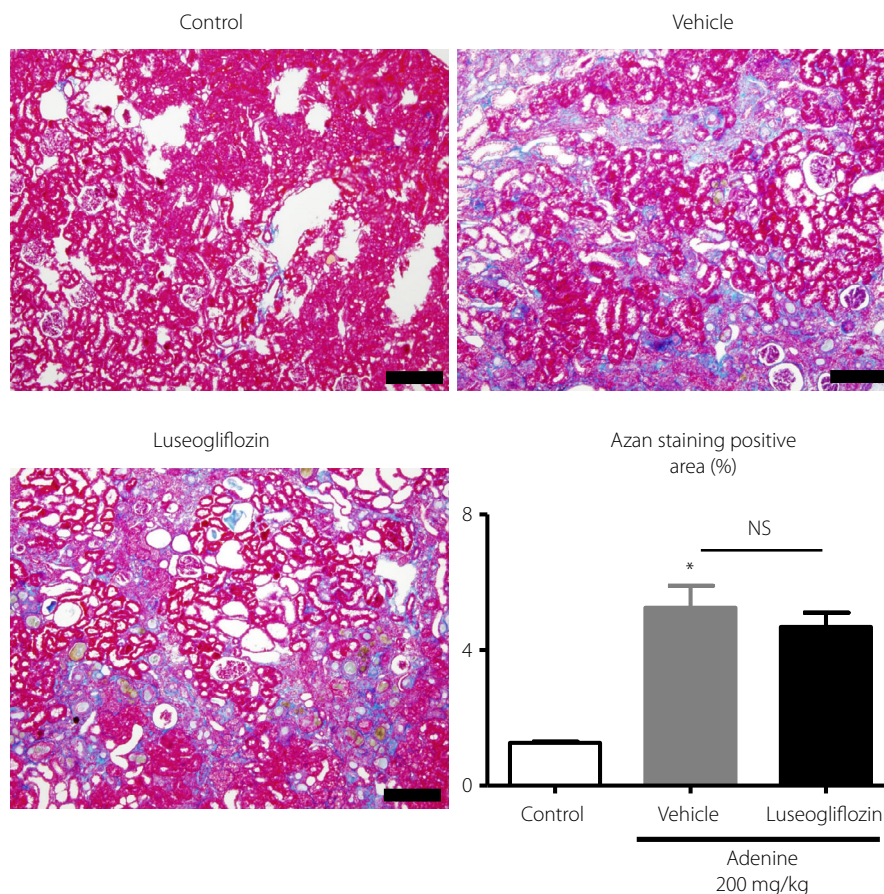
Figure 2 | Effect of luseogliflozin on tubulointerstitial injury in non-diabetic Wistar–Kyoto rats with renal anemia. Interstitial fibrosis was evaluated by a semiquantitative analysis of azan staining. Scale bar, 100 μm. *P < 0.05, adenine + vehicle versus control.

Table 2 | Hemodynamic effect of luseogliflozin in adenine (600 mg/kg/day)-treated Wistar rats

	Control	Adenine (600 mg/kg) + vehicle	Adenine (600 mg/kg) + vehicle
Bodyweight at 0 week (g)	221 ± 5.6	119 ± 4.0*	113 ± 4.9
Bodyweight at 2 weeks (g)	277 ± 9.0	121 ± 8.5*	114 ± 5.3
Bodyweight at 6 weeks (g)	359 ± 15.8	166 ± 18.1*	151 ± 6.9
Urine volume at 2 weeks (mL/day)	9.4 ± 1.2	34.5 ± 3.1*	36.4 ± 2.1
Water intake at 2 weeks (mL/day)	20.5 ± 1.5	44.0 ± 3.2*	48.2 ± 2.7
Food intake at 2 weeks (g/day)	14.7 ± 1.3	9.0 ± 1.1*	10.2 ± 1.0
Blood pressure at 1 week (mmHg)	149 ± 6.6	147 ± 8.3*	142 ± 2.4
BUN level at 0 week (mg/dL)	22 ± 1	140 ± 1*	137 ± 2
BUN level at 6 weeks (mg/dL)	25 ± 1	140 ± 1*	130 ± 4
Plasma Cre level at 0 week (mg/dL)	0.48 ± 0.02	1.80 ± 0.12*	1.62 ± 0.12
Plasma Cre level at 6 weeks (mg/dL)	0.43 ± 0.02	1.40 ± 0.06*	1.50 ± 0.07

Values are the mean ± standard error of the mean. †*P < 0.05, adenine + vehicle versus control. Bodyweight at 0, 2 and 6 weeks after luseogliflozin treatment in adenine (600 mg/kg/day)-treated rats, respectively. Urine volume, water intake, food intake and blood pressure after luseogliflozin treatment in adenine (600 mg/kg/day)-treated rats. Blood urea nitrogen (BUN) and plasma creatinine (Cre) levels at 0 and 6 weeks after luseogliflozin treatment in adenine (600 mg/kg/day)-treated rats, respectively.

increase food intake in adenine-treated Wistar rats (Table 2). There was no significant difference in blood pressure among the groups (Table 2).

Adenine-treated Wistar rats had a significantly lower hematocrit (38 ± 1%) and significantly lower Hb levels (12.9 ± 0.2 g/dL), as compared with control rats (hematocrit

47 ± 1%, Hb 15.9 ± 0.3 g/dL; Figure 3a,b). The administration of luseogliflozin for 6 weeks did not induce any change in the hematocrit (40 ± 1%) or the Hb levels (13.6 ± 0.4 g/dL), as compared with vehicle treatment (hematocrit 38 ± 2%, Hb 12.8 ± 0.6 g/dL; Figures 3a,b).

Adenine-treated Wistar rats had a lower plasma erythropoietin level (15.3 ± 2.6 pg/mL), as compared with control rats (52.8 ± 6.5 pg/mL; Figure 3c). The additional administration of luseogliflozin for 6 weeks did not alter the plasma erythropoietin level (9.27 ± 0.80 pg/mL), as compared with vehicle treatment (8.91 ± 1.75 pg/mL; Figure 3c). Similarly, luseogliflozin-treated rats did not show any difference in the water balance gap, as compared with vehicle-treated rats (luseogliflozin-treated rats 11.8 ± 4.6 vs vehicle-treated rats 9.5 ± 4.9 mL/day).

Adenine-treated Wistar rats had higher BUN (139 ± 2 mg/dL) and Cre levels (1.70 ± 0.09 mg/dL), as compared with control rats (BUN 22 ± 1 mg/dL, Cre 0.48 ± 0.02 mg/dL; Table 2).
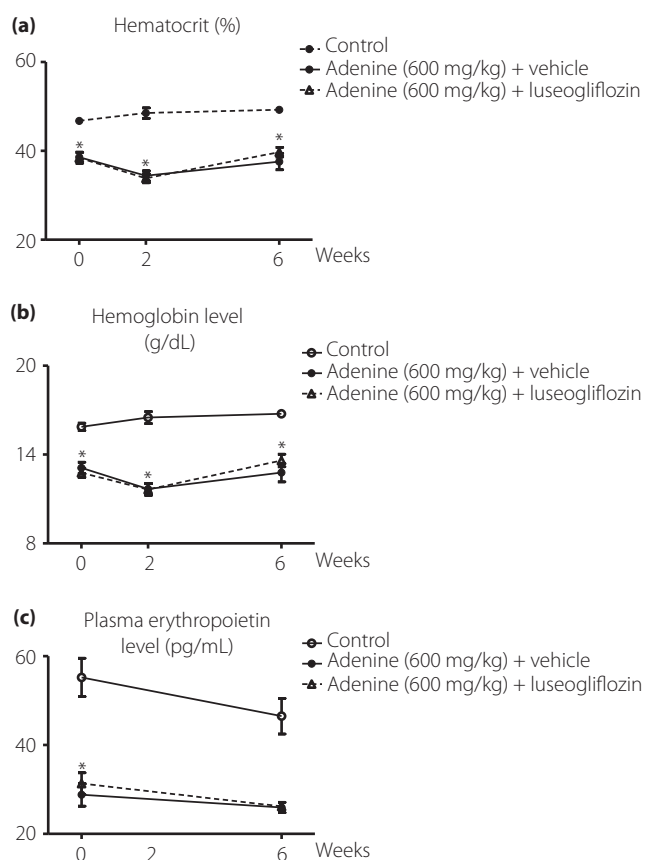

Figure 3 | Effect of luseogliflozin on the hematocrit and hemoglobin, and plasma erythropoietin levels in non-diabetic Wistar rats with renal anemia. Wistar rats were treated with adenine (600 mg/kg/day) for 10 days to induce renal anemia. The (a) hematocrits and (b) hemoglobin levels at 0, 2 and 6 weeks after luseogliflozin treatment. (c) The plasma erythropoietin levels before and after luseogliflozin treatment in adenine (600 mg/kg/day)-treated Wistar rats. *$P < 0.05$, adenine + vehicle versus control.

The additional administration of luseogliflozin for 6 weeks did not alter the BUN (130 ± 4 mg/dL) or plasma Cre (1.50 ± 0.07 mg/dL) level, as compared with vehicle treatment (BUN 140 ± 1 mg/dL, Cre 1.40 ± 0.06 mg/dL; Table 2). Furthermore, adenine-induced renal interstitial fibrosis, as evaluated by the azan-positive area, was not affected by luseogliflozin treatment (Figure 4).

Effects of an SGLT2 inhibitor on erythropoietin production in erythropoietin-producing cells

Consistent with the findings of previous studies[15], treatment of hiPSC-derived erythropoietin-producing cells with 50 µmol/L FG-4592 significantly increased erythropoietin levels in the medium (108 ± 28 mIU/mL), as compared with vehicle treatment of these cells (54 ± 3 mIU/mL). However, luseogliflozin (100 or 500 nmol/L) did not increase the erythropoietin levels in the medium (100 nmol/L luseogliflozin, 61 ± 8 mIU/mL; 500 nmol/L luseogliflozin, 57 ± 14 mIU/mL; Figure 5a, the data are shown as values that are normalized to the erythropoietin level that was associated with vehicle treatment).

Studies were also carried out to verify whether the difference in glucose concentration affects the ability of luseogliflozin to produce erythropoietin. However, erythropoietin levels in the medium were not significantly different between 25 and 50 mmol/L glucose. Additionally, treatment with lusegliflozin did not change the erythropoietin levels during 25 or 50 mmol/L glucose treatment (Figure 5b).

DISCUSSION

Clinical studies have shown that the hematocrit and the Hb levels were significantly elevated in patients with type 2 diabetes who were treated with SGLT2 inhibitors[9,10,12,21–23]. Furthermore, the complication of CKD promotes an elevation of the hematocrit in diabetes patients treated with an SGLT2 inhibitor[11]. Based on these clinical observations, several investigators hypothesized that SGLT2 inhibitors directly induce a hematopoietic effect through the stimulation of erythropoietin production in the kidney[7,8,24]. In the present study, we carried out studies to examine the hematopoietic effect of the SGLT2 inhibitor, luseogliflozin, in non-diabetic rats with adenine-induced CKD modeling renal anemia. Previous studies have shown that luseogliflozin at doses of 0.3–3 mg/kg dose-dependently increased urinary glucose excretion in non-diabetic and diabetic rats[25]. Furthermore, administration of 10 mg/kg/day luseogliflozin exerted renoprotective effect in rats with type 2 diabetes[26]. As the present study also showed that treatment with luseogliflozin at 10 mg/kg/day significantly increased urinary glucose excretion in non-diabetic adenine-induced CKD rats, it seems likely that luseogliflozin at 10 mg/kg/day effectively inhibits SGLT2 in these animals. However, the blood glucose, hematocrit and Hb, and plasma erythropoietin levels were not changed by luseogliflozin treatment. Further *in vitro* studies in hiPSC-derived erythropoietin-producing cells showed no change in erythropoietin release into the medium after the
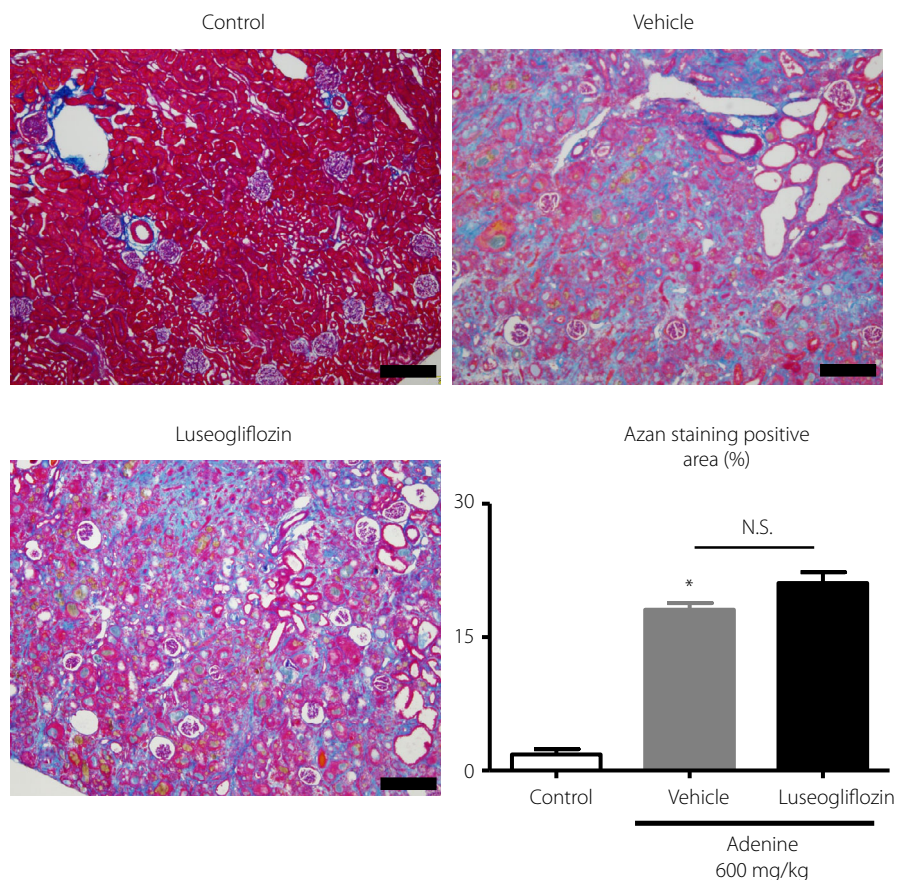
Control

Vehicle

Luseogliflozin

Azan staining positive area (%)

Figure 4 | Effect of luseogliflozin on tubulointerstitial injury in non-diabetic Wistar rats with renal anemia. Interstitial fibrosis was evaluated by a semiquantitative analysis of azan staining. Scale bar, 100 μm. *$P < 0.05$, adenine + vehicle versus control.

addition of luseogliflozin. Collectively, these data fail to support the hypothesis[7,8,24], based on previous clinical studies[9,10,12,21–23], that SGLT2 inhibitors directly stimulate erythropoietin production.

Although clinical observations have suggested that SGLT2 inhibitors have a hematopoietic effect in patients with type 2 diabetes, no studies have examined such an effect in non-diabetic patients; experiments addressing this issue are particularly important to exclude the possibility that kidney erythropoietin production is merely enhanced by the SGLT2 inhibitor-induced reductions in plasma glucose levels in patients with diabetes. Importantly, recent clinical studies have shown that erythropoietin deficiency is induced during the early phase of DKD, but is not induced in non-diabetic CKD of similar severity[13]. Here, the effect of an SGLT2 inhibitor was examined in non-diabetic rats treated with adenine, a model representing renal anemia. The present data show that treatment with an SGLT2 inhibitor did not change the hematocrit or the Hb and plasma erythropoietin levels in non-diabetic rats with adenine-induced renal anemia. These data are consistent with those of previous studies in rodents that SGLT2 inhibition did not change the hematocrit or the Hb levels[27–29]. Given the lack of change in the

hematocrit and the Hb levels in these experiments[27–29], the discrepant data regarding the hematopoietic effect of SGLT2 inhibition between humans and rodents might be due to a species difference. Further clinical studies including non-diabetic patients with CKD, such as EMPA-KIDNEY and DAPA-CKD, will clarify the issue of whether SGLT2 inhibitors increase hematocrit and Hb levels in non-diabetic CKD patients (EMPA-KIDNEY: https://www.boehringer-ingelheim.com/EMPA-KIDNEY, DAPA-CKD: https://www.astrazeneca.com/media-centre/press-releases/2016/astrazeneca-announces-two-new-phase-IIIb-trials-for-Forxiga-in-chronic-kidney-disease-and-chronic-heart-failure-120920161.html#).

Clinical studies have also shown that the SGLT2 inhibitor-induced reduction in plasma volume contributes to the increased hematocrit[30]. Indeed, treatment with SGLT2 inhibitors induces weight loss[31,32], which is associated with increases in urine volume and urinary sodium excretion[33,34]. In the present study, we observed no difference in the urine volume, bodyweight or water balance gap between vehicle- and luseogliflozin-treated rats with adenine-induced renal anemia. It is important to note that luseogliflozin should be pharmacologically effective in these CKD model animals, because their urinary glucose excretion
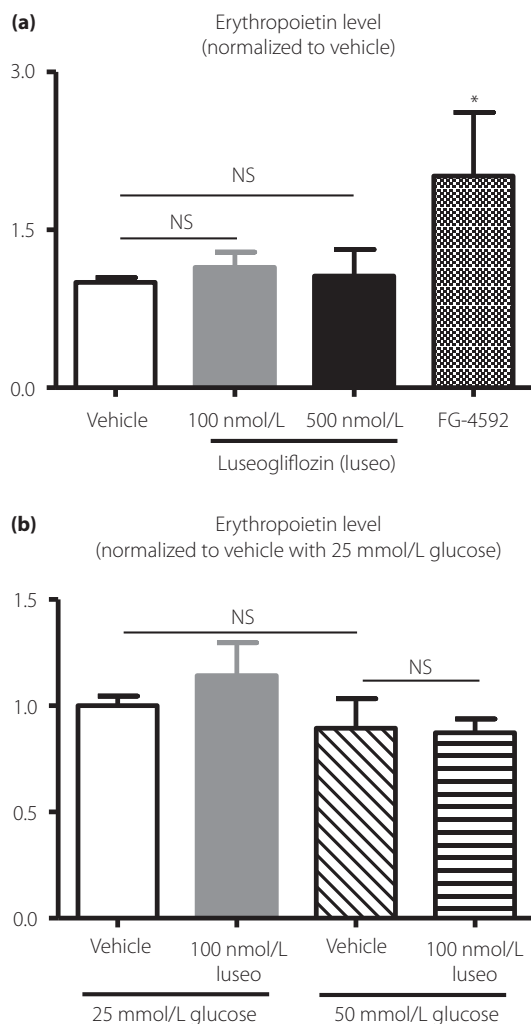
Figure 5 | Effect of luseogliflozin (luseo) on erythropoietin production in human induced pluripotent stem cell-derived erythropoietin-producing cells. (a) Erythropoietin levels in the culture media after treatment with vehicle, luseogliflozin (100 and 500 nmol/L), or FG-4592 (50 μmol/L) in human induced pluripotent stem cell-derived erythropoietin-producing cells. (b) Effects of luseogliflozin (100 nmol/L) on erythropoietin levels in the culture medium under 25 mmol/L or 50 mmol/L glucose concentrations in human induced pluripotent stem cell-derived erythropoietin-producing cells. *$P < 0.05$, FG-4592 versus control.

was significantly increased. The present data also show that treatment with an SGLT2 inhibitor does not induce a diuretic effect or hemoconcentration in rats with adenine-induced CKD. In agreement with previous studies[14], adenine-treated CKD model rats showed markedly higher basal urinary volume and water intake compared with control rats, and this might affect the SGLT2 inhibitor effects on urine volume, bodyweight and water balance. Recent studies have shown that ipragliflozin did not change the total body water content in non-diabetic and diabetic rats when free access to food and water was allowed[29]. These data suggest that the SGLT2 inhibitor does

not change total body water balance in non-diabetic rodents under conditions of ad libitum food and water intake.

Lambers et al.[12] reported a significant elevation of the plasma erythropoietin level after SGLT2 inhibitor treatment in patients with type 2 diabetes. Interestingly, the SGLT2 inhibitor-induced enhancement in the plasma erythropoietin level was transient, and the level of plasma erythropoietin gradually returned to normal. In contrast, the observed increase in the hematocrit continued over time and did not return to its basal value[12]. These different time-dependent changes between the hematocrit and plasma erythropoietin levels indicate that SGLT2 inhibitors functionally improve erythropoietin production in the kidneys, as hypothesized by other investigators[7,8,24]. However, there have not yet been any other clinical reports showing that the plasma erythropoietin level is directly increased by treatment with an SGLT2 inhibitor. Furthermore, the present study failed to observe a significant increase in the plasma erythropoietin level after treatment with an SGLT2 inhibitor in non-diabetic rats with renal anemia. Further studies should be carried out in diabetic animals; however, there is no available animal model of DKD that shows renal anemia with a low erythropoietin level[35,36].

It is difficult to directly analyze erythropoietin production in the kidneys in vivo by using currently available methods, such as immunohistochemistry. Therefore, we carried out in vitro experiments to investigate whether an SGLT2 inhibitor directly stimulates erythropoietin production in erythropoietin-producing cells differentiated from hiPSCs[15]. The present data showed that treatment with an SGLT2 inhibitor did not increase the amount of erythropoietin release into the medium in hiPSC-derived erythropoietin-producing cells. However, because hiPSC-derived erythropoietin-producing cells were generated through hepatic lineage[15], future studies should examine the effect of SGLT2 inhibitors in erythropoietin-producing cells from the kidneys. Our data also showed that luseogliflozin did not stimulate erythropoietin production under a high glucose concentration (50 mmol/L) in hiPSC-derived erythropoietin-producing cells. Thus, these data also do not support the clinical study-based hypothesis that SGLT2 inhibitors might directly stimulate erythropoietin production in the kidney[7,8,24]. However, a weak point of the present study was that our in vitro studies with hiPSC-derived erythropoietin-producing cells did not allow an accurate comparison between low- and high-glucose levels, because they are not able to exist under conditions where the glucose levels are <25 mmol/L. Further investigation should be carried out using improved hiPSC-derived erythropoietin-producing cells that can grow under low-glucose conditions.

In conclusion, the data from the present study in non-diabetic CKD model rats with adenine-induced renal anemia failed to support the hypothesis proposed by other investigators[7,8,24], based on their clinical studies in patients with diabetes[9,10,12,21–23], that SGLT2 inhibitors directly stimulate erythropoietin production. Further clinical studies including non-diabetic patients

with CKD, such as EMPA-KIDNEY and DAPA-CKD, will clarify the issue of whether SGLT2 inhibitors increase hematocrit and Hb levels in non-diabetic CKD patients. The examination of the hematocrit and plasma erythropoietin levels by this clinical trial could reveal whether the previously observed SGLT2 inhibitor-induced increase in the hematocrit is independent of its blood sugar-lowering effect.

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DISCLOSURE

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