Induction of experimental diabetes and diabetic nephropathy using anomer-equilibrated streptozotocin in male C57Bl/6J mice

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ABSTRACT

Streptozotocin (STZ) is widely used to induce experimental diabetes in murine models. However, the ability to induce diabetic nephropathy (DN) is more challenging. It has been recommended to inject STZ at multiple low doses within 15 min after dissolution due to its alleged instability. However, some studies suggest that STZ is stable for days due to equilibration of its two anomers (α and β), 90 min after dissolution, and that this anomer-equilibrated STZ leads to higher survival rates and persistent hyperglycaemia with minimal weight loss. The aim of this study was to determine an optimal dose of anomer-equilibrated STZ to induce kidney tubular damage and compare it with the more commonly used freshly prepared STZ. We hypothesised that anomer-equilibrated STZ provides a better, reproducible experimental model of diabetes-induced kidney damage with improved animal welfare. Body measurements, fasting glycaemia, insulinemia and renal histology were assessed in male C57Bl/6J at two and six months of age treated with fresh (50 mg/kg) or anomer-equilibrated (dose ranging 35–50 mg/kg) STZ or vehicle control. We demonstrated a dose-dependent effect of anomer-equilibrated STZ on the induction of hypoinsulinaemia and hyperglycaemia, as well as body weight in two-month-old mice. Interestingly, in six-month-old mice STZ leads to body weight loss, independently of STZ preparation mode. Anomer-equilibrated STZ provoked moderate to severe kidney tubule structural damage, resulting in significant kidney hypertrophy, whereas freshly prepared STZ only caused mild alterations. In conclusion, our study proposes that anomer-equilibrated STZ provides a robust murine model of diabetes and early-stage diabetic nephropathy, which can be used to test therapeutic approaches to treat and/or prevent renal damage.

1. Introduction

Diabetic Nephropathy (DN) is a multi-stage renal complication of type 1 and/or type 2 diabetes and the leading cause of end-stage renal disease (ESRD), where the only option for treatment is dialysis or transplantation [1]. DN is characterised by renal structural changes including tubular injury, mesangial expansion, glomerular hypertrophy and glomerulosclerosis resulting from hyperglycaemic conditions [2–4].

To investigate the pathogenesis and treatment for DN, murine models have been used for decades due to their commonalities with human disease [5]. Streptozotocin (STZ) is an alkylating antineoplastic agent selectively toxic to insulin-secreting pancreatic β-cells [6]. Due to its analogy with glucose, STZ enters the pancreatic cells through glucose transporter 2 (GLUT2) and induces alkylation of DNA hence cellular death [7,8]. STZ is composed of two anomers: α-anomer and β-anomer, with the α-anomer being the most toxic [9]. To induce experimental diabetes in murine models, the Diabetic Complications Consortium [10] specifies the administration of 5 daily low-doses of STZ intraperitoneally within 15–20 min post dissolution as it is commonly accepted that STZ in sodium citrate buffer solution degrades rapidly after that time point.

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However, STZ has been demonstrated to be stable for days at room temperature [11,12]. When STZ is dissolved in sodium citrate buffer, the concentration of the α-anomer is 3 to 20-fold greater than that of the β-anomer [9] and it takes 60—90 min to reach anomer equilibrium [11,13]. Garza-Rodea et al. [13] have shown that mice injected with anomer-equilibrated STZ displayed a higher level of survival and persistent hyperglycaemia with minimal body weight loss compared to freshly injected STZ. However, the use of anomer-equilibrated STZ to induce kidney damage and diabetic kidney disease has not yet been reported. As induction of pathogenic DN using STZ can be challenging, this is important to test to better define a robust model.

The aim of this study was to determine the optimal dose of anomer-equilibrated STZ to induce renal damage representative of DN, then compare both freshly prepared and 90-min anomer-equilibrated STZ in mice to further refine the protocol. We hypothesised that mice administered with 90-min anomer-equilibrated STZ would provide a more reproducible experimental model of diabetes and diabetes-induced kidney damage with improved animal welfare which would comply with the principles of 3Rs (replace, reduce, refine).

2. Materials and methods

2.1. Ethics

All animal procedures were performed by trained staff under a project license (PPL P1ECEB2B6), approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and complied with the ARRIVE guidelines.

2.2. Animals

Male wild type (WT) C57Bl/6J mice were purchased from Charles River (USA) at the age of four weeks. Male C57/Bl/6J mice at

six months of age were bred in-house. Animals were group housed in cages with *ad libitum* access to standard CHOW diet unless otherwise specified (9% fat, 22% protein and 69% carb) and water. Animals were kept at constant temperature of 22—24 °C with a 12h light/dark cycle. Thirty-two two-month-old mice were split into four groups: vehicle control treated mice (vehicle) and mice injected with 35, 40 or 50 mg/kg 90-min anomer-equilibrated STZ (respectively Eq. STZ 35, 40 and 50 mg/kg). Fourteen six-month-old mice were separated into three groups: vehicle control treated mice (vehicle), mice injected with 50 mg/kg of fresh STZ (Fresh STZ 50 mg/kg), and mice treated with 50 mg/kg 90-min anomer-equilibrated STZ (Eq. STZ 50 mg/kg). Animals were injected intraperitoneally (IP) with either vehicle or STZ for five consecutive days, followed by monitoring of body weight, body composition and fasted glycemia for six weeks.

2.3. STZ preparation

STZ (Sigma-Aldrich, USA) contains ≥75% of the α-anomer as specified by the manufacturer. Pre-weighed STZ was dissolved in sterile 0.05 M sodium citrate buffer (pH 4.5) at desired concentration. STZ-sodium citrate buffer solutions were vortexed for 30 s and IP injected within 15 min (fresh STZ), or vortexed for 30 s every 10 min for 90 min prior to IP injection (90-min anomer-equilibrated STZ).

2.4. Body measurements

Body weight was monitored weekly using a digital benchtop scale. Body weight variations were calculated with the weight on the first day of injection as initial value.

Body composition was assessed at weeks 0, 3 and 6 using a magnetic resonance imaging-3-in-1 scanner (EchoMRI, Echo Medical Systems, USA) to determine total body fat and lean mass. Three consecutive scans were taken, and average values calculated.

A.

2-month-old male C57Bl/6J
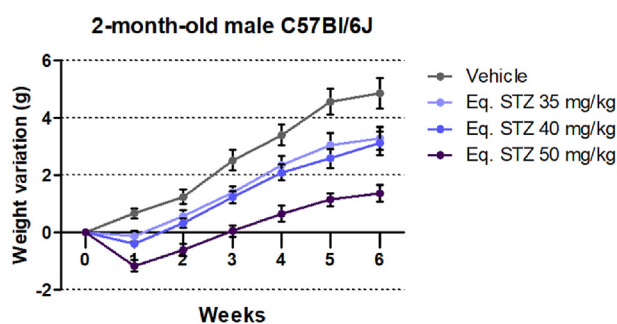
	Weeks						
	0	1	2	3	4	5	6
Vehicle vs eq. STZ 35 mg/kg	n.s.	n.s.	n.s.	n.s.	n.s.	**	**
Vehicle vs eq. STZ 40 mg/kg	n.s.	n.s.	n.s.	n.s.	*	****	***
Vehicle vs eq. STZ 50 mg/kg	n.s.	***	***	****	****	****	****
Eq. STZ 35 vs 50 mg/kg	n.s.	n.s.	n.s.	*	**	***	****
Eq. STZ 40 vs 50 mg/kg	n.s.	n.s.	n.s.	n.s.	*	*	***

B.

6-month-old male C57Bl/6J
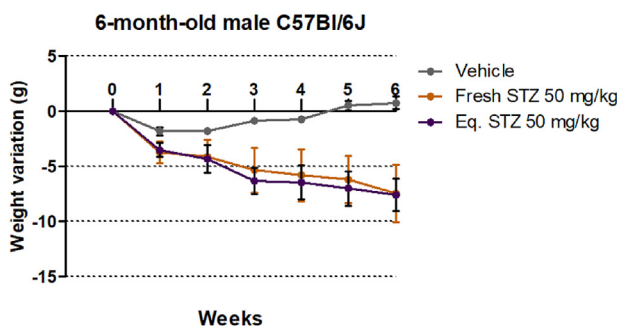
	Weeks						
	0	1	2	3	4	5	6
Vehicle vs Fresh STZ 50 mg/kg	n.s.	n.s.	n.s.	n.s.	n.s.	*	**
Vehicle vs eq. STZ 50 mg/kg	n.s.	n.s.	n.s.	n.s.	*	**	***

Fig. 1. Body weight variation of male C57Bl/6J injected with different doses of STZ at two months of age (A) or injected with 50 mg/kg of STZ prepared freshly or after a 90-min anomer equilibration at six-months of age (B). Statistical analyses were performed using two-way ANOVA, followed by Bonferroni multiple comparison post-hoc test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

2.5. Blood glucose measurement

5-h fasted blood glucose levels were monitored weekly. Needle tail-prick blood drops were obtained using a single-use sharp needle and glucose determined using a veterinary glucometer (AlphaTRAK, UK).

2.6. Humane sacrifice and tissue collection

Mice were humanly sacrificed by CO_2 inhalation followed by cervical dislocation. Trunk-derived blood was collected into a BD Microtainer SST Tube (BD Biosciences, USA).

Kidneys were collected and individually weighed using a four decimal place precision scale. Left kidneys were sliced into 3 equal cross-sectional pieces. Middle sections were immersed in 10% formalin solution at 4 °C for 24 h, then stored at 4 °C in PBS. Other sections were snap frozen in liquid nitrogen, then stored at −80 °C.

2.7. Serum insulin ELISA

Serum insulin from trunk-derived blood samples was determined using commercially available mouse insulin ELISAs (Cat# 90,080, Crystal Chem Inc., USA) according to the manufacturer's instructions.

2.8. Kidney histology

Kidneys were fixed in 10% formalin, embedded in paraffin,

sectioned, and stained with haematoxylin and eosin (H&E) by the Aberdeen Royal Infirmary Histopathology Department (Aberdeen, UK). Slides were scored by Dr Paul AJ Brown, consultant pathologist, Aberdeen Royal Infirmary, who was blinded to the study. 100 random intersections were scored, using a graticule, for each kidney for tubule dilation, tubular atrophy or vacuolation; and a mark of 0 (normal histology) or 1 (abnormal profile) was given. The total mark for each kidney profile was calculated by the addition of 100 graticule intersections per tissue section, and then scored as follows: 1 = none; 2 = mild; 3 = moderate; 4 = severe. Images were taken using a light microscope EVOS XL (Thermofisher Scientific, UK).

2.9. Data and statistical analyses

GraphPad Prism Software (v5 GraphPad, USA) was used for all statistical analyses. Data are shown as means ± SEM, and significances were determined by one-way or two-way ANOVA, followed by Bonferroni multiple comparisons post-hoc test, or Kruskal-Wallis test followed by Dunn's multiple comparisons post-hoc test. P values f < 0.05 (*) were considered statistically significant.

3. Results

3.1. Anomer-equilibrated STZ affects body weight

To establish the most effective dose of anomer-equilibrated STZ to induce disease with minimal weight loss, mice weights were
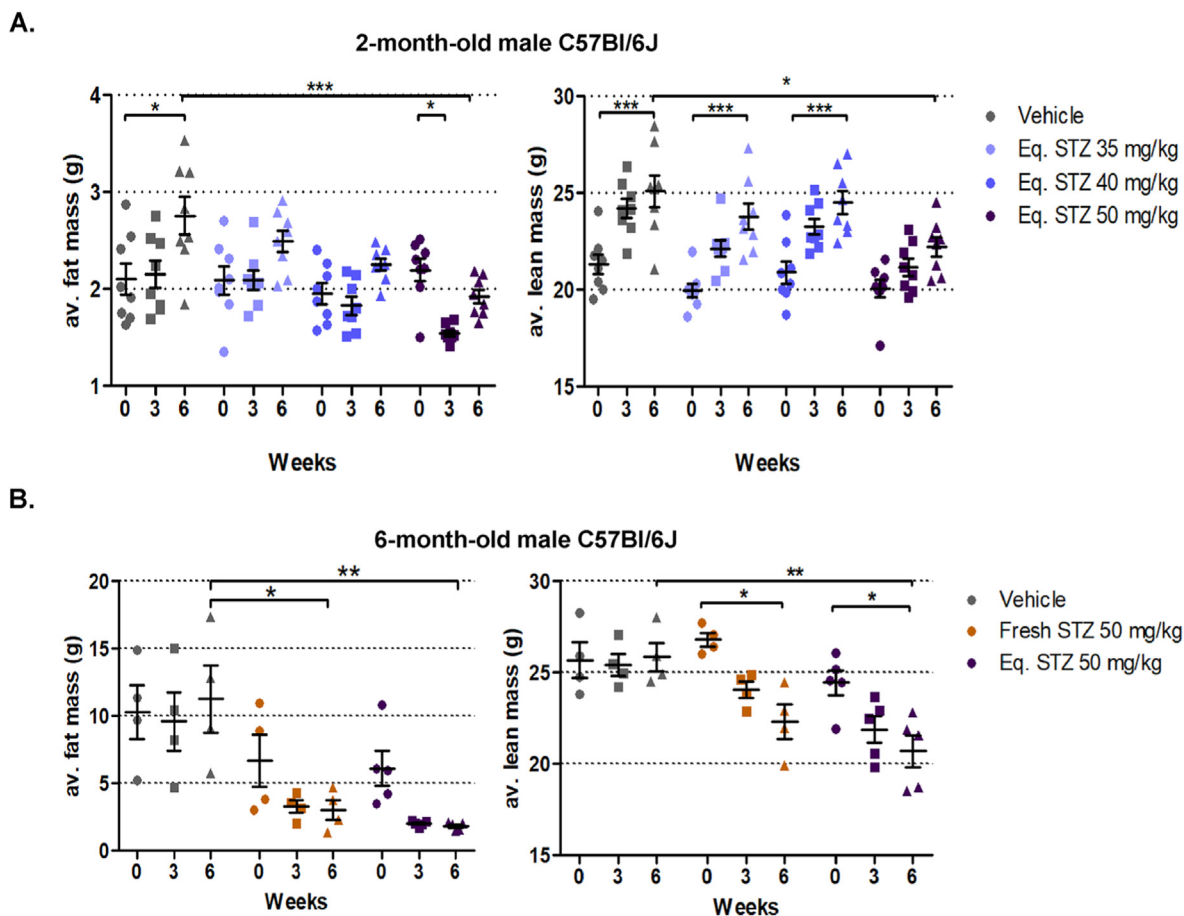

Fig. 2. Body composition using echoMRI 3-in-1 scanner in male C57Bl/6J injected with different doses of STZ at two months of age (A) or injected with 50 mg/kg of STZ prepared freshly or after 90-min anomer equilibration at six-months of age (B). Statistical analyses were performed using two-way ANOVA, followed by Bonferroni multiple comparison post-hoc test. *P < 0.05; **P < 0.01; ***P < 0.001.
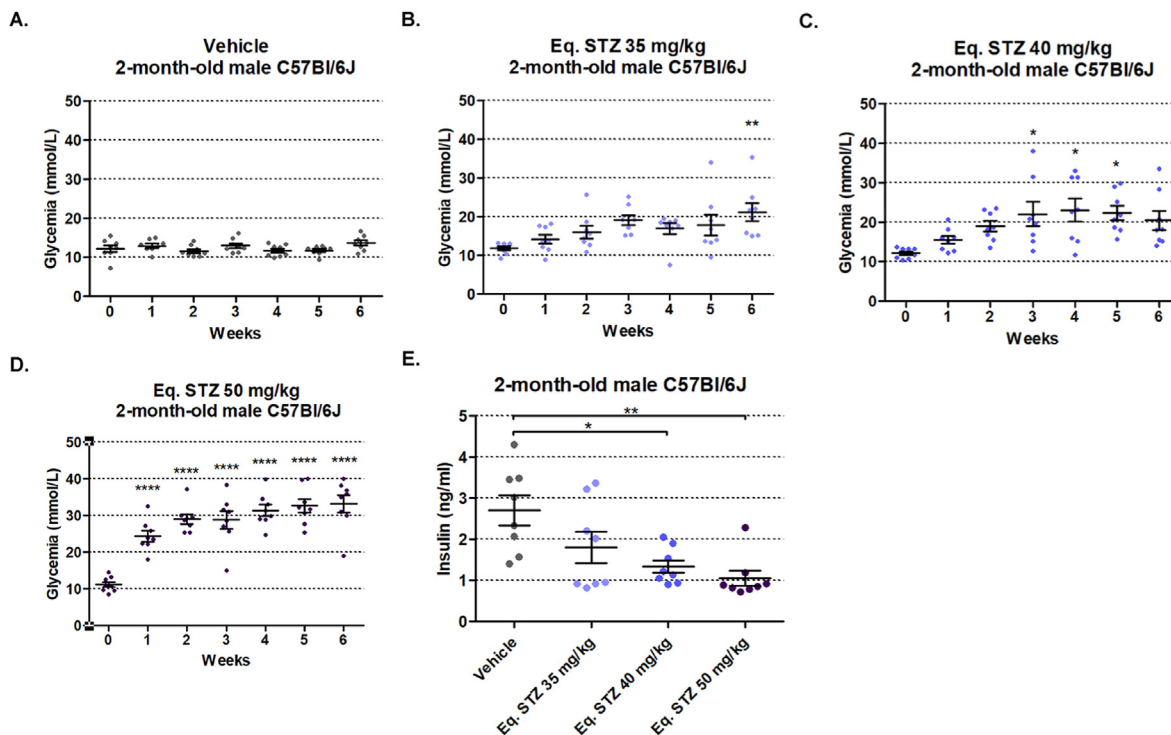
Fig. 3. Two -month-old C57Bl/6J mice treated with vehicle (A) or anomer-equilibrated STZ at 35 mg/kg (B), 40 mg/kg (C) and 50 mg/kg (D) were fasted for 5 h prior to weekly basal glucose monitoring. At termination of the study, serum from trunk-derived blood sampling was isolated and used to determine the concentration of insulin by ELISA (E). Statistical analyses were realised using one-way ANOVA, followed by Bonferroni multiple comparison post-hoc test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

monitored for 6 weeks and compared to weights of mice without STZ injection (controls).

In young two-month-old mice, the body weight of the control group was not impacted by the procedure. However, anomer-equilibrated STZ injected mice lost weight during the first week of study where daily injection occurred (Fig. 1A), but gradually gained weight from week 2 onwards. Weight gain in mice treated with either 35 mg/kg or 40 mg/kg anomer-equilibrated STZ was significantly lower compared to the control group (Fig. 1A week 6, eq. STZ 35 mg/kg: p < 0.01; eq. STZ 40 mg/kg: p < 0.001). The weight gain difference with the control group was even greater in animals injected with 50 mg/kg anomer-equilibrated STZ (Fig. 1A week 6, p < 0.0001), but also compared to animals injected with lower STZ doses (Fig. 1A week 6, eq. STZ 35 vs 50 mg/kg: p < 0.0001; eq. STZ 40 vs 50 mg/kg: p < 0.001).

In mice at six months of age, a weight drop was observed in mice injected with either freshly prepared or 90-min anomer-equilibrated STZ, but also within the control group (Fig. 1B, week 1). The latter regained weight back to the starting value and this was reached at 6-weeks post injection. In contrast, mice injected with 50 mg/kg STZ continued to exhibit a significant weight loss compared to the vehicle, independently of its method of preparation (Fig. 1B week 6, vehicle vs fresh STZ 50 mg/kg: p < 0.01; vehicle vs eq. STZ 50 mg/kg: p < 0.001).

3.2. Anomer-equilibrated STZ alters body composition

To determine whether the STZ-induced body weight loss was due to a lean or a fat mass loss, whole body scans were used to quantify the body composition over the course of the study.

EchoMRI scanning performed in two-month-old mice at 3 weeks post-STZ injection revealed that fat mass was negatively affected by the action of anomer-equilibrated STZ at 40 mg/kg and

50 mg/kg doses (Fig. 2A left, eq. STZ 50 mg/kg week 0 vs week 3: p < 0.05), whereas it remained unaltered by either the lowest STZ dose or vehicle control. In concordance with the body weight data, all mice had gained adiposity after week 3 post injection. However, animals treated with the highest dose of anomer-equilibrated STZ did not reach their initial lipidaemic composition and it was significantly lower from the control vehicle-injected group (Fig. 2A right week 6, vehicle vs eq. STZ 50 mg/kg: p < 0.001).

In six-month-old mice, echoMRI scanning revealed that the decline in body weight in STZ-treated animals was mainly due to a loss in fat rather than lean mass (Fig. 2B, left). At termination of the study, both 50 mg/kg of freshly prepared or 90-min anomer-equilibrated STZ-treated groups displayed a significantly lower fat composition compared to the vehicle group (respectively, p < 0.5 and p < 0.01). However, lean mass was also significantly lower in STZ treated animal albeit at a lower level (Fig. 2B right, fresh STZ 50 mg/kg week 0 vs week 6: p < 0.05; eq. STZ 50 mg/kg week 0 vs week 6: p < 0.5).

3.3. Anomer-equilibrated STZ steadily decreases fasting circulating insulin

As stated previously, STZ acts as a toxin for insulin-secreting pancreatic β-cells. Serum insulin levels from trunk-derived blood were measured to determine whether different doses and/or methods of STZ preparation had an impact on fasting insulinemia and the extent of pancreatic injury.

Two-month-old animals injected with vehicle or 35 mg/kg anomer-equilibrated STZ displayed a broad range of serum insulin concentrations, ranging from approximately 1 to 4 ng/mL (Fig. 3E). In the 40 mg/kg anomer-equilibrated STZ group, the spread of serum insulin was significantly reduced, ranging from 1 to 2 ng/mL (Fig. 3E, vehicle vs eq. STZ 40 mg/kg: p < 0.05). Finally, in the

highest dose group, most of the animals exhibited hypo-insulinaemia with insulin levels lower than 1 ng/mL (Fig. 3E, vehicle vs eq. STZ 50 mg/kg: $p < 0.01$).

Similarly, fasting insulinaemia was significantly lower in six-month-old animals treated with 50 mg/kg fresh and/or anomer-equilibrated STZ (Fig. 4D, vehicle vs fresh STZ 50 mg/kg: $p < 0.05$). However, equilibrated STZ group had least variability.

3.4. Higher dose of anomer-equilibrated STZ promotes severe hyperglycaemia

To determine whether the insulin-lowering effect of STZ induces a substantial hyperglycaemic response in animals treated with the toxin, blood glucose was monitored weekly with a veterinary glucometer following a 5-h fasting period.

Fasted blood glucose levels in vehicle treated two-month-old animals remained unchanged during the course of the study, as expected (Fig. 3A). Low doses of anomer-equilibrated STZ provoked a mild, but significant, elevation in fasted blood glucose levels during the first three weeks post injection, then stabilised between 15 mM and 30 mM until termination of the study (Fig. 3B and C, eq. STZ 35 mg/kg week 0 vs week 6: $p < 0.01$; STZ 40 mg/kg week 0 vs week 3, 4 & 5: $p < 0.5$). However, mice injected with 50 mg/kg equilibrated STZ exhibited a rapid elevation of fasting blood glucose

followed by a slow rise of fasted glycaemia, until most individual mice reached glycaemic values between 30 mM and 40 mM (Fig. 3D, eq. STZ 50 mg/kg week 0 vs all weeks: $p < 0.0001$).

Six-month-old animals exhibited similar profile to younger animals. Fasted glycaemia in the control group remained unchanged (Fig. 4A) but underwent a rapid increase upon injection with 50 mg/kg anomer-equilibrated STZ (Fig. 4B, eq. STZ 50 mg/kg week 0 vs week 1, 4, 5 & 6: $p < 0.0001$). By contrast, 50 mg/kg of freshly injected STZ induced a slow increase in fasted blood glucose levels during the first two weeks post-injection. Thereafter, glycaemic values demonstrated a broad range of values from 15 mM to 40 mM at the termination of the study (Fig. 4C).
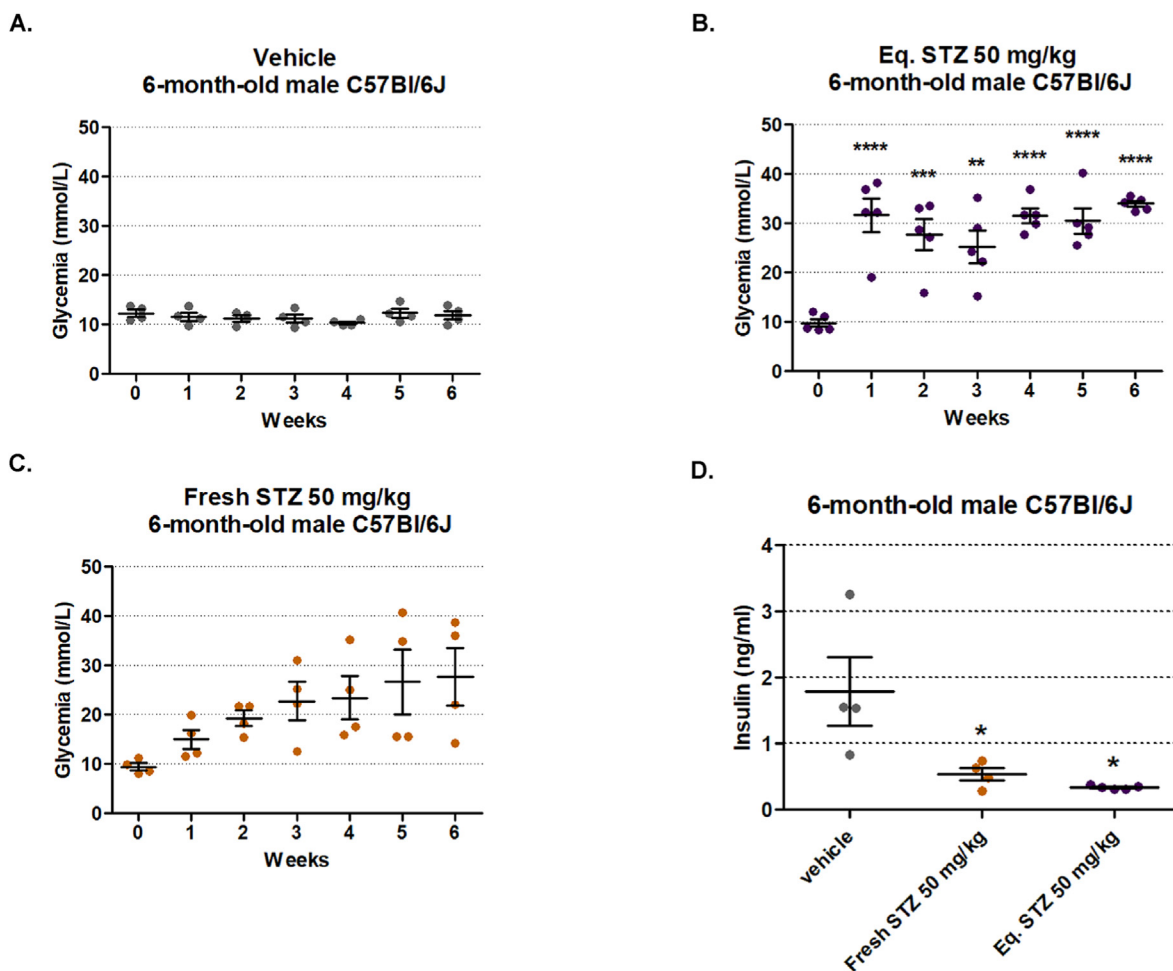
Table 1
Weight of kidneys and kidney-to-body weight ratio in two-month-old male C57Bl/6J treated with vehicle (n = 4) or 50 mg/kg fresh (n = 4) and anomer-equilibrated STZ (n = 5). Statistical analyses were analysed using one-way ANOVA, followed by Bonferroni multiple comparison post-hoc test. Asterisk: vehicle vs all, **$P < 0.01$.

	Kidney weight (g)		Kidney-to-bodyweight ratio (g/kg)	
	M	SD	M	SD
Vehicle	0.3301	0.0276	9.2317	0.7722
Fresh STZ 50 mg/kg	0.4004	0.0426	11.1976	1.1910
Eq. STZ 50 mg/kg	0.4285 (**)	0.0375	11.9827 (**)	1.0480

Fig. 4. Six -months old C57Bl/6J mice treated with freshly prepared or 90-min anomer-equilibrated STZ were fasted for 5 h prior to weekly basal glucose monitoring (A, B & C). At termination of the study, serum from trunk-derived blood sampling was isolated and used to determine the concentration of insulin by ELISA (D). Statistical analyses were realised using one-way ANOVA, followed by Bonferroni multiple comparison post-hoc test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.
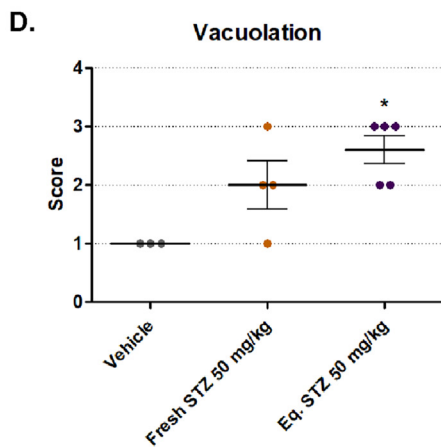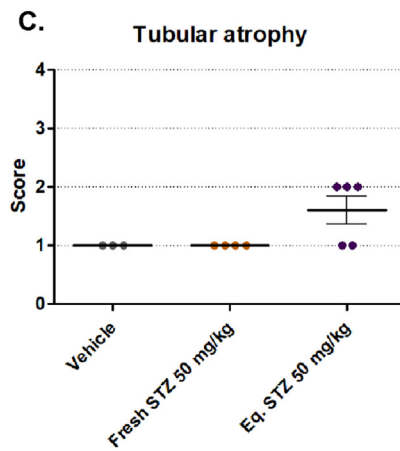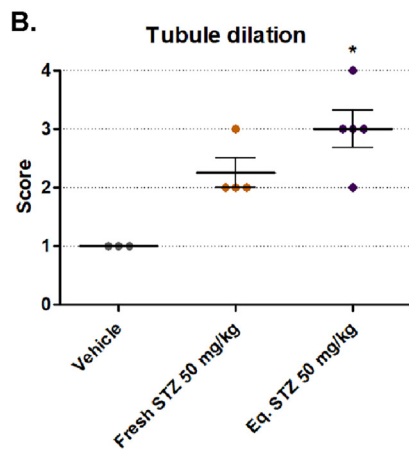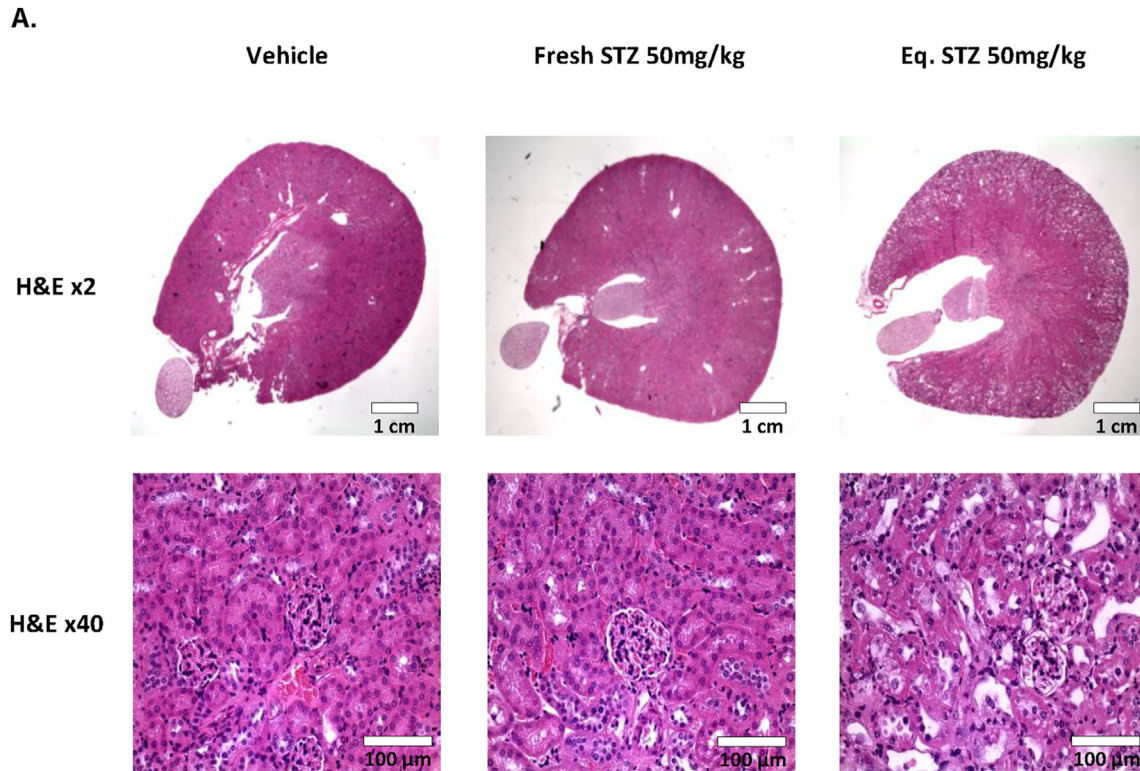
Fig. 5. Formalin-fixed kidney sections from 6-months old male C57Bl/6J injected with vehicle (n = 3), fresh (n = 4) or equilibrated (n = 5) STZ were mounted onto microscope slides and stained with haematoxylin and eosin to reveal changes in renal tissue structure (A). Tubule dilation (B), tubular atrophy (C) and vacuolation (D) were scored by Dr Paul Brown as followed: 1 = none; 2 = mild; 3 = moderate; 4 = severe. Statistical analyses were realised using Kruskal-Wallis test, followed by Dunn's multiple comparison post-hoc test. Asterisks: vehicle vs all. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Anomer-equilibrated STZ causes renal hypertrophy

To determine the effects of hyperglycaemia on kidney damage, kidneys were first weighed and normalised to the body weight to evaluate and compare renal enlargement in two-month-old mice injected with anomer-equilibrated or fresh STZ.

STZ at 50 mg/kg induced substantial increases in kidney weight and kidney-to-body weight ratio six weeks post injection, with significant differences observed between animals injected with equilibrated STZ versus control group (Table 1, vehicle vs Eq. STZ 50 mg/kg: p < 0.01). Increases in kidney-to-body weight indicated signs of renal hypertrophy upon anomer-equilibrated STZ injection.

3.6. Ninety-minute equilibrated-anomer STZ provokes tubular damage

Renal tissue damage was assessed as an indicator of early diabetic nephropathy using haematoxylin and eosin (H&E) staining to reveal cellular structure.

At six months of age, anomer-equilibrated STZ provoked substantial changes in tubular cell structures compared to the vehicle control group. Microscopy photography revealed many visible lumens in H&E-stained slides (Fig. 5A), consequently to the presence of moderate to severe tubule dilation (Fig. 5B, vehicle vs eq. STZ 50 mg/kg: p < 0.05) and tubular atrophy (Fig. 5C). Macrovesicles in renal tubular cells, or vacuolation were also present in large number upon anomer-equilibrated STZ treatment (Fig. 5A and D, vehicle vs eq. STZ 50 mg/kg: p < 0.5). In contrast, same dose of freshly prepared STZ induced merely mild tubule dilation (Fig. 5B) and mild tubular atrophy (Fig. 5C). Vacuolation events were also observed although at non constant level (Fig. 5D). However, no visible glomerular lesions were observed under the microscope in either of the STZ groups.

4. Discussion

A robust and reproducible murine model of DN has been challenged using STZ. Here we addressed whether this could be improved by the mode of STZ preparation and thus the ratio of α- and β-anomers. Five daily consecutives low doses of freshly prepared STZ are recommended to generate pharmacologically induced model of diabetes in mice [10]. However, we have now demonstrated that 5 consecutives daily injections of anomer-equilibrated STZ at 50 mg/kg induced severe fasted hyperglycaemia (>30 mM) in wild-type C57Bl/6J mice at two and six months of age, whereas freshly injected STZ failed to develop stable and consistent hyperglycaemia. STZ selectively targets insulin-secreting pancreatic β-cells and causes cell death [6,8]. We found that anomer-equilibrated STZ significantly decreased fasting insulin levels, in a dose-dependent manner and consequently had a greater diabetogenic effect, as has been suggested by others [12].

Although two-month-old mice did not lose weight 6 weeks after injection of anomer-equilibrated STZ at different doses, six-month-old animals exhibited an immediate and continuous weight loss over the course of the study. Such variation in body weight could be due to the fact that older animals have higher fat mass and therefore are impacted more significantly than younger animals. Moreover, studies have shown age-related changes in behaviour in C57Bl/6J mice, suggesting that pain [14] and anxiety [15] increase with age, which can contribute to weigh loss in older mice. However, findings by Shoji et al. [16] rejected the age-pain/anxiety correlation, but authors admitted that divergent conclusion might be due to test experience and strains.

The severe hyperglycaemia subsequent to STZ injection of both, anomer-equilibrated and freshly prepared STZ, caused structural changes within the kidney. We demonstrated that kidneys were enlarged due to tubule dilation, tubular atrophy and vacuolation. However, animals treated with anomer-equilibrated STZ displayed greater kidney hypertrophy and tubular damage but without glomerular alterations. These changes are likely due to hyperfiltration, and increased fibrosis as seen in other murine models of diabetic nephropathy [5,17]. These are all consistent with the early phases of DN that have been more challenging to induce in a consistent manner [18,19].

The potential of anomer-equilibrated STZ to induce experimental diabetes has previously been suggested [13]. We demonstrated that anomer-equilibrated STZ also leads to signs of early diabetic kidney disease and diabetic nephropathy. Further investigations should now explore how/if alterations in dietary composition could worsen the pathogenesis of our reproducible early-stage diabetic kidney disease as a more advanced DN model. A study where diabetic *db/db* mice were fed a high protein diet (60% kcal from protein) revealed a rapid progression of nephropathy, including increases in urine readouts and renal structural changes [20].

In conclusion, our approach provides a better, reproducible experimental model of diabetes and early-stage kidney damage due to diabetes. The animal welfare is improved, which complies with the principles of 3Rs in that the anomer-equilibrated STZ effects are more reproducible and therefore refined. This refined model should provide further opportunities for testing of therapeutic approaches to prevent and/or revert pathogenesis of early diabetic kidney disease.

Declaration of competing interest

The authors declare that they have no conflicts of interests.

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