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ORIGINAL RESEARCH

Type 2 diabetes mellitus-related non-alcoholic steatohepatitis in the Zucker Diabetic Sprague Dawley rat

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Non-alcoholic steatohepatitis (NASH) is a severe form of non-alcoholic fatty liver disease that is highly prevalent in Type 2 diabetes mellitus (T2DM). NASH progresses into cirrhosis and hepatocellular carcinoma and is known to worsen the prognosis and mortality in T2DM. Our understanding of the mechanisms underlying NASH development in T2DM is hindered by the absence of a good animal model that can physiologically develop T2DM and NASH. This study investigated the potential of the Zucker Diabetic Sprague Dawley (ZDSD) rat as a suitable model for studying T2DM-related NASH. Eight, twenty-week old ZDSD rats which became diabetic at week sixteen, were compared with six age-matched, non-diabetic Sprague Dawley (SD) rats. We measured body mass gain, fasting glucose, fasting triglycerides and glucose handling pre and post diabetic onset. We also measured circulating levels of the liver function enzymes; alanine transaminase and alkaline phosphatase, and other surrogate markers of kidney and pancreatic function. Liver samples were also scored for histopathological markers of NASH. ZDSD rats developed frank T2DM and exhibited impaired glucose handling, chronic hyperglycaemia, deranged lipid metabolism and impaired kidney function compared to SD rats. Histopathological analyses of the diabetic ZDSD rat liver showed the presence of steatosis, inflammation, hypertrophy and fibrosis. The co-occurrence of both T2DM and advanced NASH in the ZDSD rat compared to SD rats validates our hypothesis of its potential as a model for studying the pathogenesis of these two closely related diseases.

Keywords: ZDSD rat, hyperglycaemia, liver function, steatohepatitis, fibrosis

Introduction

Non-alcoholic steatohepatitis (NASH) is an advanced and severe form of non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of the metabolic syndrome that is highly prevalent in Type 2 diabetes mellitus (T2DM).[1-3] NASH is characterised by steatosis, inflammation, ballooning of hepatic cells and advanced fibrosis and with time may develop into cirrhosis and hepatocellular carcinoma.[4] There is evidence that the coexistence of NAFLD/NASH with T2DM worsens the prognosis of either disease.[3,5-7] The metabolic syndrome and T2DM can occur as either a precursor or consequence of NASH[8,9] but the mechanistic basis of this precursor-consequence relationship remains incompletely understood. Ethical issues surrounding the use of human subjects for studying the complex pathophysiology of these diseases necessitate the development of appropriate animal models.

While several rodent models may be available for studying T2DM and NASH, one of the greatest challenges in studying the pathogenesis of T2DM-related liver disease has been that of finding an animal model that correctly mirrors the development, progression and coexistence of both conditions in humans (see Takahashi et al.[10] for review). Artificially-induced T2DM models (e.g. alloxan or streptozotocin-induced T2DM) cannot be used reliably to study the development of T2DM-related NASH because they do not show the progressive changes and distinct physiological phases typical of the development of T2DM. Although these chemically-induced diabetic models show evident hyperglycaemia, the high glucose levels are primarily the result of the cytotoxic effects of the chemicals on pancreatic β-cells. Thus, these models typically represent insulin insufficiency as opposed to the insulin resistance that characterises T2DM. Other diet-induced obesity rodent models also exist that may successfully develop NASH but these usually require prolonged periods of dietary treatment before they develop insulin resistance and frank diabetes.[11]

Of the rodent models that spontaneously develop diabetes, the Zucker Diabetic Fatty (ZDF) rat has been commonly used for studying T2DM. However, the development of T2DM in ZDF rats is the result of a recessive homozygous mutation in leptin receptor signalling, which is not common in humans.[12] Although traditionally recognised only for its role in mediating satiety, recent research has shown that leptin plays a critical role in the development of advanced hepatic disease. In hepatic disease, leptin is a potent activator of hepatic stellate cells, which secrete fibrillar collagen during fibrogenesis.[13-15] With missing leptin signalling, the ZDF rat therefore becomes unsuitable for studying T2DM-related NASH. An improvement of the ZDF rat, the Zucker Diabetic Sprague Dawley (ZDSD) rat which was derived from a cross breed between a diet-induced obese Sprague Dawley-derived rat and lean ZDF$^{fa/-}$ rats[12] may be more applicable for

NASH studies. The ZDSD rat is predisposed to spontaneously develop modest obesity and frank diabetes.

T2DM has often been associated with complications such as retinopathy, nephropathy, cardiovascular complications and hepatic dysfunction.[16-18] Several studies have characterised these comorbidities in the ZDSD rat and demonstrated that it is a good model for studying the pathophysiology and for testing treatments for these diabetic sequelae.[12,19] The ZDSD rat has been shown to develop neuropathy,[20] nephropathy[21] osteoporosis and other skeletopathic changes[12,19] but reports on the development of NAFLD or NASH in this model are scanty in refereed literature. Thus, we have chosen to investigate if the ZDSD rat can physiologically develop both T2DM and NASH. Identifying a model in which T2DM and NASH develop, progress and coexist as they do clinically, could be useful for studying the mechanistic link between the two conditions; the knowledge of which is critical in the development of effective treatments. Our results show that the ZDSD rat develops metabolic derangement, frank diabetes and liver disease characterised by steatosis, inflammation, fibrosis and hypertrophy.

Methods

Animals

Fourteen, 8-week-old, male rats (ZDSD; *n* = 8; PreClinOmics, Indianapolis, USA and Sprague Dawley; *n* = 6; Central Animal Services, University of the Witwatersrand, Johannesburg, RSA) were individually housed in acrylic cages lined with wood shavings at an ambient temperature of 22 ± 2 °C. A 12h:12h light/dark cycle was maintained (lights on 6 am clock time). All rats were given food (Purina 5008 rodent chow; LabDiet, St Louis, Missouri, USA) and water *ad libitum*. Animal welfare and all experimental procedures were done in accordance with the principles of the Animal Ethics and Control Committee of the University of the Witwatersrand and were approved by its Animal Ethics Screening Committee (Ethics Clearance Number 2015/07/28C).

Experimental procedures

Blood glucose and triglycerides

Fasting levels of glucose and triglycerides were measured fortnightly. To measure circulating levels of these metabolites, the rats were fasted for 12 hours overnight after which tail vein blood was collected using the pinprick method and glucose was measured using a glucometer (Accutrend®Plus, Roche Diagnostics, Germany). Circulating levels of triglycerides were measured using a GCT meter (Accutrend®Plus, Roche Diagnostics, Germany). Synchronisation of diabetes in ZDSD was done according to the supplier's instructions.[20] The definition of permanent onset of diabetes in the ZDSD rats was confirmed by two subsequent fasting glucose readings above 13.9 mmol/L or 250 mg/dL.[21] Glucose handling was monitored through oral glucose tolerance tests, which were performed once every four weeks. To measure glucose handling, rats were fasted for 12 hours overnight and their fasting glucose levels recorded (T_0) before receiving an oral glucose load (2 g/kg). Glucose levels were then measured from blood collected from tail vein blood at 15, 30, 60 and 120 min after the glucose load.

Terminal procedures and hepatic tissue preparation

Rats were euthanised by terminal anaesthesia of 1 ml sodium pentobarbital (Euthanase, 200 mg/ml; Kyron Laboratories (Pty) Ltd, South Africa). Blood was collected by cardiac puncture into serum separating tubes, centrifuged at 3913 rcf (4 °C for 10 minutes) and stored at -20 °C for biochemical analyses. The rats were transcardially perfused using ice cold saline (0.9%) for 2 min and caudate liver samples were collected and preserved in 10% phosphate-buffered formalin for histopathological analyses.

The formalin-fixed liver samples were processed for routine histology through ascending grades of ethanol using an automatic processor (Microm STP 120, Thermo Fischer Scientific USA). The samples where then embedded in paraffin wax and sectioned at 4 µm thickness using a rotary microtome (Leica RM2125 RTS, Leica Biosystems, USA). The tissue sections were subsequently stained with haematoxylin and eosin (H&E) for visualising steatosis, inflammation and hypertrophy; and Masson's trichrome stain (MT) for collagen fibres (fibrosis). Morphological changes were analysed using a compound microscope (Leica DM 500, Leica Biosystems, USA) and photomicrographs acquired using a high definition video camera (Leica ICC50, Leica Biosystems, USA). Images were prepared with CorelDraw X7 Software (Version 13, Corel Corporation, Ottawa, Canada) and were only adjusted for contrast and brightness.

Haematoxylin and eosin-stained sections were semi-quantitatively scored for microsteatosis, macrosteatosis, inflammation (necro-inflammatory cell aggregation), and hypertrophy.[22] Macrovesicular steatosis, microvesicular steatosis and hepatocellular hypertrophy were analysed by determining the percentage area affected in each camera field at x20 magnification. Macrosteatosis, microsteatosis, and hepatocellular hypertrophy were graded as follows: Grade 0 = < 5%; Grade 1 = 5–33%; Grade 2 = 33–66%; Grade 3 = > 66% area affected per camera field of the parenchyma. Inflammation was scored by counting the number of inflammatory cell aggregates in the liver parenchyma and graded as follows: Grade 0 = no foci; Grade 1 = 0.5–1.0 foci; Grade 2 = 1–2 foci; Grade 3 = > 2 foci, per camera field at x100 magnification. Two independent individuals who were blinded to treatments performed scoring.

Fibrosis was measured from photomicrographs captured from MT-stained liver sections at x20 magnification using ImageJ software.[23] The area [A = ap X ∑p] and area fraction ($A_{fraction}$) of each liver section (x20) occupied by collagen fibres were measured using the point counting method, where ap is the area per point (0.002 mm^2); ∑p is the sum of the points falling on the collagen fibres within a camera field (0.149 mm^2) of each liver section and $A_{fraction}$ = [A ÷ 0.149 mm^2 X 100]. A total of 20 camera fields were used for each section.

Liver, kidney and pancreatic function tests

Serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine

(CREA), amylase, lipase and albumin, were measured using an IDEXX VetTest® Chemistry Analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) according to the manufacturer's instructions.

Data analysis

All data are expressed as mean and standard deviation (SD) and were analysed using GraphPad Prism (version 7, GraphPad Software Inc, USA). Body mass was plotted against time and analysed using a repeated measures (RM) two-way analysis of variance (ANOVA) with treatment and time as main effects. Bonferroni's test was used to identify differences whenever the ANOVA detected significant main effects or interactions. Fasting levels of glucose and TGs were analysed using a RM two-way ANOVA with treatment and time as main effects followed by Bonferroni *post hoc* test. Serum levels of ALP, ALT, BUN: CREA, amylase, lipase and albumin were compared using a two-tailed, unpaired t-test with a level of significance of $P < 0.05$.

Results

Body mass

Figure 1 shows the body mass of ZDSD and control SD rats recorded over 10 weeks. The main effects of time ($F_{(13, 247)} = 95.25$, $P < 0.0001$), treatment ($F_{(13, 247)} = 95.25$, $P < 0.0001$) and their interaction ($F_{(13, 247)} = 95.25$, $P < 0.0001$) were such that the body mass of the control SD rats increased from week 10 to week 20 while the body mass of the ZDSD rats increased only up to week 16. Thereafter, the average body mass of ZDSD rats progressively decreased and was significantly lower than that of control SD rats.

Fasting glucose, triglycerides and cholesterol

Figure 2A shows the fasting glucose levels of ZDSD and control SD rats. No rats were diabetic at the beginning of the study and by week 14 there were no significant differences between the fasting blood glucose levels of the ZDSD and the control SD rats ($P > 0.9999$; Bonferroni). The main effects of time ($F_{(3, 36)} = 6.98$, $P = 0.0008$), treatment ($F_{(1, 12)} = 224$, $P < 0.0001$) and their interaction ($F_{(3, 36)} = 8.54$, $P = 0.0002$) were such that fasting glucose levels of ZDSD progressively increased between week 14 and 20 when compared to those of controls.

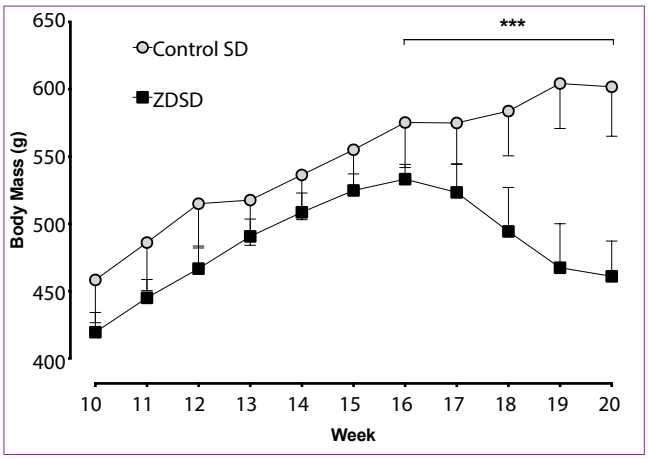

Figure 1: Body mass (mean and SD) of Zucker Diabetic Sprague Dawley (ZDSD) rats and normal Sprague Dawley rats (control SD) over ten weeks. Diabetes was induced by high fat feeding in ZDSD rats commencing from week 15. *** ZDSD significantly different from control SD rats ($P < 0.0001$; Bonferroni).

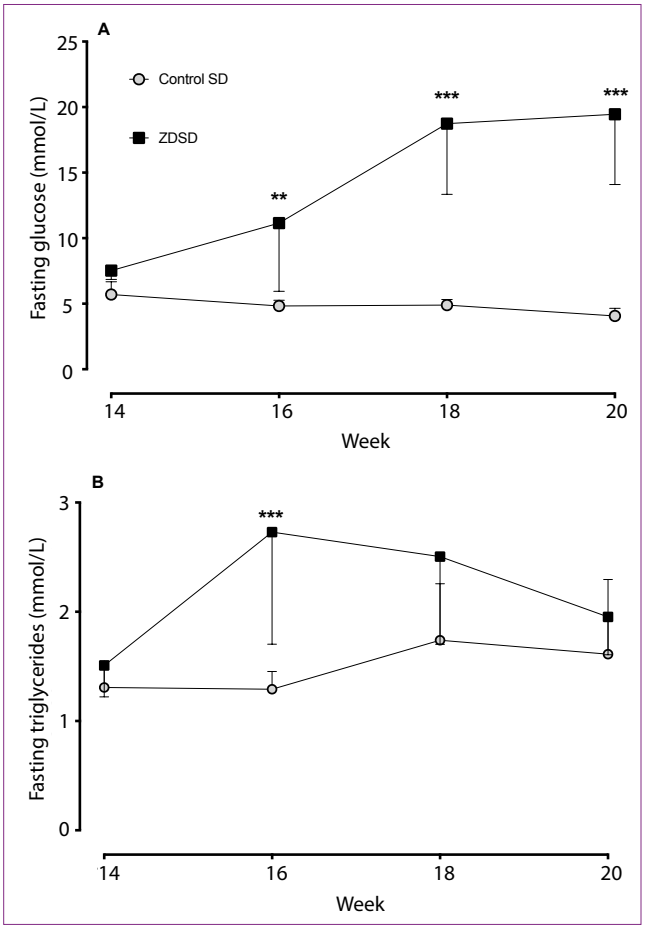

Figure 2: Fasting glucose (**A**) and triglycerides (**B**) (mean and SD) of Zucker Diabetic Sprague Dawley (ZDSD) rats and normal Sprague Dawley rats (control SD). Diabetes was induced by high fat feeding in ZDSD rats commencing from week 15. *** ZDSD significantly different from control SD ($P < 0.001$; Bonferroni). ** ZDSD significantly different from control SD ($P < 0.01$; Bonferroni).

Figure 2B shows a comparison of fasting TGs of ZDSD and control SD rats. There were no significant differences in the fasting TG levels at week 14 ($P > 0.9999$; Bonferroni) but TG levels of the ZDSD rats were significantly greater than those of controls at 16 weeks [main effects of time ($F_{(3, 36)} = 4.84$, $P = 0.0062$), treatment ($F_{(1, 12)} = 10.72$, $P = 0.0067$) and their interaction ($F_{(3, 36)} = 3.75$, $P = 0.0019$)].

Oral glucose tolerance tests

Figure 3 shows oral glucose tolerance test results of ZDSD rats compared to control SD rats at week 20. The basal levels of glucose of ZDSD rats were significantly greater than those of controls ($P < 0.0001$; Bonferroni). The circulating levels of glucose in ZDSD rats rose to peak at 28.14 ± 4.14 mmol/L; 30 min after an oral glucose load while the circulating levels of glucose in control SD rats rose to peak at 7.08 ± 0.78 mmol/L; 60 min after the oral glucose load.

Glucose levels remained significantly raised in ZDSD rats (25.79 ± 2.35 mmol/L) as compared to control SD rats (6.82 ± 0.61 mmol/L) 120 min after the glucose load. Overall, ZDSD rats showed

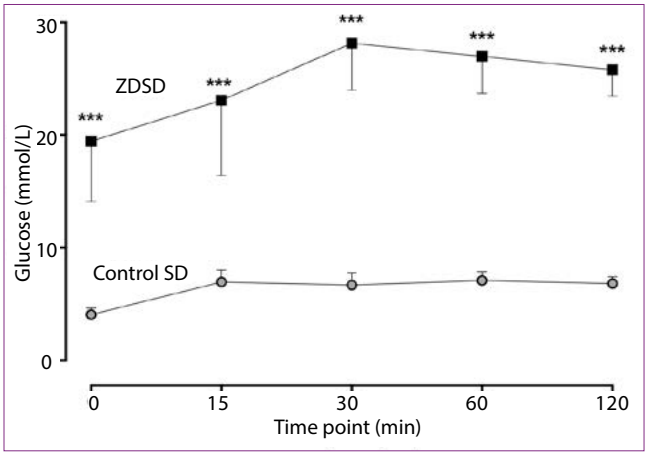

Figure 3: Glucose handling (mean and SD) in Zucker Diabetic Sprague Dawley (ZDSD) rats and normal Sprague Dawley rats (control SD) following a 2 g/kg oral glucose load. *** ZDSD significantly different from control SD ($P < 0.0001$; Bonferroni).

impaired glucose handling when compared with controls [main effects of time ($F_{(4, 48)} = 10.80$, $P < 0.0001$), treatment ($F_{(1, 12)} = 155.2$, $P < 0.0001$) and their interaction ($F_{(4, 48)} = 3.38$, $P = 0.0162$)].

Liver, kidney and pancreatic function tests

Table I shows serum levels of surrogate markers of liver, kidney and pancreatic function. Serum ALP levels were significantly increased in the ZDSD rats when compared to controls ($t = 6.12$; $P < 0.0001$). Although ALT levels tended to be higher in ZDSD rats than their control SD counterparts, there was no statistical difference between mean ALT levels for the two treatments ($t = 1.34$; $P = 0.2045$). Similarly, we observed no significant differences between serum levels of albumin for the two groups ($t = 0.074$; $P = 0.9421$). ZDSD rats exhibited lower serum levels of amylase when compared to control SD rats ($t = 5.81$;

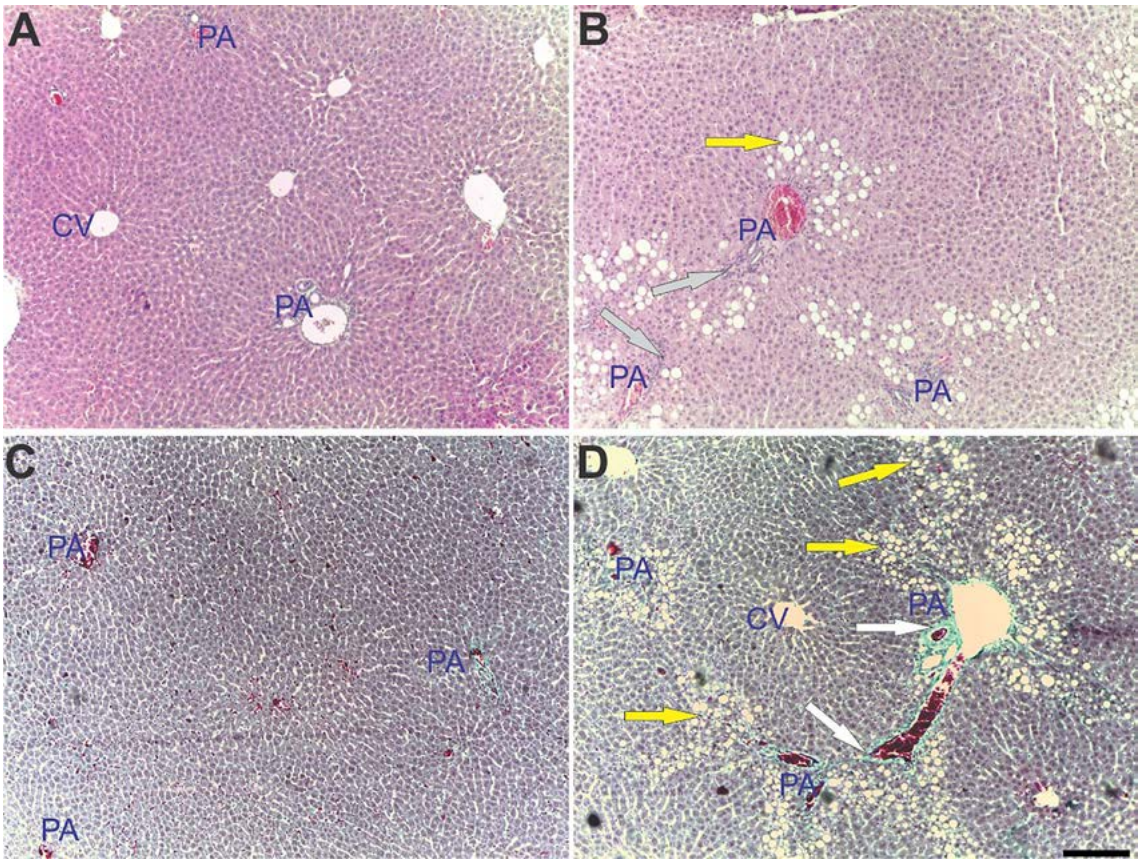

Figure 4: Histological photomicrographs of NASH in ZDSD vs control SD rat livers. Histological cross sections of control SD (**A**) and ZDSD (**B**) livers stained with haematoxylin and eosin stain (x10), or control SD (**C**) and ZDSD (**D**) livers stained with Masson's trichrome stain (x10). Yellow arrows show steatosis, grey arrows in **B** show necro-inflammatory cell aggregates and white arrows show in **D** collagen deposition. PA = portal area and CV= central vein. The scale bar in **D** represents 100 µm.

Table I: Serum levels of surrogate markers of liver, kidney, and pancreatic function

	Control SD	ZDSD	*P* value
Alanine aminotransferase (U/L)	84.00 ± 50.84	114.88 ± 35.58	0.2045
Alkaline phosphatase (U/L)	178.50 ± 20.19	366.40 ± 72.51	**< 0.0001**
Albumin (g/dL)	29.00 ± 2.28	28.88 ± 3.60	0.9421
Total bilirubin (µmol/L)	7.00 ± 2.10	6.63 ± 1.69	0.8825
BUN:CREA ratio	55.67 ± 5.43	83.13 ± 10.67	**< 0.0001**
Amylase (U/L)	2165.00 ± 206.24	1500.00 ± 216.33	**< 0.0001**
Lipase (U/L)	268.00 ± 190.76	204.00 ± 201.88	0.5593

Data are represented as mean ± SD. ZDSD – Zucker Diabetic Sprague Dawley rat, BUN – blood urea nitrogen, CREA – creatinine. Bold *P* values show significant difference between ZDSD and control SD.

Table II: Non-alcoholic fatty liver disease (NAFLD) scores for ZDSD and control SD rat livers

	Control SD	ZDSD	*P* value
Macro steatosis	0.0 (1.0–0.0)	3.0 (3.0–2.0)	**0.001**
Micro steatosis	0.0 (0.0–0.0)	1.0 (2.0–1.0)	**0.001**
Hypertrophy	0.0 (0.0–0.0)	1.0 (2.0–1.0)	**0.001**
Inflammation	0.0 (0.5–0.0)	2.0 (2.0–1.0)	**0.010**
Fibrosis area fraction (%)	1.61 ± 1.12	8.50 ± 1.63	**0.002**

Data are represented as median (interquartile range) for macrosteatosis, microsteatosis, hypertrophy and inflammation. $P < 0.05$. ZDSD – Zucker Diabetic Sprague Dawley rat. Bold *P* values show significant differences between ZDSD and control SD.

$P < 0.0001$) but serum levels of lipase were not different ($t = 0.6006$; $P = 0.5593$).

Histopathology

Figure 4 shows representative photomicrographs of NASH in ZDSD rat livers and Table II shows the corresponding scores/grades for the histopathological features observed. No steatosis was evident in the livers of control SD rats (Figures 4A and 4C). ZDSD rats developed severe (grade 3) macrovesicular steatosis ($P = 0.001$) around the portal areas (Figures 4B and D). Mild to moderate (grade 1–2) microvesicular steatosis was also observed in ZDSD rats ($P = 0.001$) around the portal areas (Figures 4 B and D). We observed periportal, bridging fibrosis in ZDSD rats (Figure 4D) but no fibrosis was evident in the livers of control SD rats (Figure 4C). Fibrosis was significantly greater in the livers of ZDSD rats as compared to control SD rats ($P = 0.002$). Diabetic ZDSD rats also developed mild to moderate (grade 1–2) lobular and portal inflammation (Figure 4B) which was absent in the livers of normal control SD rats.

Discussion

Understanding the pathophysiology of T2DM-related NASH has been hindered by the absence of an appropriate animal model that correctly mirrors the development, progression and coexistence of the condition in humans. Our results show that ZDSD rats developed frank diabetes and non-alcoholic steatohepatitis by 20 weeks of age. Diabetes in ZDSD rats was characterised by hyperglycaemia (fasting glucose; 19.45 ± 5.36 mmol/L) (Figure 2A) and weight loss (Figure 1). The development of T2DM and the accumulation of lipids in the liver in the ZDSD rat has been previously reported but to our knowledge, our study is the first to describe the co-occurrence of non-alcoholic steatohepatitis with T2DM in this diabetic rat model. In diabetic ZDSD rats, NASH was characterised by both microvesicular and macrovesicular steatosis, inflammation, hypertrophy and fibrosis (Figure 4). We also observed a derangement of alkaline phosphatase, amylase and BUN:CREA ratio but no significant changes in alanine transaminase, albumin and lipase in ZDSD rats (Table I).

The ZDSD rat is a highly translational model for T2DM, which lends its credibility to the spontaneous nature in which diabetes develops; very similar to how it develops clinically. Unlike in chemically-induced diabetic models, the ZDSD model develops a clear prediabetic phase followed by overt diabetes. In our study, the prediabetic or metabolic syndrome phase was characterised by hyperglycaemia before the onset of frank diabetes; as evidenced by the mean fasting glucose of 7.5 mmol/L at 14 weeks. It is interesting to note however, that the progression of diabetes and the associated weight loss in the ZDSD from week 16–20 was rapid. Weight loss associated with overt diabetes, albeit more gradual (8.2% between weeks 23–31) was similarly reported in a study[21] that characterised T2DM in the ZDSD rat. However, in our study, the onset of diabetes was synchronised to occur early (week 16) unlike in their study where full diabetes was only observed between weeks 19–21. The ZDSD model spontaneously develops diabetes without the need for feeding with a diabetogenic diet[21] but a synchronising diet (Purina 5SCA, LabDiet) can help synchronise the onset of diabetes to ensure little variation of diabetic states between the experimental animals.[24-26]

By week 20, the disease had progressed to frank diabetes characterised by severe and chronic hyperglycaemia (fasting glucose > 13.9 mmol/L), significant abnormalities in glucose handling (see Figure 3) and impaired pancreatic and renal function (see Table I). As occurs in humans, the ZDSD model has been shown to exhibit insulin resistance, compensatory hyperinsulinaemia (which peaks at week 19) followed by beta cell loss and overt diabetes.[21] The dysregulation of insulin followed by beta cell dysfunction as previously described[21] could explain abnormalities in glucose handling and overt diabetes observed in our study. Our results showed reduced serum levels of pancreatic amylase reflecting a derangement of pancreatic function consistent with what others have described.[27]

Metabolic derangements characteristic of T2DM predispose one to the development of NAFLD and NASH. The pathogenesis of NASH is believed to follow a two hit hypothesis: (1) the "first hit" characterised by an accumulation of fat in hepatocytes resulting from hyperglycaemia, hypertriglyceridaemia and insulin resistance and (2) a number of "second hits" which include oxidative stress and an excessive production of pro-inflammatory cytokines.[28-30] In NASH, oxidative stress is the result of overproduction of reactive oxygen species by the mitochondria, microsomes and peroxisomes in response to the accumulation of free fatty acids in the cell.[31] Mitochondrial β-oxidation when saturated gives way to peroxisomal β-oxidation increasing the production of hydrogen peroxide and subsequently highly reactive hydroxyl radicals through the Haber–Weiss and Fenton reactions.[32] Although this study does not investigate the mechanisms linking T2DM and NASH, it provides the necessary preliminary evidence of the coexistence

of the two diseases and the potential of the ZDSD rat model for investigating mechanisms underlying T2DM-related NASH.

Oxidative stress in NASH is also exacerbated by the activation of cytochrome P450 2E1, an inducible, xenobiotic-metabolising enzyme present in the liver whose expression and activity are reportedly increased in both NASH and diabetes mellitus.[33,34] The increase in pro-inflammatory cytokines is usually a response to oxidative stress but cytokines are also produced by macrophages, natural killer cells and activated hepatic stellate cells.[31] Cytokine production in the liver has been shown to induce insulin resistance and to exacerbate oxidative stress.[35,36] Thus, a vicious cycle of oxidative stress, inflammatory cytokine production and insulin resistance is triggered ultimately leading to necroinflammation and steatohepatitis.

For humans, NASH scoring focusses on three main histopathological changes in the liver namely: steatosis, inflammation and ballooning with or without fibrosis.[37,38] Because we were working with rats, we employed the modified Kleiner scoring system which has been developed and validated for scoring NAFLD and NASH in small rodents.[22] Our results show that ZDSD diabetic rats developed both macrovesicular and microvesicular steatosis where steatosis is defined as fat accumulation in more than 5% of hepatocytes.[38] Macrovesicular steatosis in ZDSD rats was characterised by large intracellular lipid vacuoles that displaced the nucleus to the side while smaller lipid vesicles that did not displace the nucleus were observed in microvesicular steatosis. Both macro and microvesicular steatosis are key markers of NAFLD in humans[39] and thus the occurrence of these markers in the ZDSD diabetic rat further supports our hypothesis that it could be useful as a transitional model for T2DM-related liver disease.

Steatohepatitis describes a condition in which steatosis is accompanied by inflammation, ballooning and at advanced stages with fibrosis.[38] Diabetic ZDSD rats in our study developed lobular and portal inflammation. Inflammation was scored as the number of aggregates of necro-inflammatory cells (foci) found within a camera field. One limitation of this study, however, was that we did not characterise the types of inflammatory cells recruited in T2DM-related NASH in the ZDSD diabetic rat. In their mouse model of NASH, however, others found different inflammatory cells which include monocytes, Kupffer cells and other polymorphonuclear cells.[22] Further studies intending to use the ZDSD model could further characterise the different types of inflammatory cells.

In addition to observing steatosis and inflammation, we also observed ballooning of hepatocytes, another marker considered as important when scoring for NASH in humans. While this feature may be clear in humans, it appears less apparent in our model T2DM-related NASH. The presence or absence of ballooning in rodent models is subjective and subject to inter-observer variation.[22,37,40] For this reason, we did not score for ballooning in diabetic ZDSD rats but we chose to score for hypertrophy instead. By week 20, diabetic ZDSD rats exhibited clear, mild to moderate (grade 1–2) hypertrophy characterised by abnormally enlarged hepatocytes with or without apparent steatosis.

Lipid peroxidation resulting from the accumulation of fat in the liver produces malonaldehyde, a potent activator of hepatic stellate cells, which produce collagen thus causing fibrosis. A prevalence of 80% NASH and 40% advanced fibrosis has been reported in T2DM patients.[41,42] The presence and degree of fibrosis in a T2DM-NASH patient is of critical clinical relevance as it dictates the long-term prognosis of the condition. From our findings, diabetic ZDSD rats showed periportal fibrosis with portal-to-portal bridging and little-to-no centrilobular fibrosis. The zonal distribution of fibrosis we observed in diabetic ZDSD rats is similar to that reported for other rodent models of NASH.[10] The presence of the bridging, borderline cirrhotic fibrosis in the ZDSD model is a very relevant finding and further strengthens its suitability as a model for studying the pathophysiology and treatments outcomes in advanced T2DM-related NASH. Advanced fibrosis and cirrhosis usually precede the development of hepatocellular carcinoma.

Serum ALT levels have historically played a predictive role in the diagnosis of NASH. Although there was a tendency to increase in the serum levels of liver enzymes, we observed no statistical significance in the serum levels of ALT in diabetic ZDSD rats compared to SD controls. Additionally, serum levels of bilirubin and albumin were not significantly changed. However, we observed an isolated elevation of serum levels of ALP. It is not uncommon (even clinically) for patients to develop NASH without apparent derangement of ALT[43,44] or with isolated ALP elevation.[45] Although not entirely produced in the liver, ALP is an important surrogate marker of liver function in NASH.[46] Overall, with a limiting sample size like ours, it is very difficult to make solid conclusions on liver function tests in the ZDSD rat model. Limitations of sample size coupled with variability in the degree of manifestation of diabetes and NASH in the ZDSD rats may have played a part in the lack of statistical difference observed between liver function tests of diabetic ZDSD and control SD rats.

Conclusion

We have identified a model, which shows great potential as an experimental model for studying the pathophysiology of T2DM-related liver disease. Understanding the pathogenesis of T2DM-related NASH is clinically important given its prevalence and contribution to morbidity and mortality in both developed and developing regions of the world today. We have shown that the ZDSD rat develops both diabetes and non-alcoholic steatohepatitis; mirroring the development of these diseases in humans. In addition to the metabolic derangement (hyperglycaemia and hyperlipidaemia), the livers of diabetic ZDSD rats show classical histopathological patterns of NASH (steatosis, inflammation, hypertrophy and fibrosis) which may progress to cirrhosis. The ZDSD rat therefore may be the much-needed "many diseases one-rat" model for characterising the pathophysiological mechanisms underlying the development T2DM-related NASH. The model could also be more useful than

existing rodent models for testing the efficacy of T2DM-related NASH treatments.

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Conflict of interest

The authors declare no conflict of interest.

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