Severe Insulin Resistance and Moderate Glomerulosclerosis in a Minipig Model Induced by High-Fat/ High-Sucrose/ High-Cholesterol Diet

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Abstract: To develop a minipig model of type 2 diabetes that simulates the common manifestations of the metabolic abnormalities and resembles the kidney pathology of type 2 diabetes in the human population, male Chinese Bama minipigs were divided into 2 groups (5 in each) and fed with a control diet (CD) or high-fat/ high-sucrose/ high-cholesterol diet (HFSCD) for 5 months. The biochemical parameters of blood and urine, and the oral glucose tolerance test were monitored after the feeding program. The insulin resistance was estimated by the HOMA-IR index and the glucose elimination constant (K_G), and beta-cell function by the HOMA-beta index and the acute insulin response (AIR). Glomerulosclerosis index (GSI) was semi-quantitated by the degree of glomerular lesions in kidney sections stained with Masson trichrome. Extracellular matrix deposition in the kidney was examined by the protein expression of type IV collagen, connective tissue growth factor (CTGF) and matrix metalloproteinases 2 (MMP-2) using immunohistochemistry. Feeding HFSCD to minipigs markedly caused hyperglycaemia, hyperinsulinaemia and dyslipidaemia. HOMA-IR was significantly increased while HOMA-beta, AIR and K_G were obviously decreased in the HFSCD group compared with control group. Microalbuminuria, glucosuria and moderate glomerulosclerosis were exhibited in HFSCD-fed minipigs. The expression of type IV collagen and CTGF was elevated whereas that of MMP-2 was reduced in the kidneys of HFSCD group compared with the CD group. We concluded that feeding HFSCD to Chinese Bama minipigs for 5 months can induce humanoid type 2 diabetes and early-stage diabetic nephropathy, and accelerate extracellular matrix deposition and glomerulosclerosis.

Key words: diabetic nephropathy, diabetogenic diet, extracellular matrix, minipig model, type 2 diabetes

Introduction

Although type 2 diabetes is ten times more prevalent than type 1 diabetes, the progression of renal disease in type 2 diabetes has been studied less intensively and is not as well understood. Type 2 diabetes, unlike type 1 diabetes, is a more insidious disorder. Peripheral insulin resistance exacerbates and blood insulin increases to compensate for the insulin resistance. Hyperglycaemia and islet beta-cell degeneration take place progressively. Microalbuminuria may occur during the phase of insulin resistance. However, for ethical reasons, the pathogenesis of diabetic nephropathy during this phase cannot be studied with biopsies. In view of the incompletely understood pathogenesis, additional studies on nephropathy related to type 2 diabetes are required. Most animal models do not fulfill study requirements, since they are based on monogenic disorders of little relevance to human diabetes or on chemical destruction of beta-cells, which is also less clinically relevant [19]. It has been shown by several groups that type 2 diabetes can be induced in the mouse [19], rat [5], hamster [16] and minipigs [8, 20] by diabetogenic diet enriched in high-fat and high-energy. High saturated fat also induced type 2 diabetes and diabetic nephropathy in C57BL/6J mice [17] and Golden Syrian hamsters [12]. Diet-induced hypercholesterolaemia developed renal injury in rats and domestic crossbred pigs [6, 18]. These models are more similar to the human condition and can be used in studies on the pathophysiology of type 2 diabetes and renal complications.

To our knowledge, the current study is the first to demonstrate that a high-fat/ high-sucrose/ high-cholesterol diet induces renal structural and functional changes similar to human type 2 diabetes in Chinese Bama minipigs. Chinese Bama minipigs are a characteristic strain of miniature pigs in China which have been produced by inbreeding of half-siblings, resulting in the animal's characteristic small size, stable genetics and uniform phenotype of "two black ends" in which the fur of the head and rump is black and that of the rest of the body is white.

Materials and Methods

Animal and diets

Ten male Chinese Bama miniature pigs, 3 months of

age, were obtained from the Laboratory Animal Center of the Third Military Medical University (Chongqing, China). A cohort of animals was randomized into 2 groups of similar body weight: n=5 in the normal control diet (CD) group; n=5 in the high-fat/ high-sucrose/ high-cholesterol diet (HFSCD) group. The HFSCD used in this study was 51% normal miniature pig's diet supplemented with 10% lard, 37% sucrose and 2% cholesterol [20]. On a caloric basis, the HFSCD consisted of 38.26% fat from lard, 53.52% carbohydrate, and 8.22% protein (total 21.55 MJ/kg), whereas the normal diet contained 10.35% fat, 68.99% carbohydrate, and 20.66% protein (total 13.13 MJ/kg). The amount of daily fodder was 4% weight of minipigs which were fed three times daily with either CD or HFSCD. Water was available *ad libitum*. Body weights were recorded every two weeks. The total study period was 5 months. All experiments were performed according to the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

Biochemical assays

Blood samples were obtained from the orbital sinus of the animals at the end of each month after an overnight fast, and the plasma specimens were used for measuring glucose, insulin, triglycerides, total cholesterol, creatinine, blood urea nitrogen (BUN) and N-acetyl-beta-glucosaminidase (NAG). Glucose, triglycerides and total cholesterol were determined by enzymatic kits (Rongsheng Biotech., Shanghai, China). Insulin was assayed by a radioimmunoassay kit (China Institute of Atomic Research, Beijing, China).

The homeostatic model assessment (HOMA) is widely used to assess longitudinal changes in beta cell function and insulin resistance (IR) in order to examine the natural history of diabetes. The following equations are used in HOMA: Homa-beta = $(20 \times \text{FINS}) / (\text{FPG} - 3.5)$ [15], Homa-IR = $(\text{FPG} \times \text{FINS}) / 22.5$ [2] for beta cell function and IR, respectively, where FINS is the fasting plasma insulin concentration (U/l) and FPG is fasting plasma glucose (mmol/l).

Randomly obtained urine samples in the morning were used for determination of glucose, microalbumin, creatinine, BUN and NAG at months 0, 2, 4 and 5. Urinary microalbumin was determined using a turbidatex-based immunoassay. Glucose in the urine was assayed by the hexokinase method [7] (albumin

and glucose kits from Mingdian Bio., Shanghai, China). Plasma and urinary creatinine, BUN and NAG were measured using the Jaffe, Fearon and PNP methods, respectively (kits from Jianchen Biotech., Nanjing, China). The urinary glucose/creatinine ratio, albumin/creatinine ratio [11] and NAG/creatinine ratio [1] were calculated. The NAG clearance, expressed as per cent of creatinine clearance, was calculated according to the formula: (urinary NAG/plasma NAG) $\times$ (plasma creatinine/urinary creatinine) $\times 100$.

Oral glucose tolerance test (OGTT)

For assessment of glucose tolerance, an OGTT was performed on the minipigs at monthly intervals. After an 18-h overnight fast, animals were given 2 g/kg D-glucose by gavage through a gastric tube which was inserted into the stomach. Blood samples were obtained to determine plasma glucose and insulin *via* the auricular veins at 0, 15, 30, 60, 90, and 120 min after glucose administration.

In the OGTT, the acute insulin response (AIR) was estimated as the mean of the increase in plasma insulin at 15 min above the baseline, and the glucose elimination was quantified as K_G (*i.e.*, the glucose elimination constant), the slope of the natural logarithm of the glucose values at 15, 30 and 60 min after the glucose load, and expressed as percent elimination of glucose per minute [1].

Pancreatic islet histopathology

To examine the effect of the diet on pancreatic islets, samples of pancreas were fixed in phosphate-buffered 10% formalin (pH 7.4) for 24 h, paraffin-embedded, sectioned (4 μ m) and stained with hemotoxylin and eosin (H&E).

Kidney histopathology and semiquantitation

Each left kidney removed from anesthetized minipigs was weighed and the kidney weight index (left kidney weight/body weight ratio, g/kg) was calculated at the end of month 5. Left kidneys were sliced transversely in the same place, fixed in 10% formaldehyde in 0.1 mol/l PBS (pH 7.4), embedded in paraffin, and sectioned at 4 μ m. Sections stained with H&E and Masson trichrome were used to evaluate the general histological changes in glomerular structure.

In kidney sections stained with Masson trichrome, at

least 50 glomeruli from each minipig were randomly examined. The degree of lesions in each glomerulus was graded on a scale of 0 to 4+ by the method of Saito *et al* [13]. Grade 0 represents a normal glomerulus; grade 1, 1–25% loss of capillaries in the tuft (minimal sclerosis); grade 2, 26–50% loss (moderate sclerosis); grade 3, 51–75% loss (moderate-severe sclerosis) and grade 4, 75–100% of the tuft sclerosed (severe sclerosis). An average score (glomerulosclerosis index, GSI) was calculated for each animal. Glomerulosclerosis was defined as GBM thickening, mesangial hypertrophy, hyalinosis, and capillary occlusion [4].

All sections were examined by technicians without prior knowledge of the experimental protocol.

Immunohistochemistry

Kidney sections were evaluated immunohistochemically using the EliVision™ plus horseradish peroxidase (HRP) System (Maixin Bio., Fuzhou, China). Briefly, deparaffinized and hydrated sections were pretreated with 0.1% trypsin for 30 min at room temperature. After quenching the endogenous peroxidase by immersion in 3% hydrogen peroxide for 10 min, sections were incubated at 4°C overnight with polyclonal antibodies against type IV collagen, connective tissue growth factor (CTGF) or matrix metalloproteinases 2 (MMP-2) (above primary antibodies from Boster Bio., Wuhan, China) diluted to 1:100 each. Then, the sections were incubated with polymer enhancer for 20 min and with polymerized HRP-labeled goat anti-rabbit IgG for 30 min at room temperature. The antigens were visualized using 3, 3'-diamino-benzidine chromogen solution. Finally, tissue sections were counterstained with hematoxylin, dehydrated and coverslipped for viewing. For all studies, negative controls were included with substitution of the primary antibodies with 10% non-immune goat serum.

Statistical analysis

All values are expressed as mean $\pm$ SD. HOMA scores, urine glucose/creatinine ratio, albumin/creatinine ratio and NAG/creatinine ratio were log-transformed to approximate a normal distribution. Quantitative data were assessed by Student's *t*-test and semi-quantitative data were compared by Wilcoxon tests. Significance was accepted at the $P < 0.05$ level.

Results

Effect of HFSCD on homeostasis

Figure 1 shows that the body weights were similar in the two groups at the beginning of the experiment (CD 6.04 ± 1.24 kg vs. HFSCD 6.04 ± 2.13 kg), and linearly elevated with feeding duration. The weight gain was significantly higher in the HFSCD-fed minipigs than in CD-fed minipigs at week 18 and week 20 ($P < 0.01$).

As shown in Table 1, plasma glucose concentrations in the HFSCD group were linearly elevated with the time reaching 10.27 ± 2.25 mmol/l at month 5, which was 2.15 folds higher than that of the control group.

Insulin levels of HFSCD-fed minipigs rapidly elevated at month 2 and continued to increase to a peak of 24.43 ± 3.55 U/l at month 4 in the HFSCD group, and dropped off thereafter.

Plasma triglyceride and total cholesterol concentrations were significantly increased from the first month of the experimentation, and the significant increase persisted for the study period in miniature pigs fed the HFSCD compared with those fed the control diet.

Effect of HFSCD on glucose disposal and insulin secretion

Table 2 shows the results of AIR and K_G in the two groups of minipigs for OGTT performed every month. At 3 months after the start of HFSCD, miniature swine were already glucose intolerant and had impaired glucose-stimulated insulin secretion. Glucose elimination,

as estimated by K_G , was $2.43 \pm 0.58\%/min$ in CD-fed pigs versus only $1.04 \pm 0.26\%/min$ in HFSCD-fed pigs ($P < 0.05$). Similarly, AIR was markedly lower in HFSCD-fed pigs (24.39 ± 6.17 U/l) than in control diet-fed pigs (43.73 ± 8.40 U/l; $P < 0.05$). From the glucose clearance curve at the 5th month of the experiment, the plasma glucose level was highest at 30 min and rapidly declined, approaching the starting values at 120 min for pigs of the CD group; hence, glucose removal for these pigs was efficient and rapid. In contrast, there was no glucose elimination but a gradual increase between 15 and 60 min in the glucose curve of the HFSCD group (HFSCD, K_G , $-7.38 \pm 1.36\%/min$; CD, $1.95 \pm 0.21\%/min$), which suggests deficient glucose removal and severe glucose intolerance (Fig. 2a). The curve of plasma insulin levels exhibits that AIR to the

Fig. 1. Changes of the body weight in HFSCD- and CD-fed minipigs during the 5-month experimental period. Values are mean $\pm$ SD. (# $P < 0.01$ vs. CD group).

Table 1. Effects of HFSCD on glucose and lipid metabolism parameters in Chinese Bama minipigs (n=5)

Parameters	0	1 month	2 months	3 months	4 months	5 months
Plasma glucose (mmol/l)						
CD	4.71 $\pm$ 0.39	4.24 $\pm$ 0.90	4.83 $\pm$ 0.87	5.24 $\pm$ 1.00	4.65 $\pm$ 0.71	4.77 $\pm$ 0.57
HFSCD	5.12 $\pm$ 0.84	5.97 $\pm$ 0.73*	6.88 $\pm$ 0.57*	8.89 $\pm$ 2.05#	9.54 $\pm$ 2.12#	10.27 $\pm$ 2.25#
Plasma insulin (U/l)						
CD	6.97 $\pm$ 0.33	7.31 $\pm$ 0.94	7.50 $\pm$ 0.93	8.68 $\pm$ 0.82	7.25 $\pm$ 0.80	7.65 $\pm$ 1.33
HFSCD	7.47 $\pm$ 1.16	9.12 $\pm$ 1.63	15.12 $\pm$ 3.22*	21.16 $\pm$ 5.08*	24.43 $\pm$ 3.55#	17.43 $\pm$ 3.80#
Plasma triglycerides (mmol/l)						
CD	0.59 $\pm$ 0.04	0.59 $\pm$ 0.06	0.63 $\pm$ 0.10	0.69 $\pm$ 0.16	0.71 $\pm$ 0.15	0.73 $\pm$ 0.13
HFSCD	0.59 $\pm$ 0.05	0.94 $\pm$ 0.07#	1.50 $\pm$ 0.18#	1.87 $\pm$ 0.14#	2.03 $\pm$ 0.42#	2.10 $\pm$ 0.32#
Plasma total cholesterol (mmol/l)						
CD	2.06 $\pm$ 0.34	1.99 $\pm$ 0.33	2.01 $\pm$ 0.35	1.68 $\pm$ 0.21	1.82 $\pm$ 0.49	2.07 $\pm$ 0.46
HFSCD	2.31 $\pm$ 0.44	6.24 $\pm$ 1.77#	9.06 $\pm$ 1.44#	11.20 $\pm$ 2.02#	15.23 $\pm$ 2.00#	20.45 $\pm$ 2.83#

Values are means $\pm$ SD; * $P < 0.05$ HFSCD vs. CD; # $P < 0.01$ HFSCD vs. CD.

Fig. 2. Effects of the high-fat/high-sucrose/high-cholesterol diet fed to minipigs for 5 months on the levels of glucose (2a) and insulin (2b) in response to oral glucose loading examined by the oral glucose tolerance test. (2a) Pigs from the HFSCD group exhibited marked glucose intolerance compared with those from the CD group. (2b) The deficient glucose removal seen in the HFSCD group could be caused by impairment of acute insulin secretion in response to the glucose load. Values are mean $\pm$ SD. (* P <0.05, # P <0.01, HFSCD vs. CD)

oral glucose challenge was blunted after HFSCD feeding (1.51 ± 0.32 U/l) compared with after control diet feeding (32.71 ± 6.16 U/l; P <0.01), and the insulin secretion plateau was delayed, indicating that HFSCD feeding induced beta cell dysfunction in minipigs (Fig. 2b).

HOMA-IR indices were significantly higher in the HFSCD group than in the CD group after 2 months feeding, whereas HOMA-beta indices for HFSCD group were only decreased at the end of month 5 (Table 2).

Effect of HFSCD on pancreatic islet

The majority of pancreatic islets in the HFSCD group were normal as in the control group (Fig. 3B, $\times 400$). A few pancreatic islets in the HFSCD group became atrophied with a loss in number of the islets and cells within them (Fig. 3A, black arrow, $\times 400$). No inflammatory cell infiltration was observed in the pancreas of either of the two groups.

Effect of HFSCD on renal function

As shown in Table 3, the urinary glucose/creatinine ratio and albumin/creatinine ratio were significantly increased at the 4th month of HFSCD-feeding (P <0.05). Urinary NAG: creatinine ratio and NAG clearance in the HFSCD group were higher (about 1.73- and 1.69-fold, respectively) than those in the CD group only at month 5 of the study. No significant changes were observed in creatinine and BUN levels of plasma and

Table 2. Effects of HFSCD on glucose tolerance and beta cell function in Chinese Bama minipigs (n=5)

Indices	0	1 month	2 months	3 months	4 months	5 months
HOMA-IR						
CD	1.38 ± 0.18	1.54 ± 0.50	1.63 ± 0.47	1.97 ± 0.63	1.51 ± 0.32	1.64 ± 0.45
HFSCD	1.59 ± 0.26	2.29 ± 0.39	$4.38 \pm 1.05^*$	$7.04 \pm 1.05^\#$	$10.40 \pm 1.75^\#$	$7.83 \pm 1.63^\#$
HOMA-beta						
CD	168.54 ± 30.67	128.14 ± 25.69	176.19 ± 14.78	170.89 ± 17.16	171.20 ± 10.12	136.52 ± 21.55
HFSCD	130.61 ± 36.44	85.94 ± 21.94	102.50 ± 24.72	111.30 ± 26.31	87.63 ± 17.65	$56.48 \pm 11.48^*$
AIR (U/L)						
CD	48.61 ± 5.72	49.57 ± 6.81	46.52 ± 7.77	43.73 ± 8.40	40.26 ± 6.63	32.71 ± 6.16
HFSCD	49.24 ± 5.85	48.63 ± 7.04	35.83 ± 5.82	$24.39 \pm 6.17^*$	$13.01 \pm 3.17^\#$	$1.51 \pm 0.32^\#$
K_G (%/min)						
CD	2.54 ± 0.75	2.45 ± 0.66	2.52 ± 0.61	2.43 ± 0.58	2.37 ± 0.56	1.95 ± 0.21
HFSCD	2.69 ± 0.70	2.41 ± 0.58	1.38 ± 0.47	$1.04 \pm 0.26^*$	$-3.26 \pm 0.78^\#$	$-7.38 \pm 1.36^\#$

Values are means $\pm$ SD; * P <0.05 HFSCD vs. CD; # P <0.01 HFSCD vs. CD.

AIR is the acute insulin response. K_G is the glucose elimination constant.

Fig. 3. Effects of the high-fat/high-sucrose/high-cholesterol diet fed to minipigs for 5 months on the morphology of pancreatic islets. A few pancreatic islets from the HFSCD group became atrophied with a loss in the number of the islets and cells within them (Fig. 3A, black arrow, in HE staining, $\times 400$) compared with control group (Fig. 3B, $\times 400$). No inflammatory cell infiltration was observed in the pancreas of either of the two groups.

urine between HFSCD and CD pigs.

Effect of HFSCD on renal morphological changes

There was no significant difference in the left kidney weight index between the two groups (CD, 3.42 ± 0.51 g/kg vs. HFSCD, 2.89 ± 0.71 g/kg, $P=0.259$). Histological analysis of the kidneys in HFSCD-fed minipigs with HE-staining revealed that the predominant form of glomerular alteration is hypertrophy with hypercellularity (black arrow) and focal segmental glomerulosclerosis with destroyed structure of the glomerular capsule (white arrow) (Fig. 4A, $\times 400$). Some distal tubules and macula densae were vacuolated and degenerated, accompanied with inflammatory cell infiltration. Masson trichrome staining of the kidneys of the HFSCD group showed an overt increase in the mesangial area and proliferation of collagen fiber (Fig. 4C, blue dye, $\times 400$). There were no obvious changes in renal histology of CD-fed minipigs (Fig. 4B, 4D, $\times 400$). The grade of the glomerulosclerosis index was statistically significantly higher in the HFSCD group than that in the CD group (1.40 ± 0.21 vs. 0.45 ± 0.14 , $P=0.001$), representing on average, moderate damage to all glomeruli (Table 4).

Type IV collagen, a major component of basement membranes and extracellular matrix, was overexpressed in the glomeruli and tubulointerstitium of the HFSCD pigs (Fig. 4E, $\times 400$, brown staining, same as below) compared with the CD pigs (Fig. 4F, $\times 400$). Tubular and glomerular expression of CTGF was increased in

the HFSCD pigs (Fig. 4G, $\times 600$) in comparison with the CD pigs (Fig. 4H, $\times 400$). Immunoreactivity of MMP-2 showed a significant decrease in the HFSCD group (Fig. 4I, $\times 400$) *vis-à-vis* the CD group (Fig. 4J, $\times 400$).

Discussion

This study characterizes the high-fat/high-sucrose/high-cholesterol diet-fed minipig as a robust model for impaired glucose tolerance (IGT) and early-stage type 2 diabetes-associated nephropathy. In the present study the data for Chinese Bama minipigs show that HFSCD results in: (1) increased body weight gain; (2) stable hyperglycemia but progressive hyperinsulinemia with significantly increased HOMA-IR index but reduced glucose elimination, indicating progressive worsening of insulin resistance; (3) damaged islet structure and impaired insulin secretion by the results of HOMA-beta index and OGTT (particularly AIR), suggesting beta-cell dysfunction; (4) early-onset alteration of renal function including microalbuminuria, glucosuria and elevated excretion of urinary NAG, and excessive deposition of extracellular matrix inducing moderate glomerulosclerosis.

The high-fat/high-sucrose/high-cholesterol diet caused a number of perturbances of homeostasis in miniature swine including a progressive increase in fasting plasma glucose and insulin concentrations accompanied by augmented plasma total cholesterol and triglycer-

Table 3. Effects of HFSCD on renal function in Chinese Bama minipigs (n=5)

Parameters	0	2 months	4 months	5 months
Urinary glucose/creatinine ratio (mmol/mmol)				
CD	0.0365 ± 0.0057	0.0397 ± 0.0098	0.0411 ± 0.0054	0.0408 ± 0.0078
HFSCD	0.0378 ± 0.0066	0.0429 ± 0.0085	0.0625 ± 0.0079*	0.0662 ± 0.0107#
Urinary albumin/creatinine ratio (mg/g)				
CD	12.26 ± 2.54	12.77 ± 3.33	13.52 ± 4.67	12.60 ± 3.27
HFSCD	12.73 ± 3.14	14.16 ± 3.53	25.13 ± 4.65*	48.08 ± 11.77#
Urinary NAG/creatinine ratio (U/g)				
CD	7.15 ± 2.27	6.37 ± 1.91	6.10 ± 1.87	6.28 ± 1.58
HFSCD	6.44 ± 1.87	6.47 ± 1.35	7.05 ± 1.64	10.87 ± 1.71#
NAG clearance				
CD	6.27 ± 1.55	7.12 ± 2.27	7.73 ± 1.62	8.69 ± 1.29
HFSCD	7.93 ± 1.37	6.51 ± 1.72	9.86 ± 2.11	14.72 ± 3.39*
Plasma creatinine (μ mol/l)				
CD	28.56 ± 5.54	28.97 ± 7.44	28.81 ± 6.02	29.69 ± 3.81
HFSCD	26.67 ± 6.53	27.71 ± 6.36	30.76 ± 8.91	33.38 ± 9.10
Urinary creatinine (mmol/l)				
CD	3.77 ± 0.53	4.08 ± 0.84	4.13 ± 1.00	4.43 ± 0.87
HFSCD	4.05 ± 0.72	4.20 ± 0.92	4.08 ± 0.76	4.71 ± 0.69
Plasma BUN (mmol/l)				
CD	5.89 ± 1.05	6.20 ± 1.77	6.31 ± 1.36	6.57 ± 1.24
HFSCD	6.06 ± 1.10	6.35 ± 1.07	6.55 ± 1.87	6.79 ± 1.58
Urinary BUN (mmol/l)				
CD	206.67 ± 32.36	220.46 ± 39.23	217.45 ± 32.31	231.03 ± 40.47
HFSCD	221.74 ± 40.38	215.35 ± 41.62	238.62 ± 61.24	223.49 ± 50.13

Values are means ± SD; * $P < 0.05$ HFSCD vs. CD; # $P < 0.01$ HFSCD vs. CD.

NAG is N-acetyl-beta-glucosaminidase. BUN is blood urea nitrogen.

ides. The results of AIR and K_G from OGTT revealed an obvious suppression of insulin secretion and marked deterioration of glucose elimination at 3 months after the start of HFSCD. Moreover, HOMA-IR indices significantly increased in the HFSCD group from the second month, which longitudinally reflects aggravated insulin resistance, whereas HOMA-beta indices were obviously decreased only at month 5, when the level of fasting insulin tended to drop off. Thus, it is tempting to speculate from these observations that after feeding HFSCD, exogenous hyperglycaemia and dyslipidaemia cause a progressive decline in insulin sensitivity at an early stage. Subsequently, insulin secretion increases gradually to compensate for insulin resistance and hyperglycaemia, and then beta-cell failure results in the reduction of baseline insulin due to maladaptation of the beta cells caused by glucotoxicity and lipotoxicity. Hence, the model shows two important mechanistic

characteristics of IGT and type 2 diabetes: insulin resistance and islet dysfunction, which is very similar to the human pathophysiology of type 2 diabetes [3].

In this study, we also show that the model is suitable for study of diabetic nephropathy because humans and pigs share important similarities in the renal anatomy, physiology and pathophysiology [14]. The excretion of urinary albumin in HFSCD pigs, which is a parameter of glomerular and tubular alterations, increased after 4 months of feeding with HFSCD. Measurement of the protein:creatinine ratio in a single morning urine specimen is an excellent alternative to measurement of protein in 24-h urine collection [11]. As a marker of tubular injury, we assessed the total urinary activity of NAG, a lysosomal enzyme of tubular origin [1]. NAG activity was significantly higher in animals of the HFSCD group, indicating an initial malfunction of the proximal tubular epithelial cells. Creatinine and BUN

Fig. 4. Effects of the high-fat/high-sucrose/high-cholesterol diet fed to minipigs for 5 months on the renal morphology. Compared with the CD group (Fig. 4B, 4D), glomeruli of the HFSCD group showed hypertrophy with hypercellularity (black arrow), atrophy with destroyed structure of the glomerular capsule (white arrow) (Fig. 4A, in HE staining, $\times 400$), an expanded mesangial area and proliferation of collagen fiber (Fig. 4C, blue dye, Masson trichrome staining, $\times 400$). The protein expression of type IV collagen (brown staining, same as below) was increased in the glomerulus and tubulointerstitium in HFSCD-fed minipigs (Fig. 4E, $\times 400$) compared with that in control minipigs (Fig. 4F, immunostaining for type IV collagen, $\times 400$). More intensive expression of connective tissue growth factor (CTGF) in epithelial cells of Bowman's capsule and distal convoluted tubules were observed in the HFSCD group (Fig. 4G, $\times 600$) than in the CD group (Fig. 4H, immunostaining for CTGF, $\times 600$), whereas the intensity of matrix metalloproteinase-2 (MMP-2) immunostaining was decreased in the glomerulus and tubulointerstitium of HFSCD-fed minipigs (Fig. 4I, $\times 400$) compared with that in control minipigs (Fig. 4J, immunostaining for MMP-2, $\times 400$).

in plasma and urine of HFSCD-fed pigs, which are important parameters in the assessment of end-stage renal failure, did not worsen within the experimental period as compared with the control group.

The present study characterized the morphological features of a diet-induced miniature swine model that exhibits hypertrophy of the glomeruli, proliferation of collagen fiber, expansion of the mesangial matrix, and moderate glomerulosclerosis. In addition, overexpression of type IV collagen occurred in glomeruli and tubulointerstitium in minipigs of the HFSCD group, implying that extracellular matrix proteins excessively accumulated in the mesangium and basement membrane of the glomeruli and in the renal tubulointerstitium. Regulation of extracellular matrix formation is a dynamic phenomenon involving synthetic and degradative processes. The former involves the induction of various growth factors such as CTGF [9]. CTGF is a crucial mediator for transforming growth factor-beta-stimulated extracellular matrix synthesis (e.g. type IV collagen, fibronectin and laminin) [9]. The latter involves matrix metalloproteinases (MMPs), the major factors that degrade extracellular matrix and display substrate specificity. MMP-1 and -13 degrade interstitial collagens, whereas MMP-2 and -9 degrade type IV collagen and fibronectin [10]. In our study, increased expression of type IV collagen and CTGF and decreased immunoreactivity of MMP-2 in HFSCD-fed pigs indicated that HFSCD might not only promote extracellular matrix deposition and renal fibrosis through increasing the production of fibrogenic factors such as CTGF but might also attenuate extracellular protein degradation and removal by inhibiting the expression of MMP-2. These pathological features in the kidneys of pigs are similar to those of human diabetic nephropathy [4].

In conclusion, we have shown here that the Chinese Bama miniature swine fed with high-fat/high-sucrose/high-cholesterol diet for 5 months is prone to insulin resistance and type 2 diabetes, and is a robust and valuable model for early-stage diabetic nephropathy. It may therefore be used both for studying the pathogenic features of diabetogenic diet and for investigating the pathogenesis of diabetic nephropathy. We also showed specifically that the diet increased the protein expression of CTGF, promoted excessive extracellular matrix accumulation, and suppressed extracellular protein degradation, eventually resulting in moderate

Table 4. Effect of HFSCD on glomerulosclerosis index in Chinese Bama minipigs (n=5)

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
CD	3	2	0	0	0
HFSCD	0	2	3	0	0

glomerulosclerosis in the minipig kidney.

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