

Research Article

Long-Term Type 1 Diabetes Enhances In-Stent Restenosis after Aortic Stenting in Diabetes-Prone BB Rats

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Type 1 diabetic patients have increased risk of developing in-stent restenosis following endovascular stenting. Underlying pathogenetic mechanisms are not fully understood partly due to the lack of a relevant animal model to study the effect(s) of long-term autoimmune diabetes on development of in-stent restenosis. We here describe the development of in-stent restenosis in long-term (~7 months) spontaneously diabetic and age-matched, thymectomized, nondiabetic Diabetes Prone BioBreeding (BBDP) rats ($n = 6-7$ in each group). Diabetes was suboptimally treated with insulin and was characterized by significant hyperglycaemia, polyuria, proteinuria, and increased HbA_{1c} levels. Stented abdominal aortas were harvested 28 days after stenting. Computerized morphometric analysis revealed significantly increased neointima formation in long-term diabetic rats compared with nondiabetic controls. In conclusion, long-term autoimmune diabetes in BBDP rats enhances in-stent restenosis. This model can be used to study the underlying pathogenetic mechanisms of diabetes-enhanced in-stent restenosis as well as to test new therapeutic modalities.

1. Introduction

In-stent restenosis (ISR) is the most common complication associated with coronary stenting and is histologically characterized by occlusive neointima formation. As yet, no adequate treatment modalities are available to treat or prevent development of ISR [1–3]. The use of drug-eluting stents has significantly reduced the incidence of ISR when compared with bare metal stents, but also resulted in increased rates of late stent thrombosis [4]. Diabetes mellitus (DM) has been associated with increased risk to develop (in-stent) restenosis both after percutaneous transluminal

coronary angioplasty as well as coronary stenting [5–14]. DM is a risk factor for ISR development after using both drug-eluting and bare metal stents as revealed by various meta-analyses (primarily including type 2 diabetic patients) [15–17]. The beneficial effects on ISR of drug-eluting stents over bare metal stents as observed in non-diabetic patients appear to be less clear in diabetic patients. Although the exact pathogenetic mechanism underlying increased ISR development in diabetic patients is as yet unknown, it at least appears to be due to an exaggerated neointimal response after coronary stent placement as determined by intravascular ultrasound [18]. Many studies thus favor for a deleterious

effect of DM on the development of ISR, although the precise magnitude of this effect is not clear. Especially age may act as a confounder as older age is associated with increased restenosis rates independent of DM [17].

To study the molecular and cellular mechanism(s) underlying DM-enhanced development of ISR, clinically relevant animal models may be of great value. However, despite the increasing numbers of diabetic patients worldwide together with coronary artery disease being a significant source of morbidity and mortality in these patients, relevant animal models to study ISR are scarce. Although the Zucker diabetic fatty (ZDF) rat has been used as a model to study type 2 DM-associated restenosis [19–21], a reliable model to study the long-term effects of type 1 diabetes on ISR development is still lacking. Whereas type 1 DM represents only 5–10% of all diabetics it may be associated with severe coronary artery disease at a relatively young age as demonstrated in a selected population of type 1 diabetics eligible for kidney and/or pancreas transplantation [22]. Various rodent models of type 1 DM have been used to study the effects of diabetes on mechanically induced restenosis in mice, rats, or rabbits, in which diabetes is chemically induced using streptozotocin or alloxan and in which contradicting results were obtained [23]. In addition to rodent models, also a streptozotocin-induced diabetes porcine model has been used to study the development of ISR. In this model, a high mortality rate (~45%), a relatively short course of diabetes (12 weeks), and most likely high costs may actually hamper extensive use of this model [24]. Both streptozotocin and alloxan are toxic compounds sharing structural similarities with glucose which explains their selective uptake in cells expressing the GLUT2 glucose transporter. As pancreatic β -cells have relatively high levels of GLUT2, streptozotocin and alloxan are relatively, but not solely, toxic to β -cells. Because of the possible toxic side effects of these chemicals and to model more accurately human type 1 (autoimmune) DM, the availability of a spontaneous type 1 DM model for the development of (in-stent) restenosis is needed. To this end, we here describe the use of inbred Diabetes Prone BioBreeding (BBDP/Wor) rats as a model to study diabetes-enhanced development of ISR. Inbred BBDP rats are derived from a Canadian colony of outbred Wistar rats (i.e., the BB Wistar rat) in which diabetes developed spontaneously in the 1970s [25]. BBDP/Wor rats develop autoimmune diabetes spontaneously due to severe lymphopenia and preferential lack of immunoregulatory T cells [26–28]. Using the rat abdominal aorta stenting model described previously [29], we tested the hypothesis that long-term suboptimally treated hyperglycaemic BBDP rats develop enhanced ISR compared with non-diabetic age-matched control rats.

2. Materials and Methods

2.1. Rats. Specified pathogen-free Diabetes Prone BB (BBDP/Wor) and Diabetes-Resistant (BBDR/Wor) rats were bred at the Central Animal Facility of the University Medical Center Groningen, University of Groningen, Groningen, The Netherlands. Original breeding stocks were obtained from

Biomedical Research Models (BRM Inc., Worcester, MA, USA). Rats were kept under clean conventional conditions and were fed standard rat chow and acidified water *ad libitum*. All animals received humane care in compliance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1996) and the Dutch Law on Experimental Animal Care.

2.2. Diabetes Development in BBDP Rats. In the BBDP/Wor colony maintained at the University Medical Center Groningen, 80–90% of the rats spontaneously develop diabetes from ~70 days of age. Rats were considered diabetic when blood glucose levels exceeded 15 mmol/L as measured in peripheral blood obtained by tail vein puncture and by using a glucose sensor (Accu-Chek Sensor Comfort, Roche Diagnostics Nederland B.V., Almere, The Netherlands). In this study, 9 recent onset diabetic BBDP rats were initially included (both male and female) which received a 1/2 (~3–4 mm length) Linplant sustained release insulin implant (LinShin Canada Inc, Toronto, ON, Canada) subcutaneously at diagnosis using a trocar. According to the manufacturer the implants had an estimated insulin release of ~1 U/24 h/(1/2) implant for >40 days. We aimed at maintaining the blood glucose values in insulin-treated diabetic rats between 15–20 mmol/L during the entire follow-up period (up to 10 months of age). During follow-up diabetic rats were weighed 2–3 times a week. In case of weight loss, blood glucose levels were measured as described above. When blood glucose levels exceeded 20 mmol/L or blood glucose levels were between 15–20 mmol/L in the presence of substantial weight loss, rats were reimplanted with an insulin implant as described above.

In addition to the insulin-treated diabetic BBDP rats also a control group of age-matched non-diabetic BBDP rats (initially $n = 9$) was included. Diabetes development was prevented by performing thymectomy at the age of 21 days as we described in detail elsewhere [30]. Weight and blood glucose levels of thymectomized non-diabetic BBDP rats were measured once every two weeks to obtain basal values.

2.3. HbA_{1c} Measurements. To determine whether long-term suboptimal insulin treatment of diabetic BBDP rats is associated with increased glycated hemoglobin (HbA_{1c}) levels indicative of poor glycaemic control, we analyzed HbA_{1c} levels in a separate cohort of long-term diabetic BBDP rats ($n = 5$), as well as in spontaneously protected non-diabetic BBDP rats ($n = 2$) and non-diabetic BBDR/Wor rats ($n = 6$). HbA_{1c} levels were determined in freshly obtained peripheral blood (tail vein puncture) using the BIO-RAD in2it A1C analyzer (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Values are expressed as DCCT- HbA_{1c}%.

2.4. Renal Function Measurements. Two weeks before stenting renal function of the long-term diabetic and non-diabetic BBDP rats was determined by housing the rats in individual, urine-collecting metabolic cages for 24 hours and by collection of blood plasma. Plasma and urine creatinine levels were determined using the enzymatic colorimetric

TABLE 1: Characteristics of the diabetic (DM) and thymectomized non-diabetic (non-DM) BBDP rats that were stented and evaluated for the development of in-stent restenosis.

	<i>N</i>	Gender ♀/♂	Diabetes onset (median in days)	Age at stenting (median in days)	Diabetes duration at stenting (median in days)	Serum creatinine ^a (mean ± SEM in μmol/L)
Non-DM BBDP	6	4/2	N.A. ^b	298 [270–412] ^c	N.A. ^b	51 ± 7
DM BBDP	7	3/4	82 [72–140] ^c	270 [270–290] ^c	198 [150–204] ^c	56 ± 9

^a Serum creatinine levels were determined 2 weeks prior to stenting.

^bN.A.: not applicable.

^cValues between brackets indicate minimum and maximum values.

assay CREA plus (Roche Diagnostics GmbH, Mannheim, Germany) [31]. Total urinary protein excretion was determined using the Roche Diagnostics Urinary/CSF Protein assay (Roche Diagnostics GmbH, Mannheim, Germany) [32, 33].

2.5. Stent Implantation. At a median diabetes duration of 28 weeks (7 months) 9 BBDP rats were stented in the abdominal aorta as described previously [29]. As a control, 9 age-matched, thymectomized non-diabetic BBDP rats were included. Briefly, under anesthesia (2% isoflurane (Abbott, Hoofddorp, The Netherlands), 0.4 L/min O₂ and 0.4 L/min N₂O) the abdominal cavity was opened. The aorta was dissected, and surrounding connective tissue was removed. Next, two vascular clips were placed onto the aorta distal to the renal arteries and proximal to the aortic bifurcation. A small incision was then made in the distal abdominal aorta, and the balloon catheter was inserted and inflated to 9 atm pressure to deploy a premounted 2.5 × 9 mm BeStent 2 bare metal stent (Medtronic-Bakken Research, Maastricht, The Netherlands). After deflation and removal of the balloon, the aortic incision was closed with a 9-0 suture. Reperfusion was established by removing the clips, and the abdomen was closed with 4-0 sutures. Starting 5 days before stenting until the end of the experiment, rats were fed chow containing 0.33 mg clopidogrel/gram chow (Plavix, Sanofi-Aventis, Gouda, The Netherlands) in order to prevent platelet aggregation and to mimic the human clinical setting. Four weeks after stenting, rats were anesthetized and heparinized systemically with 500 IU i.v. (Leo Pharma, Breda, The Netherlands). The stented aortas were harvested, fixed in 4% formaldehyde, and embedded in methyl metacrylate for further histological analysis. During stenting, 3 non-diabetic control BBDP rats died because of perforation of the aorta, resulting in an overall mortality of 17%. In addition, 2 stents from long-term diabetic BBDP rats were lost during histological processing. Overall, stents from 7 diabetic and 6 non-diabetic BBDP rats were included for histological analysis.

2.6. Quantification In-Stent Restenosis (ISR). To quantify the severity of ISR, computerized morphometric analysis was performed on Lawson (elastin) stained sections obtained from the proximal, middle, and distal parts of each stent. The neointimal area was calculated by measuring the total area within the internal elastic lamina and the remaining lumen

using an Olympus BX-50F4 microscope equipped with an Olympus c-3030 zoom digital camera and Olympus DP-Soft version 3.0 software (Olympus, Tokyo, Japan). Total surface neointima (in μm²) was then calculated by subtracting surface remaining lumen from the total surface area within the internal elastic lamina.

2.7. Quantification Vessel Injury. The mean vessel injury score was determined as described previously [29] using a method originally developed by Schwartz et al. [34]. Vessel injury at every stent strut within a cross-section (9–10 struts/cross-section) was determined based on the anatomic vessel structures penetrated by each strut. This value ranged from 0 (least injury) to 3 (most injury). For each cross-section the mean injury score was calculated. From each stent 6–8 cross-sections (taken from the proximal, middle, and distal parts of the stent) were analyzed. The total mean injury score was expressed as the mean of the injury scores of all cross-sections analyzed within one stent.

2.8. Statistical Analysis. Data are expressed as mean ± SEM. Differences between two groups were analyzed for statistical significance using an independent samples *t*-test. All *P* values were two-tailed, and a *P*-value <.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.00 for Windows (GraphPad Software Inc., USA).

3. Results

3.1. Glucose Homeostasis in Long-Term Diabetic BBDP Rats. Diabetes incidence in the BBDP colony maintained at the University Medical Center Groningen is 80–90%. Median age of diabetes onset in the BBDP rats (*n* = 7) that were stented and included for histological analysis was 82 days (Table 1). Insulin treatment using insulin-releasing implants resulted in an almost permanent hyperglycaemic state (blood glucose >8 mmol/L) in all diabetic rats. In individual rats blood glucose oscillations were observed that varied between 8 and 27 mmol/L. Depending on the blood glucose levels (>20 mmol/L or 15–20 mmol/L in the presence of substantial weight loss (>10% weight loss compared with previous measurement)), rats received an insulin implant. Time between reimplantations varied between 5 and 35 days. As non-diabetic age-matched controls, 6 thymectomized BBDP rats were included. Figure 1 shows the blood glucose

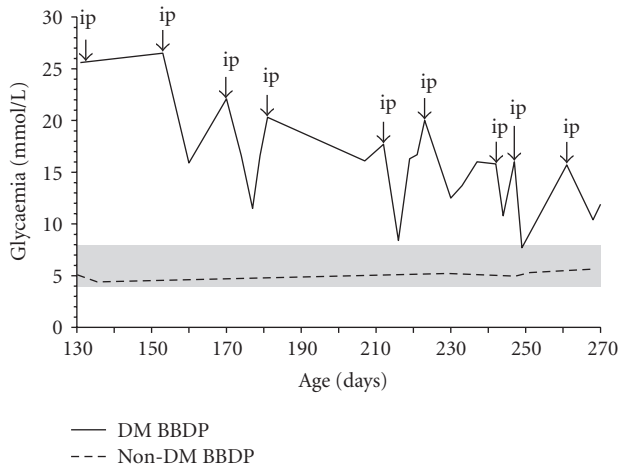


FIGURE 1: Oscillations in blood glucose levels in a representative diabetic (DM) BBDP rat treated with insulin pellets implanted subcutaneously. During a follow-up period of 140 days the DM BBDP rat received multiple insulin pellet implants (ips) guided by rapid development of hyperglycaemia (solid line) and drop in body weight (not shown). For comparison, glucose levels in an age-matched thymectomized non-DM BBDP rat are shown. The grey area indicates the normoglycaemic range (4–8 mmol/L).

oscillations observed in an individual diabetic and non-diabetic BBDP rat during a time frame of 140 days during which the diabetic rat received 9 insulin implants. The median age at stenting was 270 and 298 days for the diabetic and non-diabetic BBDP rats, respectively (not significantly different), translating in a median diabetes duration of 198 days (Table 1). During the total follow-up period till stenting mean blood glucose level after diabetes onset in diabetic BBDP rats was 15.0 ± 0.4 mmol/L compared with 5.3 ± 0.1 mmol/L in thymectomized non-diabetic BBDP rats (Figure 2(a), $P < .001$). As increased HbA_{1c} levels have been associated with increased cardiovascular risk in human diabetics, we questioned whether long-term diabetes in our BBDP rat model was also associated with increased HbA_{1c} levels. To this end, HbA_{1c} levels were determined in a separate group of long-term diabetic BBDP rats ($n = 5$) with a mean diabetes duration of 249 ± 50 days which was not statistically different from the mean diabetes duration in the stented BBDP rats (217 ± 7 days) at sacrifice. For comparison, HbA_{1c} levels were determined in spontaneously protected non-diabetic BBDP rats ($n = 2$) and non-diabetic BBDR/Wor rats ($n = 6$). As shown in Figure 2(b), long-term diabetes resulted in significantly increased HbA_{1c} levels (11.9 ± 0.2 DCCT-HbA_{1c}%) compared with age-matched non-diabetic BB rats (5.1 ± 0.1 DCCT-HbA_{1c}%).

3.2. Renal Function in Long-Term Diabetic BBDP Rats. Two weeks before stenting part of the long-term diabetic and non-diabetic BBDP rats were housed in metabolic cages for 24 hrs to collect urine. Long-term diabetes and the associated permanent hyperglycaemic state resulted in a 6-fold increased ($P < .001$) urinary volume compared with non-DM BBDP rats (Figure 3(a)). This was accompanied by severe

polydipsia (not shown). Furthermore, total urinary protein excretion (Figure 3(b), $P < .01$) and urinary creatinine excretion (Figure 3(c), $P < .001$) were significantly increased in long-term DM BBDP rats compared with non-DM BBDP rats. Increased urinary creatinine excretion in DM BBDP rats suggests the presence of glomerular hyperfiltration which is commonly observed in diabetics. Hyperfiltration was supported by the slightly increased creatinine clearance rates observed in the long-term DM rats (1.7 ± 0.3 mL/min in DM versus 1.0 ± 0.2 mL/min in non-DM, not significant). Despite the presence of proteinuria and hyperfiltration in long-term DM BBDP rats, plasma creatinine levels in these rats were similar to the levels detected in age-matched non-DM BBDP rats indicating preserved renal function in the presence of diabetes (Table 1). Histological analysis of PAS-stained renal sections did not reveal increased interstitial and glomerular matrix expansion in long-term DM BBDP rats (not shown).

3.3. Enhanced In-Stent Restenosis in Long-Term Diabetic BBDP Rats. In total, 18 rats (9 DM and 9 non-DM BBDP rats) received a bare metal stent (Figure 4(a)) in the abdominal aorta. During the stenting procedure, 3 non-DM BBDP rats died because of perforation of the aorta (mortality rate of 17%). Age of the DM and non-DM BBDP rats at stenting was similar between both groups (Table 1, median age, resp., 270 and 298 days, not significantly different). Diabetes duration (median) in DM BBDP rats at stenting was 198 days (Table 1). Stents were harvested 28 days after stenting. Stents from 2 DM BBDP rats were lost during histological processing. Finally, stents from 7 DM and 6 non-DM BBDP rats were histologically analyzed for the severity of ISR. At 28 days poststenting in both non-DM and DM BBDP rats development of ISR was detected which was characterized by neointima formation surrounding the stent struts. Figures 4(d), 4(e), 4(f), and 4(g) show representative photomicrographs of ISR in, respectively, non-DM and DM BBDP rats. Quantitative analysis revealed that long-term DM resulted in a 32% increase in surface neointima compared with non-DM BBDP rats (Figure 4(b), $P = .02$). This increase in neointima formation in DM BBDP rats was not associated with an increased mean injury score (Figure 4(c), $P = .957$).

4. Discussion

Diabetes mellitus (DM) is associated with increased risk for the development of in-stent restenosis (ISR) [15–17]. Underlying pathogenetic mechanisms are as yet unknown, and adequate treatment modalities are lacking. In order to increase our insights into the molecular and cellular mechanism(s) underlying type 1 DM-enhanced development of ISR a clinically relevant rodent model might be of great value. Yet a model as such is not available. We therefore tested the hypothesis that long-term spontaneously diabetic hyperglycaemic BBDP rats develop enhanced ISR and may be used as a suitable model to study the molecular and cellular mechanism(s) involved in DM-enhanced development of ISR.

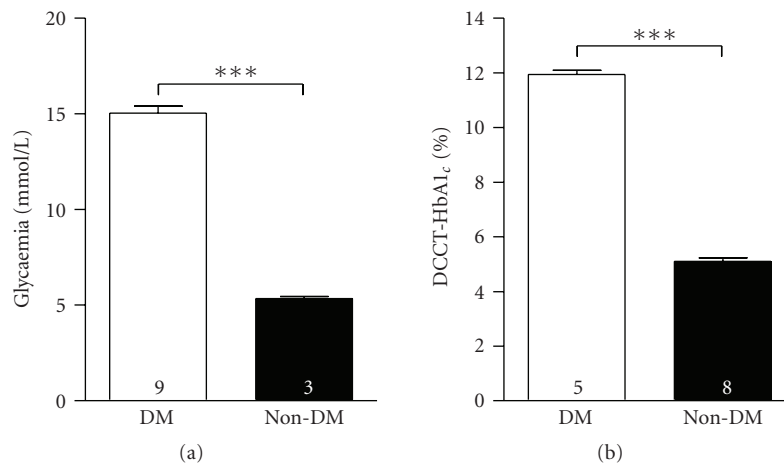


FIGURE 2: Blood glucose and HbA_{1c} levels are increased in long-term diabetic (DM) BBDP rats. (a) Average blood glucose level during the entire follow-up period starting at diabetes onset until sacrifice (ranging from 150 to >200 days) (white bar). For comparison, mean blood glucose levels in age-matched non-DM BBDP rats are shown (black bar). (b) Hyperglycaemia in long-term diabetic BBDP rats is associated with increased glycated haemoglobin (HbA_{1c}) levels (white bar) compared with non-DM rats (black bar). Data are expressed as the mean ± SEM (***) $P < .001$. Values within bars indicate the number of rats analyzed. DM: long-term diabetic BBDP rats, Non-DM: non-diabetic rats.

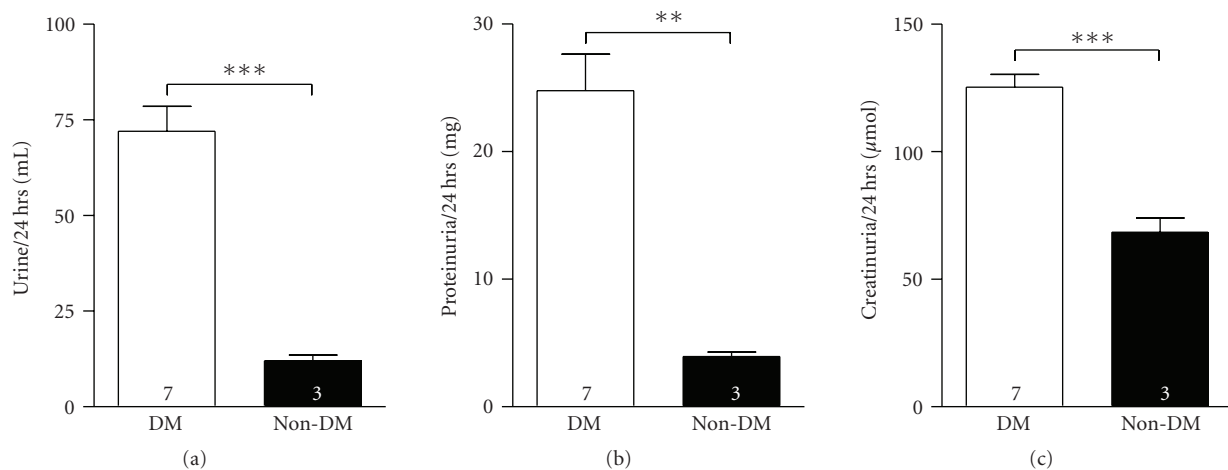


FIGURE 3: Long-term diabetes in BBDP rats is associated with significant polyuria (a), proteinuria (b), and creatinuria (c). Two weeks prior to stenting rats were housed in metabolic cages, and 24-hour urine samples were collected and analyzed as described in Section 2. Data are expressed as mean ± SEM (** $P < .01$, *** $P < .001$). Values within bars indicate the number of rats analyzed. DM: long-term diabetic BBDP rats, Non-DM: non-diabetic rats.

The hyperglycemic syndrome in BBDP rats develops spontaneously due to a disturbed balance in autoreactive and regulatory T cells [26–28]. The BBDP rat has been suggested to represent the best rodent model for human type 1 DM as in this model diabetes manifests during adolescence and involves an autoimmune disorder without the need of exogenous intervention.

To achieve suboptimally controlled DM with manifest hyperglycaemia and elevated HbA_{1c} levels, recent onset diabetic BBDP rats were treated with insulin using slow release insulin implants. By doing so, we were able to maintain DM BBDP rats in a rather permanent hyperglycaemic state for at least 8 months. The hyperglycaemic state was associated

with increased HbA_{1c} levels as well as polyuria, polydipsia, proteinuria, and glomerular hyperfiltration. Severity of proteinuria was within the range reported by Cohen et al. who also determined protein excretion in long-term DM BBDP rats that were treated with daily insulin injections [35]. As older age is associated with increased restenosis rates independent of DM [17], for our study it was important to include age-matched non-diabetic controls rather than prediabetic young BBDP rats. In order to prevent diabetes development in BBDP rats thymectomy was performed at the age of 21 days as we described previously [30]. DM and non-DM BBDP rats were followed for 7 months after which they received a bare metal stent implanted in the

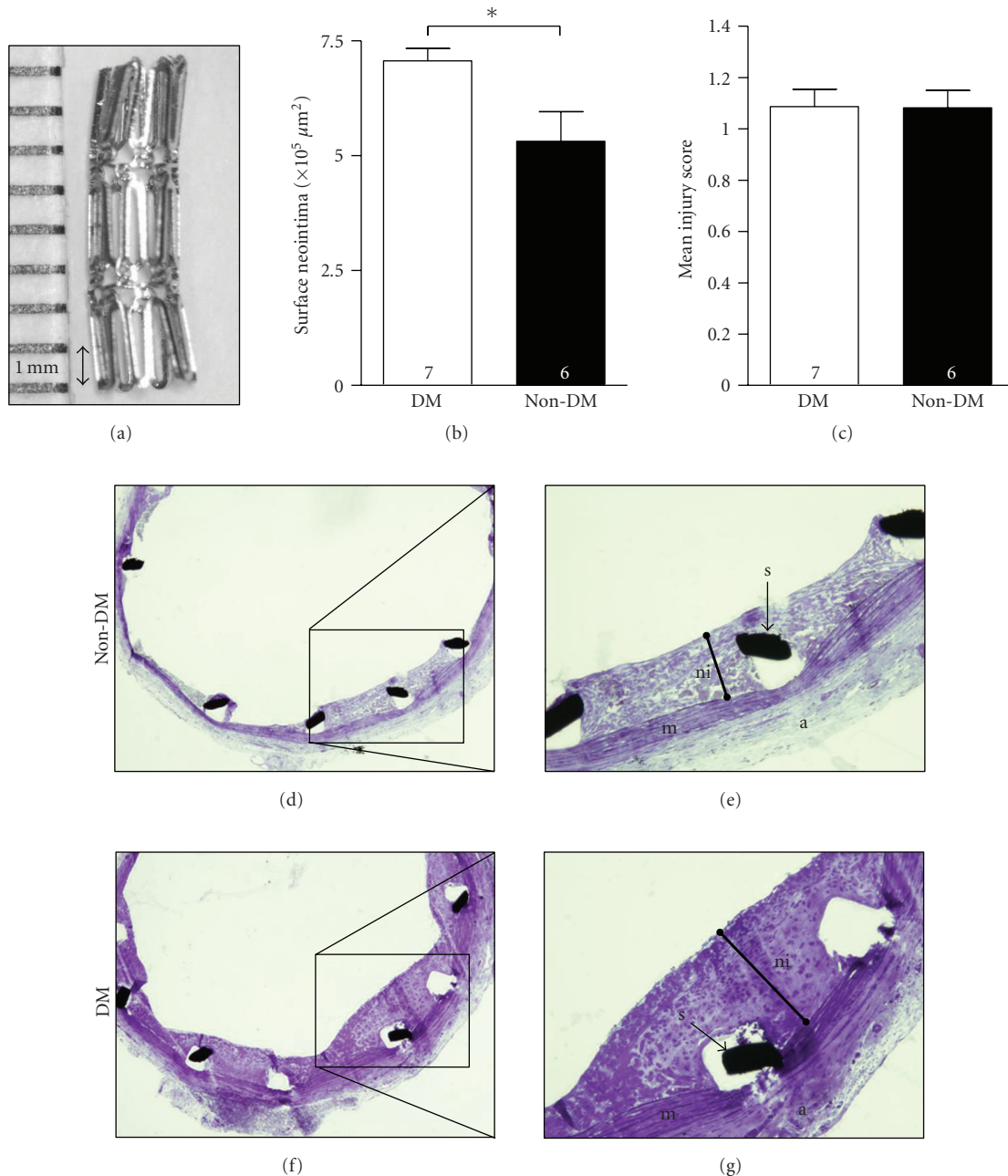


FIGURE 4: Long-term diabetes in BBDP rats is associated with enhanced in-stent restenosis 4 weeks after stenting in the abdominal aorta. (a) Photograph of a BeStent 2 bare metal stent used for implantation. The inflation balloon has been removed. (b) Surface area neointima present 28 days after stenting in long-term DM (white bar) and non-DM (black bar) BBDP rats. (c) Mean injury score at 28 days after stenting in long-term DM (white bar) and non-DM (black bar) BBDP rats. Data are expressed as the mean \pm SEM (* $P < .05$). Values within bars indicate the number of rats analyzed. DM: long-term diabetic BBDP rats, Non-DM: non-diabetic rats. ((d), (e)) Representative photomicrographs (Lawson staining) of stented abdominal aortas from a thymectomized non-diabetic (non-DM) BBDP rat 28 days after stenting. ((f), (g)) Representative photomicrographs (Lawson staining) of stented abdominal aortas from a diabetic (DM) BBDP rat 28 days after stenting. (e) and (g) are high-power magnifications (magnification $\times 100$) of the framed areas shown in, respectively, (d) and (f) (magnification $\times 40$). a: adventitia; m: media; ni: neointima; s: stent strut.

abdominal aorta. Our results clearly demonstrated that long-term DM significantly enhanced the development of ISR by 32% compared with non-DM age-matched thymectomized BBDP rats. This enhanced development of ISR in diabetic BBDP rats was not associated with increased vascular injury,

that is, increased penetration of the stent struts through the internal elastic lamina into the medial layer. The severity of ISR was previously shown to be positively correlated with the mean injury score [29]. However, in the same study we demonstrated differences in severity of ISR between bare

metal and sirolimus-eluting stents, which was independent of the mean injury score. Our data suggest that T1DM enhances the development of ISR by factors other than direct vascular injury.

The current study was performed as a proof of concept to demonstrate that long-term T1DM in BBDP rats indeed enhances the development of ISR following stenting in the abdominal aorta. Although we did not study the underlying mechanism(s) of enhanced ISR in diabetic BBDP rats yet, we suggest that long-term diabetes increases the proliferative and migratory capacity of medial and neointimal smooth muscle cells, thereby facilitating neointima formation [36, 37]. In addition, reduced endothelial repair capacity in diabetic BBDP rats might have contributed to enhanced ISR [38], but this needs to be determined.

The major advantage of the BBDP model to study the development of ISR over other T1DM rodent models is the fact that diabetes develops due to autoimmune-mediated destruction of pancreatic islets without having the toxic side effects of the use of streptozotocin or alloxan. By using slow release insulin implants we showed that long-term studies are feasible without the need of daily insulin injections. A possible weakness of the model is that BBDP rats are T cell lymphopenic and are, in that respect, not fully immunocompetent. However, despite T cell lymphopenia BBDP rats do develop autoimmune diabetes which is mediated by autoreactive T cells indicating that functional T cells are present in BBDP rats. Whether T cells are pivotal in the development of ISR is however unclear. Percutaneous transluminal coronary angioplasty has been shown to induce T cell activation in a small cohort of 10 patients with stable angina. Patients that developed restenosis had higher T cell activation levels than patients that did not develop restenosis [39]. However, treatment with the calcineurin inhibitor cyclosporine to prevent T cell activation did not reduce the development of restenosis in rabbits [40]. We therefore assume that the presence of T cell lymphopenia in BBDP rats has not been of major influence on the development of ISR.

5. Conclusions

The BBDP rat model for type 1 diabetes is suitable for studies on the long-term effects of hyperglycaemia on the development of ISR. Long-term diabetes significantly increased the development of ISR. To our opinion, future studies aiming at the identification of the molecular and cellular mechanisms involved as well as on testing the efficacy of novel therapeutic interventions in this model are warranted.

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