ORIGINAL ARTICLE

Long-term type 1 diabetes mellitus creates a pro-atherogenic environment conducive to early atherosclerosis in rats

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Abstract

Introduction: Experimental models are essential for clarifying the pathogenesis of atherosclerosis in the context of diabetes mellitus (DM). We aimed to evaluate the presence and the magnitude of several factors known to promote atherogenesis, and to assess the potential of a pro-atherogenic environment to stimulate the development of atherosclerotic lesions in a rat model of long-term type 1 DM. *Materials and Methods:* Six control and five DM Wistar rats were evaluated. DM was induced at 11 weeks of age using streptozotocin (STZ; 60 mg/kg, intraperitoneal). Animals were monitored up to 38 weeks of age, when plasma glucose, lipid profile, and markers specific for systemic inflammation, endothelial dysfunction, and oxidative stress were measured. The amount of fat within the aortic wall was assessed semiquantitatively using Oil Red O staining. *Results:* Diabetic rats presented significantly higher plasma glucose (p < 0.001), total cholesterol and triglycerides (both p = 0.02), high-sensitivity C-reactive protein (p = 0.01), and vascular endothelial growth factor (p = 0.04) levels, and significantly lower interleukin-10 (p = 0.04), superoxide dismutase (p < 0.01), and glutathione peroxidase (p = 0.01) levels than the control rats. Mild (grade 1) atherosclerotic lesions were observed in the aortic wall of 80% of the diabetic rats and in none of the control rats. *Conclusions:* This study presents a STZ-induced type 1 DM rat model with one of the longest follow-ups in the literature. In this model, long-term DM created a highly pro-atherogenic environment characterised by hyperglycemia, dyslipidemia, systemic inflammation, endothelial dysfunction, and oxidative stress that resulted in the development of early aortic atherosclerotic lesions.

Keywords: Atherosclerosis, experimental model, long-term follow-up, streptozotocin, type 1 diabetes mellitus

INTRODUCTION

Diabetes mellitus (DM), a complex metabolic disease caused by insulin deficiency or diminished effectiveness of endogenous insulin, is characterized by chronic hyperglycemia, disorders of glucose, lipid, and protein metabolisms. Globally, 8.5% of the population is affected by this disease, and it is estimated that by the year 2030, DM will become the seventh cause of death worldwide.[1] Between 5% and 10% of all diabetic cases are accounted for by type 1 DM, which results from selective destruction of pancreatic β-cells, leading to absolute insulin deficiency.[2]

Type 1 DM is linked with a multitude of chronic cardiovascular complications. Cardiovascular diseases have higher prevalence in diabetics, and type 1 DM is associated with an up to 10-fold increase in cardiovascular risk compared to the non-diabetic population of the same age.[3] Atherosclerosis, one of the macrovascular manifestations of DM, is the primary mechanism responsible for cardiovascular diseases.[4]

Considering the significant global prevalence of type 1 DM and the high morbidity and mortality

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associated with its vascular complications, the need to identify new methods of preventing and treating these complications is imperative. A thorough understanding of the pathogenic processes involved in the development of these complications cannot be gained without the development of animal models that accurately mimic the pathology present in humans.

The long-held belief that rats cannot develop atherosclerosis due to their protective lipid profile[5] represented a major disadvantage, as it limited the study of the pathogenic mechanisms of this disease, the identification of new therapeutic targets, and the testing of new therapeutic strategies. The progressive development of several rat models with atherosclerotic lesions has partially changed this view.[6-9] However, the options remain limited when it comes to the possibility of studying atherogenesis in DM rat models. While atherosclerotic lesions have been reported in type 2 DM models,[10-12] this was not the case for either transgenic or chemically-induced type 1 DM models, regardless of the duration of diabetes.[13-15]

Accordingly, we aimed to complement this gap in knowledge by evaluating the presence and the magnitude of several factors known to promote atherogenesis (e.g., dyslipidemia, inflammation, oxidative stress, and endothelial dysfunction), and to assess the potential of this pro-atherogenic environment to promote the development of atherosclerotic lesions in a rat model of long-term type 1 DM.

MATERIALS AND METHODS

Animals and housing
The study included 14 male Wistar rats (6 weeks old) purchased from the Cantacuzino Experimental Station (Bucharest, Romania). The animals were housed individually in polycarbonate cages in climate-controlled conditions (21-24°C), with a 12-hour light/12-hour dark cycle, and had free access to water and food. Following a one-week accommodation period, the animals were randomly assigned to control (n=6) or DM (n=8) groups.

All experimental protocols were in accordance with the International Council for Laboratory Animal Science guidelines (Directive 2010/63/EU) and were approved by the Ethics Committee of the University of Medicine and Pharmacy of Tîrgu Mureș.

Induction of diabetes mellitus
At 11 weeks of age, after a 12-hour fast-free period, rats assigned to the DM group were injected intraperitoneally with streptozotocin (STZ; 60 mg/kg bodyweight; Sigma-Aldrich, St Louis, MO) diluted in citrate-buffered saline (0.1 mol/L, pH 4.5; Sigma-Aldrich), as described previously.[16,17] Insulin was not administered over the course of the experimental protocol. The animals in the control group received a similar volume of citrate buffered saline, without STZ. One week after STZ administration, treated rats were again subjected to 12 hours of fasting and glycemic levels were measured to confirm the induction of DM, as described previously.[16,18] Rats were considered diabetics if their plasma glucose levels exceeded 250 mg/dL.

Blood sampling and analysis
At the age of 38 weeks, all animals were anesthetised with isoflurane. The abdominal cavity was opened, the aorta was cannulated, and blood samples were collected. The blood was left at room temperature to coagulate and was then centrifuged. The serum was distributed in Eppendorf microtubes and stored at -80°C until analysis.

White blood cells (WBC) count was performed using the direct current detection method (Sysmex XP–300 Automated Hematology Analyzer; Sysmex Corporation, Japan). Total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides were measured by enzymatic colorimetric methods using automatic analyzers (Cobas 6000 analyzer series; Roche Diagnostics, Basel, Switzerland, and Dimension RxL Max; Siemens Healthcare Diagnostics Inc., Newark, DE). High-sensitivity C-reactive protein (hs-CRP) was determined by the immunoturbidimetric method (Automated Analyzer Cobas Integra 400; Roche Diagnostics). Interleukins 1b (IL-1b), 6 (IL-6), 10 (IL-10), 18 (IL-18), and vascular endothelial growth factor (VEGF) levels were quantified with bead-based Multiplex assays using the Luminex 200 system (Luminex Corporation, Austin, TX). Von Willebrand factor (vWF), glutathione peroxidase (GPx), superoxide dismutase (SOD), and malondialdehyde (MDA) levels were measured using the enzyme-linked immunosorbent assay method (Automated Analyzer MiniBos; Adaltis Italia S.p.A., Bologna, Italy).

Tissue sampling and histological analysis
Immediately after blood sampling, the thoracic

cavity was opened, and the heart together with the large vessels were removed. A section of the ascending aorta was isolated and immersed in formalin. Oil Red O staining was performed to quantify lipid deposits within the aortic wall. Frozen, 8-10 μm sections were mounted on adhesive slides and rinsed in tap water for 10 min, then in 60% isopropyl alcohol. Freshly prepared Oil Red O solution was then added, and slides were rinsed in 60% isopropyl alcohol, counterstained with Haematoxylin stain, rinsed in distilled water, and mounted in glycerine jelly. Sections were examined with a Leica DM750 microscope (Leica Microsystems GmbH, Mannheim, Germany) and photographed using a Leica ICC50 HD camera (Leica Microsystems GmbH). The amount of fat within the aortic wall was scored on a semiquantitative scale (from 0 to 3): 0 – not stained; 1 – slightly stained; 2 – intensely stained; 3 – very intensely stained.

Statistics
Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software; San Diego, CA). Data were expressed as mean ± standard error of mean and were compared using the Mann-Whitney *U* test. A two-tailed p-value of less than 0.05 was considered statistically significant.

RESULTS

Three of the animals in the DM group died during the monitoring period and were removed from the final data analysis.

Biochemical parameters
At the end of the study, plasma glucose was significantly higher in the diabetic than in the control rats (Table 1). Diabetic rats also exhibited significantly higher levels of total cholesterol

and plasma triglycerides, and tended to present higher LDL-cholesterol levels compared to non-diabetics, while there was no significant difference in HDL-cholesterol levels between the two groups (Table 1).

Parameters of inflammation, endothelial dysfunction, and oxidative stress
Analysis of parameters characteristic for an inflammatory syndrome showed significantly higher levels of hs-CRP and lower levels of IL-10 in the diabetics, whilst IL-1b, IL-6, and IL-18 levels did not differ significantly between the two groups (Table 2). White blood cell count also tended to be higher in the diabetic rats compared to controls (p = 0.05).

Diabetic rats also presented significantly higher levels of VEGF compared to controls. Analysis of oxidative stress specific markers revealed similar MDA levels, but significantly lower GPx and SOD levels in the diabetics (Table 2).

Histological evaluation of lipid deposits
Examination of ascending aorta (Fig. 1) sections revealed complete absence of lipid deposits in the control rats (Fig. 2A). Meanwhile, mild (grade 1) lipid deposits were observed in the aortic wall of 4 (80%) of the 5 diabetic rats (Fig. 2B).

DISCUSSION

Due to their protective lipid profile, rats have long been considered immune to atherosclerosis. However, over the years, studies have demonstrated that, under certain conditions, rats can develop atherosclerosis. Atherosclerotic lesions have been described in Wistar rats fed a hyperlipidemic diet containing cholesterol, cholic acid, and thiouracil,[6] in Dahl-salt sensitive hypertensive rats that overexpress the human cholesteryl ester

TABLE 1: Biochemical parameters measured in diabetic and control rats at the end of the study

Parameters	Control (n=6)	Diabetes mellitus (n=5)	p
Plasma glucose (mg/dL)	123.5 ± 5.8	512.6 ± 20.2	**< 0.001**
Total cholesterol (mg/dL)	88.2 ± 6.9	139.3 ± 11.7	**0.02**
HDL-cholesterol (mg/dL)	69.4 ± 6.1	72.2 ± 4.5	0.42
LDL-cholesterol (mg/dL)	13.8 ± 1.6	17.8 ± 1.1	0.08
Triglycerides (mg/dL)	64.6 ± 8.6	434.4 ± 45.5	**0.02**

Values are expressed as mean ± standard error of mean. p-values refer to comparisons between groups using the Mann-Whitney *U* test. HDL: high-density lipoproteins; LDL: low-density lipoproteins.

TABLE 2: Parameters of inflammation, endothelial dysfunction, and oxidative stress measured in diabetic and control rats at the end of the study

Parameters	Control (n=6)	Diabetes mellitus (n=5)	p
Inflammatory syndrome			
WBC (x $10^3/mm^3$)	2.9 ± 0.4	5.2 ± 0.8	0.05
hs-CRP (ng/mL)	0.190 ± 0.010	0.210 ± 0.002	**0.01**
IL-1b (pg/mL)	47.8 ± 4.4	41.8 ± 5.8	0.52
IL-6 (pg/mL)	964.4 ± 168.2	1,058.0 ± 416.3	0.83
IL-18 (pg/mL)	77.1 ± 10.9	68.6 ± 12.9	0.63
IL-10 (pg/mL)	34.7 ± 4.6	23.1 ± 1.8	**0.04**
Endothelial dysfunction			
vWF (pg/mL)	30.6 ± 1.0	32.8 ± 0.3	0.21
VEGF (pg/mL)	43.5 ± 0.7	47.9 ± 1.6	**0.04**
Oxidative stress			
GPx (UI/mL)	8.5 ± 0.1	8.1 ± 0.1	**0.01**
SOD (ng/mL)	12.3 ± 0.1	12.0 ± 0.1	**< 0.01**
MDA (ng/mL)	5.3 ± 0.2	5.4 ± 0.2	0.52

Values are expressed as mean ± standard error of mean. p-values refer to comparisons between groups using the Mann-Whitney *U* test.

GPx: glutathione peroxidase; hs-CRP: high-sensitivity C-reactive protein; IL: interleukin; MDA: malondialdehyde; SOD: superoxide dismutase; VEGF: vascular endothelial growth factor; vWF: von Willebrand factor; WBC: white blood cells.
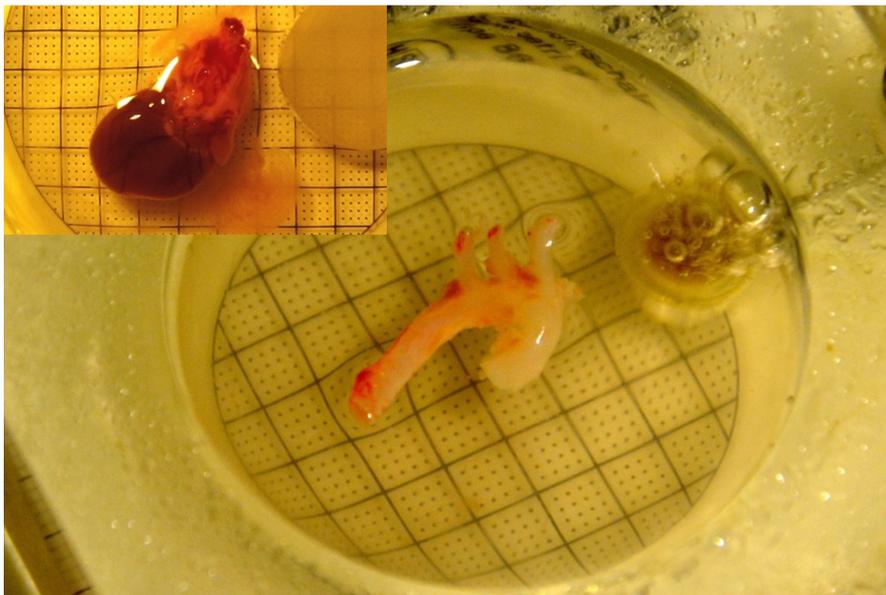

FIG. 1: A preparation of the isolated heart, together with the large vessels, from one of the studied rats (upper left corner), and the thoracic aorta subsequently isolated from the same preparation (main image). A section of the ascending aorta was then isolated from the preparation and assessed for quantification of lipid deposits using Oil Red O staining. No macroscopic abnormality was visible in any of the preparations.
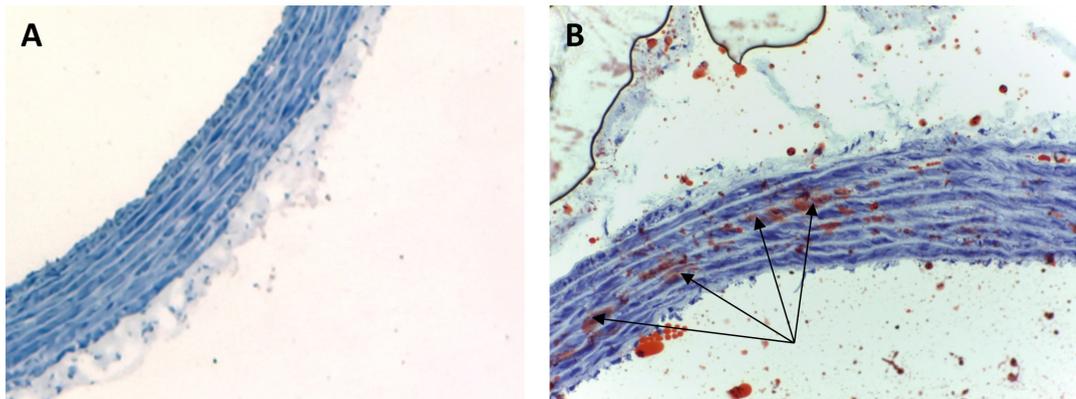
FIG. 2: Representative histology images in a control (A) and a diabetic (B) rat. Oil Red O staining reveals no lipid deposits in the aortic wall of the control rat (A), and highlights the presence of lipid deposits (arrows) in the aortic wall of the diabetic rat (B). Lipid deposits appear colored in red.

transfer protein,[7] in apolipoprotein E knockout rats fed a special high cholesterol/bile salt diet,[8] as well as in the transgenic JCR:LA-cp rat, which exhibits all aspects of the human metabolic syndrome.[9] While the development of these models has greatly benefited the study of atherosclerosis pathogenesis, evaluation of this process in the context of DM, one of the major risk factors for atherosclerosis, is still limited. Even though changes in the internal environment and complications induced by type 1 DM over longer periods have been studied both in transgenic[13,14] and chemically-induced[15-19] diabetes models, none of these studies assessed the presence and severity of the mechanisms involved in atherogenesis in such a model. The present study demonstrates that long-term type 1 DM creates a pro-atherogenic environment and leads to the development of intermediate, type III atherosclerotic lesions, as defined by the American Heart Association.[20]

Long-term type I diabetes mellitus provides the biochemical environment conducive to the development of atherosclerosis
Diabetic patients with poor glycemic control present dyslipidemia, with increased levels of triglycerides[21,22] and LDL-cholesterol,[22,23] while HDL-cholesterol levels are normal or slightly increased in those with optimal glycemic control,[21] or decreased in diabetic ketoacidosis.[24] Similarly, and consistent with previous reports,[18] diabetic rats in the present study presented significant dyslipidemia, with a more pronounced increase in triglyceride levels than in total cholesterol, while also showing a tendency towards an increase in LDL-cholesterol levels. No difference was found between diabetic and control rats with regard to HDL-cholesterol levels. In addition to changes in lipid levels, type 1 DM has also been associated with significant changes in the composition of plasma lipids.[25] Hyperglycemia-induced glycosylation of LDL-cholesterol enhances its absorption by aortic intimal cells and promotes its uptake by macrophage cells via scavenger receptors, leading to increased intracellular accumulation of cholesterol esters.[26] Glycosylated LDL-cholesterol also appears to be more susceptible to oxidation, critical for its atherogenicity.[27] While triglycerides are not directly atherogenic, their association with atherogenic remnant particles[28] and the ability of triglyceride-rich lipoproteins to suppress the anti-atherosclerotic and anti-inflammatory effects of HDL-cholesterol[29] explain the contribution of hypertriglyceridemia in the pathogenesis of atherosclerosis in this setting. This highly atherogenic lipid profile is likely to have played a major contribution to the occurrence of lipid pools in the aortic walls of our diabetic rats.

Rats with long-term diabetes present systemic inflammation, endothelial dysfunction, and oxidative stress
Inflammation, endothelial dysfunction, and oxidative stress are interconnected components of a complex spider web that promotes atherosclerosis initiation and progression; this relationship is highly exacerbated in diabetics.[30] Diabetes mellitus is recognised as a systemic inflammatory disorder. Raised levels of inflammatory proteins, coupled with decreased levels of anti-inflammatory molecules, have been reported in adults with type 1 DM.[31-34] In line with these observations, diabetic rats in the present

study presented significantly elevated levels of hs-CRP and significantly reduced circulating levels of the anti-inflammatory IL-10 protein. Together with the absolute increase in WBC count, these results confirm the presence of a systemic, pro-atherogenic inflammatory environment in these animals. The increased VEGF levels seen in the diabetic rats in our study point towards concomitant endothelial dysfunction, one of the key elements in the initiation and progression of atherosclerotic lesions.[35-37] Indeed, chronic exposure to DM has been shown to overwhelm vascular endothelial defense mechanisms and to promote atherosclerosis.[38] Increased free radicals production and altered antioxidant defense systems are often seen in both humans and animals with DM,[39-42] although higher erythrocyte SOD activity has also been reported in subjects with type 1 DM.[43] Discordant results have also been reported for GPx, with some studies showing lower[44,45] and others higher[46,47] GPx activity. In line with most of the previous studies, evaluation of oxidative stress markers demonstrated a reduction in antioxidant defense mechanisms in our diabetic rats, which displayed significantly lower antioxidant enzymes (GPx and SOD) levels than their non-diabetic counterparts.

Pro-atherogenic changes in the internal environment of diabetic subjects are well-known and documented. The highly modified internal environment of our rats with long-term type 1 DM, which displayed hyperglycemia and hyperlipidemia, systemic inflammation, endothelial dysfunction, and altered antioxidant defense mechanisms, resulted in the occurrence of intermediate aortic atherosclerotic lesions, as demonstrated by Oil Red O staining, a marker of lipid accumulation indicative of lipid-loaded macrophages.[48] Although several fat-soluble dyes, including Sudan IV and Sudan black, have been developed to quantify atherosclerotic burden in human and animal vasculature, because of its ease of use, reliability, and large amount of information provided, Oil Red O has imposed itself as the "gold standard" stain for specifically identifying lipids.[49] Using Oil Red O staining in animal models of cardiovascular diseases can provide important data regarding the impact of environmental factors on atherosclerosis initiation and progression, as well as the impact of prophylactic and therapeutic strategies.

Despite the long duration of DM in the present study, true atheromas were not observed. These results suggest that this type 1 DM rat model could be useful for assessing the pathophysiology of early atherosclerosis and the potential benefit of atherosclerosis preventive strategies, but is probably less suitable for the study of advanced atherosclerosis. On the other hand, although no true atheromas were found, this study demonstrates that, without subjecting animals to a hyperlipidemic diet, long-term type 1 DM is sufficient to induce the changes necessary to initiate atherosclerosis in an animal normally resistant to the development of this pathology.

Potential limitations

One of the potential limitations of our study was the small number of animals, especially within the DM group, which could have affected the statistical power of the study. Also, we cannot rule out the possibility that over an even longer period of DM, the observed intermediate lesions would have progressed to true atheromas. However, the duration of DM in this study was among the longest in the literature. Autopsy was not performed in the 3 diabetic rats that died during the study and the cause(s) of their deaths can only be speculated based on the results of previous studies. Such causes may include kidney damage, infectious complications, or pulmonary oedema.[50,51] Since none of the deaths occurred early (within days) after STZ administration, it is unlikely that acute STZ toxicity contributed to these deaths.[50]

CONCLUSION

The present study presents a STZ-induced type 1 DM rat model with one of the longest follow-ups in the literature. In this model, long-term DM created a highly pro-atherogenic environment characterised by hyperglycemia, dyslipidemia, systemic inflammation, endothelial dysfunction, and oxidative stress that resulted in the development of intermediate aortic atherosclerotic lesions.

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