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Apoptotic germ-cell death and testicular damage in experimental diabetes: prevention by endothelin antagonism

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Abstract This paper explores the role of endothelins (ETs) in diabetes-induced testicular damage by investigating, in a temporal manner, testes from streptozotocin (STZ)-induced diabetic rats. Testicular and epididymal weights and testicular morphology were assessed. Cell death was evaluated by light microscopy using conventional staining and morphology, and by apoptotic cell staining using the *T*erminal deoxynucleotidyl transferase-mediated d*U*TP *N*ick *E*nd-*L*abeling (TUNEL) technique. Expression of endothelin-1 (ET-1) mRNA was evaluated by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method. Furthermore, effects of a mixed ET_A and ET_B receptor antagonist, bosentan, were studied. Testicular weights did not show any change at 1 month of follow-up, but were decreased after 6 months of diabetes. However, epididymal weights were significantly decreased at the end of both time periods in the diabetic rats. Morphological evaluations of the testes from diabetic rats showed a reduction in seminiferous tubular diameter, an increase in the number of empty testicular tubules and an increase in vascular density. Furthermore, degenerated germ cells and TUNEL-positive cells were significantly higher in diabetic rats than in control animals. The changes in diabetic animals were associated with increased ET-1 mRNA expression and were prevented by bosentan treatment. Administration of bosentan prevented decreased testicular weights, reduced seminiferous tubule diameters, increased vascular densities and incidences of degenerated and apoptotic germ cells and empty tubules in diabetic rats at the long-term follow-up. These results demonstrated that an ET-1 mediated pathway might be involved in testicular injury and germ-cell apoptosis in diabetes.

Key words Apoptosis · Diabetic rats · Enthothelin-1 · Endothelin blockade · Spermatogenesis

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Introduction

Diabetes mellitus, a disease of old age, is an enormous global health problem. Due to advances in medical care, diabetic patients can anticipate a longer life span. With increased longevity, chronic complications of diabetes are of major concern [9]. Infertility is a significant problem in the diabetic population. Low testosterone levels, testicular dysfunction and impaired spermatogenesis have been demonstrated in the testes of diabetic men and experimental animals [2, 18]. Although mechanisms involved in the development of such changes have not been thoroughly characterized, increased apoptotic cell death in the seminiferous tubule of diabetic mice has been demonstrated recently [15]. Sustained hyperglycemia may cause functional and structural alterations in target organs of diabetic complications [9]. Endothelins (ETs) are a family of 21 amino acid peptides with diverse biological actions. In addition to acting as vasoactive peptides, ETs have important roles in morphogenesis [3]. Alteration of ETs is of importance in several vascular dysfunctions [16]. In addition to the vascular endothelium, ETs are produced by several non-vascular cellular components [10, 13, 16]. ET-1 is upregulated in various organs of diabetic rats, suggesting the possible involvement of ET in the pathogenesis of diabetic complications [8, 16]. We have

previously demonstrated that in the retina, overexpression of ETs and their receptors plays a key role in the pathogenesis of diabetes-induced functional and structural defects [8, 11].

ET-1 peptides and both ET_A and ET_B receptors are present in the human and the rat testis [7, 10, 13]. Increased ET-1 levels may cause vasoconstriction, leading to decreased blood flow, ischemia and tissue damage. The testis, in particular, is sensitive to hypoxia-induced cellular damage. Apoptotic cell deaths may occur during embryonic development and secondary to non-physiological stresses, such as mild ischemia, hyperthermia and radiation. In the normal testis, some germ cells undergo apoptosis during normal development. However, the rate of apoptosis may markedly increase in response to stimuli, such as gonadotrophin or androgen deficiencies, administration of certain toxic substances, hyperthermia and ischemia [5, 7]. In streptozotocin (STZ)-induced diabetic rats, an increase in germ-cell apoptosis in the earlier stages, leading to derangement of spermatogenesis, has been demonstrated [15]. To explore a possible pathogenetic role of ET-1 in the development of diabetes-induced apoptosis in the rat testis, we studied testicular structure, apoptotic germ-cell death and ET-1 mRNA expression in the STZ-induced diabetic rats. In addition, the effects of a general ET receptor antagonist, bosentan, on these alterations were investigated.

Materials and methods

Animals and treatments

All animals were cared for in accordance with the guidelines of the University of Western Ontario Council on Animal Care Committee. Male Sprague-Dawley rats of approximately 200 g were obtained from Charles River Canada Ltd (St Constant, PQ, Canada) and were fed with rat chow and water ad libitum. STZ (Sigma, St. Louis, Mo.) was administered as a single dose of 65 mg/kg intravenously (i.v.) in citrate buffer. The control animals received an injection of buffer only. Diabetic animals were monitored daily with respect to urine volume, sugar and ketone, and received a daily dose of insulin (0.5–2 U/day) to prevent ketosis. Body weight and blood glucose levels were monitored regularly. Bosentan was obtained from Actelion (courtesy of Dr M. Clozel). Bosentan is a potent ET_A and ET_B receptor blocker [6]. Bosentan was administered by daily oral gavage at a dosage of 100 mg/kg of body weight per day. Twelve randomly selected animals from each group were sacrificed after 1 ($n = 6$ per group) and 6 months ($n = 6$ per group) of follow-up. Before sacrifice, the animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal (i.p.)). The animals were sacrificed by cardiac puncture and blood was collected for glycated hemoglobin (Hb) measurement (Glycotest TM; Pierce, Rockford, Ill.). Following sacrifice, both testes and epididymides were removed and weighed. The right testes were snap frozen in liquid nitrogen and were stored at −80 °C until further analysis of mRNA production. The left testes were fixed in 4% paraformaldehyde in phosphate buffer and embedded in paraffin. They were used for assessment of testicular morphology and apoptotic cell death.

Histological assessment

Five-micrometre-thick sections from three randomly selected rats from each group were cut consecutively from the paraffin blocks and were mounted onto glass slides. The sections were deparaffinized and dehydrated. Two sections from each sample were stained using hematoxylin and eosin (H&E) to evaluate testicular morphology. The sections were analyzed as to small vessel density and diameter of seminiferous tubules, since alteration of these parameters has been established in diabetic rats [1]. For seminiferous tubule diameter, slides were examined at 40× magnification. The images were stored electronically and the shortest diameter of each testicular tubule was measured using Mocha Image Analysis Software (Jandel Scientific, Calif.). Five tubules from each specimen were measured in five separate fields. For vascular density measurements, the slides were examined at 100× magnification and ten preset, area-weighted fields (1 mm^2) of cross-sectioned tubules were scanned through an ocular grid. All vessel profiles in the outlined area were counted, disregarding those intersected by the "forbidden line" [1].

Evaluation of apoptotic cells in situ

H&E staining. The cells showing degenerating changes were counted by light microscopic examination at 400× magnification. The features of degenerated cells included nuclear pyknosis with or without cellular shrinkage, nuclear fragmentation and chromatin margination or condensation at the periphery with a sharp delineation [12].

TUNEL staining. The method of *T*erminal deoxynucleotidyl transferase-mediated d*U*TP *N*ick *E*nd-*L*abelling (TUNEL) was used for in situ staining of apoptotic cells as described in our previous study [5]. Briefly, after deparaffinization and rehydration, the slides were incubated with 20 μg/ml proteinase K for 15 min at room temperature, and then washed in deionized water. Endogenous peroxidase was inactivated with 2% H_2O_2 for 5 min. The subsequent staining was conducted according to the manufacturer's instruction (ApopTag, In Situ Apoptosis Detection Kit; Intergen, Purchase, N.Y.). The sections were rinsed, preincubated with equilibration buffer for 15 s and incubated with digoxigenin-labeled dUTP and dATP in TDT buffer in a humidified chamber at 37 °C for 60 min. The reaction was stopped by immersing slides in stop/wash buffer. After washing the sections three times with phosphate-buffered saline (PBS) (0.1 M, pH 7.4), anti-digoxigenin-peroxidase was applied to specimens and further incubated for 30 min at room temperature. Staining was developed by DAB (diaminobenizidine) and counterstained in hematoxylin. Both H&E- and TUNEL-stained slides were evaluated and quantified as to the number of apoptotic or degenerated cells per 100 nuclei.

Measurement for ET-1 mRNA

RNA isolation. TRIZOL reagent (Canadian Life Technologies, Burlington, Ontario, Canada) was used to isolate RNA from the testis as described by the manufacturer's instructions. Quantitation of RNA was performed by determining the absorbances at 260 and 280 nm. First strand cDNA synthesis was performed using the Superscript-II system (Canadian Life Technologies). RNA was added to oligo(dT) primers, denatured at 70 °C for 10 min and quenched on ice for 1 min. Reverse transcription was carried out by the addition of MMLV (Moloney murine leukemia virus)-reverse transcriptase (RT) and dNTPs at 42 °C for 50 min in a total reaction volume of 20 μl. The reaction was terminated by a 15-min incubation at 70 °C. The resulting RT products were stored at −20 °C.

Polymerase chain reaction (*PCR*). The amplification was carried out using our previously described methodologies [8]. The ET-1 primers 5′-GCTCCTGCTCCTCCTTGATG-3′ (sense) and 5′-CTCGCTCTATGTAAGTCATGG-3′ (antisense), with a predicted product size of 499 bp were used. Reactions were performed in 30-μl volumes containing 1 × PCR buffer, 1.5 mM $MgCl_2$ 250 μM dNTP mix, 1 μM of each amplification primer, 2.5 U *Taq* polymerase and 4 μl of the RT product. The initial cycle was carried out following: 3 min at 94 °C for denaturation, 1 min at 60 °C for annealing and 3 min at 72 °C for extension. Subsequent cycles of

PCR were performed using the following conditions: denaturation, 45 s at 94 °C; annealing, 45 s at 54 °C; and extension, 1 min at 72 °C and 7 min for final extension. The linearity of the PCR was established by analyzing a PCR product with a variable amount of template and variable cycle numbers. It has been previously shown that in this reaction, the PCR amplification is log-linear up to 40 cycles. In this study, we used 30 cycles of amplification. Simultaneously, a housekeeping gene (β-actin) was amplified in a separate set of tubes using the same RT product. The primer sequences for β-actin are 5′-TGGTGGTATGGGTCAGAAGG-3′ (sense) and 5′-ATCCTGTCAGCGATGCCTGGG-3′ (antisense), with a predicted product size of 813 bp. The amplification products were analyzed on a 2.5% agarose gel in 1 × TAE (trisacetate-EDTA) buffer. Ten microlitres of each PCR product was loaded in each lane and electrophoresed at 100 V for 90 min. The gels were stained with ethidium bromide and visualized under UV light.

Quantitation. Quantitation was performed by serial dilution slot-blot hybridization and densitometry of the products from the upstream of amplification onto the nylon membranes [8]. Hybridizations were performed with biotinylated amplification product specific oligoprobes (ET-1: 5′-CAAAGACCACAGACCAAGGG-3′; and β-actin: 5′-CTGACCCTGAAGTACCCCATT-3′) [8]. The detection was carried out using an NBT/BCIP system (Sure blot, Oncor, Gathisburg, Md.). The blots were analyzed by a Hewlett-Packard 4C scanner and using Mocha Image Analysis Software (Jandel Scientific). The densitometric values were expressed as arbitrary units per microgram of total RNA and the ratio of β-actin to ET-1 was obtained [8].

Statistical analysis

Data are expressed as mean ± SE in each group. The data were subject to an unpaired two-tail Student's *t*-test. A *P* value of 0.05 or less was accepted as being significant.

Results

Diabetic animals with or without bosentan treatment showed lower body weight gain, hyperglycemia and elevated glycated Hb levels compared with non-diabetic control rats (data not shown).

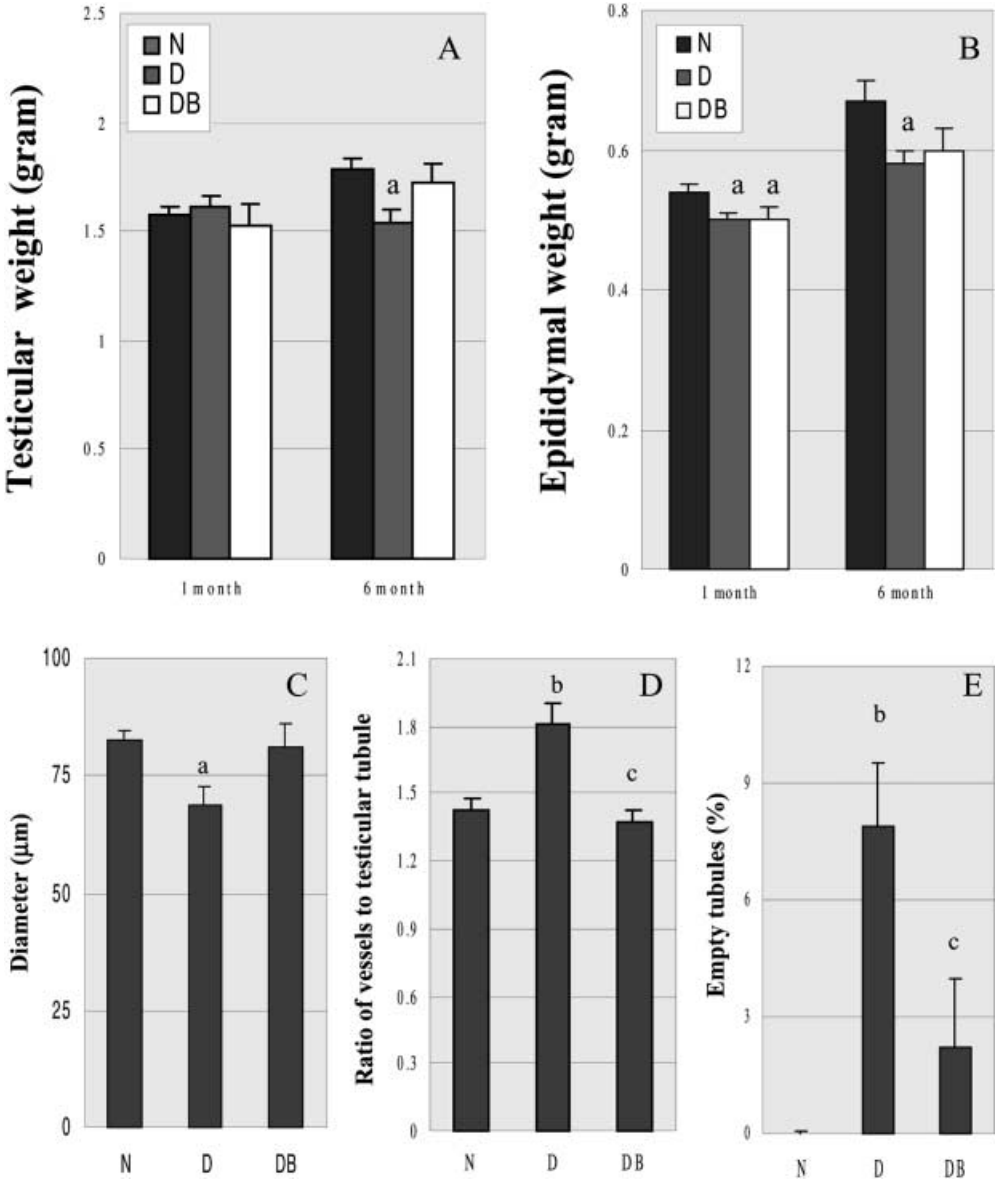

Fig. 1A–E Testicular alterations in diabetic rats and effects of ETs blocker, bosentan. Morphological analyses were performed on 6-month animals. *a*, $P < 0.05$ compared to *N*; *b*, $P < 0.01$ compared to *N*; *c*, $P < 0.05$ compared to *D*. *N* non-diabetic control rats, *D* diabetic rats, *DB* diabetic rats on bosentan

Testicular injury in diabetic rats

Figure 1A, B presents the testicular and epididymal weights in diabetic rats with or without bosentan treatment. Diabetic rats showed a decrease in testicular weight at 6 months (Fig. 1A). However, epididymal weights in diabetic rats were significantly decreased, both after short-term (1 month) and long-term (6 months) follow-up (Fig. 1B).

Results of the morphological assessment after 6 months of diabetes showed that, compared with control rats (Fig. 2A), the diabetic animals presented significantly decreased seminiferous tubule diameters (Figs. 1C, 2B) and increased testicular vascular densities (Fig. 1D). In addition, the diabetic rats demonstrated an increase in the number of seminiferous tubules with only a few or no germ cells (empty tubules) (Figs. 1E, 2B). Examination of epididymides showed the presence of immature as well as degenerated germ cells in diabetic rats but not in the non-diabetic animals.

Testicular cell death

Evaluations of both degenerated cells and TUNEL-positive cells are important for studying apoptotic cell death in the testes, as changes are not always present in the same cells [12]. Therefore, both degenerated and

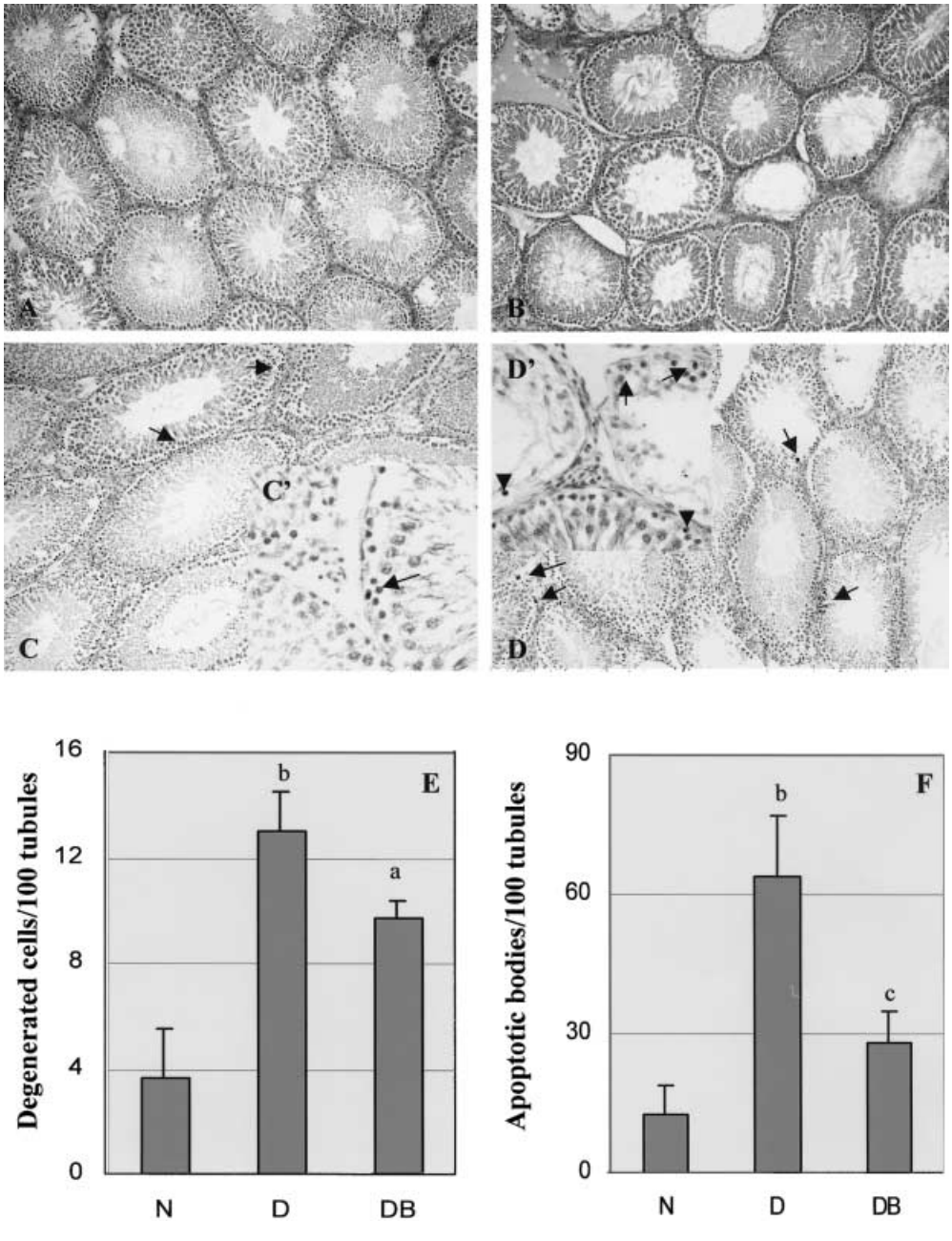

Fig. 2A–F Testicular morphology and TUNEL staining. **A** H&E stain in non-diabetic rat (100×). **B** H&E stain in diabetic rat (100×). **C** TUNEL staining in non-diabetic rat (200×), showing a few TUNEL-positive cells (*arrows*). **D** TUNEL staining in diabetic rat (200×), showing increased TUNEL-positive cells (*arrows*). **C′** and **D′** (*inserts*) TUNEL staining (400×), and **D′** showing the TUNEL positivity in degenerated cells (*arrowheads*) and TUNEL-positive cells in the empty tubules (*arrows*). **E**, **F** Quantitative evaluation for degenerated cells (on H&E staining) and TUNEL-positive cells (apoptotic bodies). *a*, $P < 0.05$ compared to *N*; *b*, $P < 0.01$ compared to *N*; *c*, $P < 0.05$ compared to *D*. *N* non-diabetic control rats, *D* diabetic rats, *DB* diabetic rats on bosentan

TUNEL-positive cells were evaluated in the present study. These changes were occasionally seen in the testes of normal rats (Fig. 2C, E, F). However, a significant increase in such changes was present in the testes of diabetic rats (Fig. 2D–F). Some degenerated cells showed TUNEL positivity (Fig. 2D′). In the empty tubules, TUNEL-positive cells could be found frequently (Fig. 2D′).

Results of ET receptor antagonist, bosentan and ET-1 mRNA expression

By morphological evaluation, the decrease in the diameter of seminiferous tubules, the increase in the vascular density and the increase in the incidence of empty testicular tubules were prevented by bosentan (Fig. 1C–E). In addition, bosentan showed a protective effect on apoptotic cell death in diabetic rats measured by H&E and TUNEL staining (Fig. 2E, F). The number of empty tubules was also significantly decreased in bosentan-treated diabetic animals as compared with diabetic animals (Fig. 1E). Furthermore, bosentan prevented decreased testicular weights in diabetic rats after 6 months (Fig. 1A). No influence of bosentan on the decrease in epididymal weight in diabetic rats was noted after 1 month of follow-up (Fig. 1B). After 6 months of follow-up, the diabetic group showed significantly reduced epididymal weight. Data from the bosentan-treated group was intermediate between the non-diabetic and diabetic groups, and were not significantly different from each group.

Semi-quantitative RT-PCR demonstrated that ET-1 mRNA was increased significantly in the testes of diabetic rats, and this enhanced expression was completely prevented by bosentan (Fig. 3).

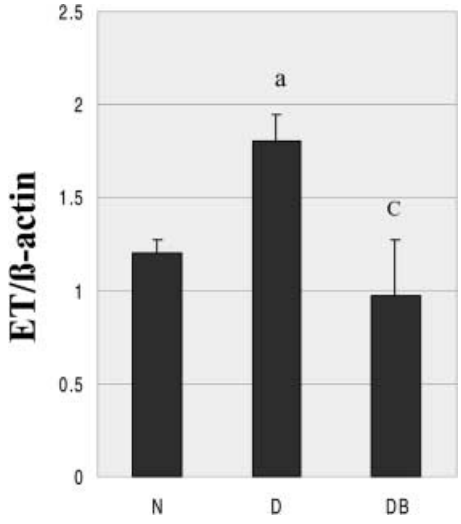

Fig. 3 mRNA analysis by semi-quantitative RT-PCR from testis of normal (N), diabetic (D) and diabetic and bosentan-treated rats (DB) after 6 months of follow-up. Data are expressed as the ratio of ET-1 gene to housekeeping gene, β-actin *a*, $P < 0.05$ compared to *N*; *c*, $P < 0.05$ compared to *D*. *N* non-diabetic control rats, *D* diabetic rats, *DB* diabetic rats on bosentan. *a*, $P < 0.05$ compared with *N*; *c*, $P < 0.05$ compared with *D*. *N* non-diabetic control rats, *D* diabetic rats, *DB* diabetic rats on bosentan

Discussion

Infertility is a well-known complication in diabetic patients, with considerable detrimental consequences. Most studies have demonstrated abnormal decreases in hormonal levels in diabetic patients and animals [2, 18]. However, the mechanisms of testicular dysfunction are not clearly understood. The present study demonstrated a decrease in testis weight, apoptotic cell death and atrophy of the seminiferous tubules in diabetic rats. These results suggest that apoptotic cell death may be a major factor in loss of testicular function.

Several conditions may cause apoptotic cell death in the testis of diabetic rats. Downregulation of hormones, which are required for normal spermatogenesis, may cause apoptotic death of germ cells [19]. In experimental diabetes, low levels of serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) have been demonstrated [18], and decreased levels of these hormones may induce germ cell apoptosis [19]. However, reduced LH or FSH levels usually cause apoptosis in stages VII–VIII of seminiferous tubules in which germ cells possess testosterone receptors [5, 19]. In this study, we have found increased germ-cell apoptosis in stages XII–XIV and earlier stages (I or II–VI). These data are similar to previous data by other investigators [15]. Increased testicular temperature may lead to ineffective spermatogenesis [17]. Increased vascular density in the testes, as seen in this study, may lead to increased scrotal temperature. Furthermore, several physical factors, including alterations in temperature, may lead to altered ET-1 expression in other organs [4]. Possible increased temperature secondary to an increase in small blood vessel density in the testis may cause apoptotic death of germ cells [19, 20]. Furthermore, an increased wall thickness of small blood vessels in diabetics may lead to hypoxia-induced cellular damage [1]. However, this notion needs to be further characterized by direct measurements.

These changes in diabetic rats were prevented by treatment with an ET receptor antagonist. In the testis, both ET-1 and ET-3, and ET_A and ET_B, receptors have been identified [7, 10, 13]. ET peptides are hormonally regulated and locally produced in the testis as autocrine/paracrine factors and may modulate testicular microcirculation [7].

Exact mechanisms of ET-1 action in the testis are not clear. Several functions of ET may be of importance. Augmented ET-1 induced vasoconstriction may reduce blood flow. Mitogenic action of ET-1 may influence cell proliferation, leading to testicular structural alterations, as seen in the present study. Increased vascular density secondary to angiogenesis is seen in other target organs of diabetic complications, such as

the retina, secondary to tissue hypoxia [14]. Several angiogenic factors are upregulated in diabetes leading to neovascularization. Vascular endothelial growth factor (VEGF) is one of the major factors leading to such neovascularization. ET-1 has a co-stimulatory relationship with VEGF [15]. The present data would suggest that ET-1 is important in causing increased testicular vascular density. Whether this action is mediated via VEGF needs further study.

The exact etiology of epididymal weight loss observed in this study is not clear. However, one may speculate that it could be secondary to ineffective spermatogenesis in the testis and a reduced number of spermatozoa in the epididymis. A partial recovery was seen after bosentan treatment, suggesting that other pathways may be of importance. Whether a timing difference exists in the recovery of these two organs remains to be examined in future studies.

Recent data indicates that hyperglycemia-induced ET overexpression in various organs is of importance in the development of target organ dysfunction in diabetes [8, 11]. We have previously demonstrated that overexpression of ET-1 and ET-3 and their receptors in diabetic rats is associated with retinal vascular dysfunction and structural abnormalities. The present study has demonstrated, for the first time, that STZ-induced diabetes can cause upregulation of ET-1 mRNA expression in association with testicular functional and structural dysfunction. It is of interest to note that bosentan treatment reduced ET-1 expression in the testis. The exact reason for such alterations is unclear. In other target organs of diabetic complications, we have not seen such phenomena [8]. The present study, however, suggests an important role of ETs in diabetes-induced infertility. The present study further suggests a potential therapeutic role of ET blockade in the treatment of infertility in diabetes.

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